EVALUATION OF CHEMICAL CONSTITUENTS AND BIOLOGICAL PROPERTIES OF EXTRACTS OF LEAVES OF *Kalanchoe pinnata* (LAM.) PERS FROM RUPANDEHI DISTRICT OF NEPAL

A DISSERTATION SUBMITTED TO THE

DEPARTMENT OF CHEMISTRY

AMRIT CAMPUS

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

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

By

DAKSHINA BHUSAL

Symbol No: CHE 1584/075

TU Regd. No: 5-2-50-265-2014

SEPTEMBER, 2022

DECLARATION

This dissertation entitled **"EVALUATION OF CHEMICAL CONSTITUENTS AND BIOLOGICAL PROPERTIES OF EXTRACTS OF LEAVES OF *Kalanchoe pinnata* (LAM.) PERS. FROM RUPANDEHI DISTRICT OF NEPAL"** submitted to the Department of Chemistry, Amrit Campus, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the partial fulfilment of the Master of Science (M.Sc.), a degree in Chemistry is a research work carried out by me under the supervision of Asst. Prof. Dr. Ram Lal (Swagat) Shrestha Department of Chemistry, Amrit Campus, Tribhuvan University, Nepal.

I, Dakshina Bhusal, with this declare that this work presented herein is genuine and initially done by me and has not been published or submitted in part or complete in this or any other form to any university or institute, here or elsewhere, to award any degree.

_____

Dakshina Bhusal

September, 2022

RECOMMENDATION LETTER

This is recommended that the dissertation work entitled "**EVALUATION OF CHEMICAL CONSTITUENTS AND BIOLOGICAL PROPERTIES OF EXTRACTS OF LEAVES OF *Kalanchoe pinnata* (LAM.) PERS. FROM RUPANDEHI DISTRICT OF NEPAL**" had been carried out by **Mrs. Dakshina Bhusal** as partial fulfilment for the Master Degree in Chemistry under my supervision. To my knowledge, no institution has submitted this material for a degree.

_____

Supervisor

Asst. Prof. Dr. Ram Lal (Swagat) Shrestha

Department of Chemistry

Amrit Campus, IOST

Tribhuvan University,

Nepal

September, 2022

LETTER OF APPROVAL

This dissertation entitled "**EVALUATION OF CHEMICAL CONSTITUENTS AND BIOLOGICAL PROPERTIES OF EXTRACTS OF LEAVES OF *Kalanchoe pinnata* (LAM.) PERS. FROM RUPANDEHI DISTRICT OF NEPAL**" submitted by **Mrs. Dakshina Bhusal**, under the supervision of **Asst. Prof. Dr. Ram Lal (Swagat) Shrestha**, Department of Chemistry, Amrit Campus, Institute of Science and Technology (IOST), Tribhuvan University, Nepal, is now approved for the partial fulfilment of the Master of Science (M.Sc.) Degree in Chemistry. This dissertation has never been submitted to another university or organization for the purpose of conferring a degree.

_____

Supervisor

Asst. Prof. Dr. Ram Lal (Swagat) Shrestha

Department of Chemistry

Amrit Campus, IOST

Tribhuvan University

_____

Internal Examiner

Asst. Prof. Dr. Deval Prasad Bhattarai

Department of Chemistry

Amrit Campus, IOST

Tribhuvan University

_____

External Examiner

Assoc. Prof. Dr. Achyut Adhikari

Central Department of Chemistry

University Campus, IOST

Tribhuvan University

_____

Head of Department

Assoc. Prof. Kanchan Sharma

Department of Chemistry

Amrit Campus, IOST

Tribhuvan University

_____

M.Sc. Coordinator

Assoc. Prof. Dr. Bhushan Shakya

Department of Chemistry

Amrit Campus, IOST

Tribhuvan University

September, 2022

ACKNOWLEDGEMENTS

To my supervisor, **Asst. Prof. Dr. Ram Lal (Swagat) Shrestha**, of the Chemistry Department at the Amrit Campus, I would like to convey my profound gratitude and appreciation for his guidance, invaluable recommendations, and practical approach throughout this study.

I want to say thank you to **Assoc. Prof. Kanchan Sharma**, Head of the Department of Chemistry, for providing me with such a great opportunity. I am grateful to **Assoc. Prof. Dr. Bhushan Shakya**, M.Sc. Program Coordinator, Department of Chemistry, Amrit Campus, for showing his incredible scientific insight, invaluable suggestions, encouragement, and regular feedback at all stages of my dissertation work.

I am very grateful to the **University Grants Commission (UGC)** which awarded me with the thesis support grant (**Grant No.: MRS-78-79-S&T-37**) and encouragement of my respected professors, lecturers, seniors, and my dual classmates. I would also like to convey my thanks to all the administrative and laboratory staff of the Department of Chemistry for their continuous assistance throughout this work.

My immense thanks to **Ms. Binita Maharjan**, Ph.D. Scholar, Amrit Campus for her continuous support and proper guidance during this work.

I owe my spouse, **Mr. Shree Ram Poudel**, a great deal of gratitude for his unwavering support and essential help in organizing till the research was finished.

Finally, but immensely, my most sincere obligation goes to my father, **Mr. Moti Prasad Bhusal**, and mother, **Mrs. Huma Devi Bhusal**, family members from the core of my heart for their never-ending love and support in every path of my efforts.
Thank you to all!

Dakshina Bhusal
September, 2022

ABSTRACT

The Crassulaceae family includes *Kalanchoe pinnata* (synonym: Bryophyllum pinnatum), also known as "Pattharchatta" in Nepali. The plant's therapeutic and medical properties contain wound-healing, antioxidant, antiproliferative, antimicrobial, anti-allergic, analgesic, nephroprotective, gastroprotective, antidiabetic, antidepressant, sedative, antilithiatic, chemoprotective, antihypertensive and immunosuppressant activities. The current study examined phytochemical screening, GC-MS, UV, FT-IR, total phenolic and flavonoid content. Extracts of *K. pinnata* were also analyzed for antimicrobial, antioxidant, antidiabetic, cytotoxicity, thin-layer, and column chromatography. The air dried powder of *K. pinnata* were extracted using seven different solvents: hexane, DCM, chloroform, ethyl acetate, acetone, methanol, and water. The aqueous extract outperformed the other six, yielding 14.50 g, whereas ethyl acetate produced only 0.21 g. Phytochemical screening discovered the presence of flavonoids, phenolic chemicals, terpenoids, tannins, steroids, and carbohydrates. The antioxidant activity was assessed by DPPH free radical scavenging method. The 3,5-dinitrosalicylic acid (DNSA) technique revealed a range of α-amylase inhibition properties. Antimicrobial susceptibility tests were tested against *Bacillus subtilis, Escherichia coli, and Candida albicans*. All the extracts showed better antimicrobial properties. The quantitative analysis of phytochemicals revealed that the TPC and TFC of the ethyl acetate is higher as comparison to the methanol extract The IC_{50} values of chloroform, ethyl acetate and methanol extracts were 2219.41 µg/mL, 365.22 µg/mL, and 293.53 µg/mL, respectively, in the antioxidant assay. The IC_{50} values of acetone, ethyl acetate and methanol extracts were 285.71 µg/mL, 504.21 µg/mL, and 195.39 µg/mL, respectively, in the α-amylase inhibition assay. The LC_{50} values of chloroform, acetone and methanol extracts were 0.181 mg/mL, 0.175 mg/mL, and 0.29 mg/mL respectively. The best TLC was shown by methanol extract in the Acetone: Hexane solvent system. From the column chromatography, single spot is obtained at 1% Ac: Hex and 5% Ac: Hex. The existence of multiple bonds, aromaticity, conjugation, and unsaturated organic molecules were confirmed through UV-visible spectrophotometer's results. The presence of O-H, C-H, C=O, $-CH_2$, $-NO_2$ and C-O were verified by FT-IR findings.

Keywords: *Kalanchoe pinnata*, extraction, chemical, biological analysis

LIST OF ACRONYMS AND ABBREVIATIONS

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

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

TABLE OF CONTENTS

CHAPTER I: INTRODUCTION

1.1 Background

Nepal is a mountainous nation in the foothills of the Himalayas. About 800 kilometers (497 miles) long and 150 to 250 kilometers (93 to 155 miles) wide, respectively, is Nepal's Himalayan axis. Nepal has a land area of roughly 1,47,516 square kilometers. India borders Nepal on three sides and surrounded by China on the other. The constrained Siliguri Corridor in West Bengal divides Nepal from Bangladesh (Nepal Tourism Board, 2018). It spans the latitudes of 26º 20' to 30º 10' north and the longitudes of 80º 15' to 88º 19' east. Nepal's diverse biodiversity is a result of the country's unique geographic location, as well as variations in altitude and climate forms (Koyama *et al*., 2012).

Despite occupying only 0.1% of the earth's surface area, Nepal is home to 3.2% of the world's flora. Nepal is a biodiversity-rich country that ranks 27th globally, 10th in Asia, and 2nd in South Asia (Chaudhary *et al*., 2016). Approximately 9,000 different flowering plant species exist, Nepal is rated 9th among Asian countries in terms of floral wealth. A total of 6,653 flowering plant species have been identified. Catalogues state that Nepal is home to 1,792 to 2,331 valuable medicinal and aromatic plants. The country's biodiversity is a significant source of money (Parajuli *et al*., 2015).

It is believed that the existence of chemical elements known as phytochemicals, which are responsible for these plants' therapeutic potential. Plants generate phytochemicals, which are non-nutritive bioactive molecules that act as stress and pathogen defence agents (Rajendran *et al*., 2017). All plants produce chemical compounds that provide them a competitive edge in the evolutionary process, such as salicylic acid, a hormone used in plant defenses (Kunwar *et al*., 2013).

An organic chemical created by a living entity in the natural world is referred to as a "natural product." Natural products have a pharmacological action that can be applied to the treatment of many disorders (Chintoju *et al*., 2015). Natural products are the primary sources of bioactive compounds, and these substances have assisted in the development of important chemicals for treating human illnesses (Saraf *et al*., 2014).

Natural products, in the broadest sense, refer to everything created by life (Hanson, 2003). The chemistry of a natural product entails biosynthesis, extraction, identification, measurement, structural clarification, physical and chemical properties, and reactions. They produce either primary metabolism or secondary metabolism. Indirectly influencing normal growth, development, and reproduction are the principal metabolites, which include carbohydrates, proteins, lipids, and oils. However, secondary metabolites (SM) do not now participate in the growth, development, or reproduction of an organization; rather, they have an ecological function (Anulika *et al.*, 2016). To classify SM in plants, the biosynthetic pathway is frequently used. This suggests that there are three major categories of SM in plants: terpenes, phenolic compounds, and alkaloids (Mera *et al.*, 2019). The largest plant SM category, consisting of almost 40,000 molecules, is terpenes (Mendoza *et al*., 2018).

Alkaloids appear to be unique among the various groups of naturally occurring organic substances, comprising lipids, proteins, amino acids, carbohydrates and flavonoids (Kurek, 2019). Alkaloids, which are present a variety of plant species, have a number of distinct functions, including insecticidal, antiparasitic, antiplasmodial, anticorrosive, antioxidative, antibacterial, anti-HIV, and anti-HIV capabilities (Fattorusso *et al.*, 2007).

Chemicals known as phenolics connect one or more aromatic rings with at least one hydroxyl group (Akarsh *et al*., 2020). Several fruits, vegetables, and beverages include flavonoids, a type of natural substance that belongs to a class of secondary plant metabolites having a polyphenolic structure (Panche *et al.*, 2016). Flavonoids possess anti-inflammatory, antibacterial, anti-cancer, antiviral, antioxidant, hepatoprotective, and anti-inflammatory effects (Kumar *et al*., 2013).

Using methods based on their size, shape, and charge, compounds can be distinguished from mixtures. Using a stationary phase, such silica gel or Sephadex, with calcium sulfate as a binder, and a mobile phase, which is the extraction solvent, is necessary for chromatography. The chromatographic methods are used to separate substances by adsorption, partition, affinity, ion exchange, or size exclusion. There are several chromatographic techniques, including paper chromatography, thin layer

chromatography (TLC), column chromatography, liquid chromatography, gas chromatography, and High performance liquid chromatography (HPLC) (Abubakar & Haque, 2020). Spectroscopy is the method that is used most frequently to measure and identify organic molecules by interpreting the spectra and comparing them to reference spectra. A very comparable technology is the hyphenated system Gas Chromatography-Mass Spectroscopy (GC-MS) (Padma *et al*., 2019).

Gas chromatography-mass spectrometry (GC-MS) combines two methods to provide a single approach for studying organic chemical combinations. The capillary column used by the gas chromatograph depends on the phase characteristics and column dimensions. Examples of GC-MS applications include drug detection, fire investigation, environmental analysis, investigating explosives, and identifying unidentified compounds (Braddick, 2015).

1.2 Introduction of the Plant *Kalanchoe pinnata* (Lam.) Pers.

Kalanchoe pinnata (Lam.) Pers. (K. pinnata) (synonym: Bryophyllum pinnatum) is a member of the Crassulaceae family and is also referred to as "Ranakalli," "Miracle leaf," "Mexican Love plant," "Katakataka," "Cathedral Bells," "Air plant," "Life plant," "Goethe plant", and so on. It is also known as the "Mother of Thousand" when new plantlets begin to emerge from the leaf (Rajsekhar *et al.*, 2016).

K. pinnata is planted as an indoor ornamental plant. It, like most succulents, cannot withstand harsh frost and will not flourish in temperatures below 10°C (50°F). It favors soil that drains well because its roots are prone to rot if not. *K. pinnata* is grown in gardens in the tropics, where it can escape and become naturalized, frequently as an invasive weed (*Kalanchoe Pinnata - Wikipedia*, 2021).

K. pinnata is native to Madagascar that is popular as a houseplant and has grown naturalized in tropical and subtropical climates. It's a perennial plant that grows to around 1 m (39") tall with thick cylindrical stems and reddish-tinged new growth that blossoms for most of the year. Flowers are primarily produced in the winter and spring (Mule *et al*., 2020).

1.2.1 Classification of the plant

Kingdom	:	Plantae
Sub kingdom	:	Tracheobionta
Super division	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Rosidae
Order	:	Saxifragales
Family	:	Crassulaceae
Genus	:	*Kalanchoe*
Species	:	*pinnata*
Binomial name	:	*Kalanchoe pinnata*
Common name	:	Pattharchatta
Synonym	:	Bryophyllum pinnatum
Sanskrit name	:	Pashanabheda (Pattewar, 2012)

Figure 1: *Kalanchoe pinnata*

Figure 2: Leaves of *K. pinnata*

1.2.2 Traditional and medicinal use

The tradition of using those herbal plants for treatment purposes remains in the Himalayan kingdom of Nepal (da Silva *et al.*, 2021). Diseases have been on the rise since the beginning of time on this planet so that researchers face a difficult task in locating a protective mechanism. They are investigated, researched, and assessed. They are used in the fight against various deadly diseases. These plant-based substances are less dangerous and have negligible to no adverse effects (Rajsekhar *et al*., 2016).

It was roughly estimated that around 1,500 of the 2,000 medicines used in Nepal and India are from plants and the rest are from animals and minerals. Among the herbal medicines used traditionally, *K. pinnata* (pattharchatta) is the one.

K. pinnata has a long history of exhibiting a broad spectrum of pharmacological activity, including treating humankind's most serious illnesses. The plant's therapeutic and medical properties contain wound-healing, antioxidant, antiproliferative, antibacterial, antiviral, antiprotozoal, anti-allergic, analgesic, antileishmanial, anthelmintic, insecticidal, nephroprotective, hepatoprotective, gastroprotective, antidiabetic, antidepressant, sedative, antilithiatic, chemoprotective, antihistamine, antihypertensive and immunosuppressant activities (Rajsekhar *et al*., 2016).

Numerous phytochemicals, including flavonoids, glycosides, carotenoids, saponin, kaempferol, and alkaloids, are present in *K. pinnata*. Additionally, it has the capacity to dissolve calcium oxalate, the primary ingredient in the production of kidney and urinary system stones (Sharma, 2020). Leaves are lightly toasted and applied to wounds, bruises, blisters, deadly insect attacks and dysentery is treated with leaf juice. The leaves treats various ailments, including sore eyes, burns, and corns (Bodakhe *et al.*, 2013).

According to ethnomedicine, *K. pinnata* has anthroposophical and tocolytic effects on expectant mothers. It was used to facilitate the placenta's delivery during childbirth. After examining the numerous plant parts, scientists were able to successfully determine the medicinal potential of the plant and its sections (Rajsekhar *et al.*, 2016).

1.2.3 Chemical constituents of *Kalanchoe pinnata*

K. pinnata (Lam.) Pers. has anticancer, antidiabetic, insecticidal, antibacterial, antiurolithiatic, and other pharmacological activities. The leaves of the plant have traditionally utilized as ethnomedicine. Free radical-induced oxidative stress was linked to various illnesses (Singh *et al*., 2019).

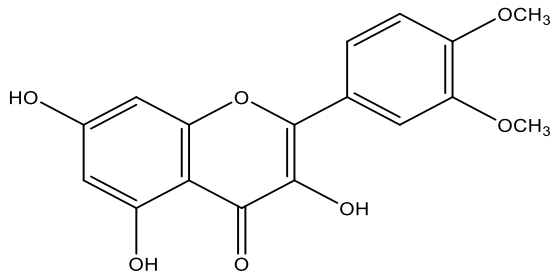
Alkaloids, triterpenes, glycosides, flavonoids, polyphenols, tannins, cardenolides, steroidal glycosides, bufadienolides, and lipids are abundant in *K. pinnata*. Bufadienolides, cardiac glycosides, a group of extremely active substances are present in the leaves. Bufadienolides have antibacterial, antitumor, cancer-preventive, and insecticidal properties and are structurally and functionally comparable to two additional cardiac glycosides, digoxin and digitoxin. These can cause cardiac toxicity in animals, especially those that graze (Pattewar, 2012).

Phenols, Phenylpropanoids and Flavonoids: p-hydroxycinnamic acid, para coumaric acid, ferulic acid, protocatechuic acid, phosphoenolpyruvate, astragalin, syringic acid, 4-hydroxy-3-methoxycinnamic acid, 4-hydroxybenzoic acid, 4,5,7-trihydroxy-3,8-dimethoxyflavone, luteolin, kaempferol, epigallocatechin-3-osyringate, quercetin, quercetin-3L-rhamonsido-L-arabinofuranoside, and quercetin-3-Odiarabinoside are found in the plant (Pattewar, 2012).

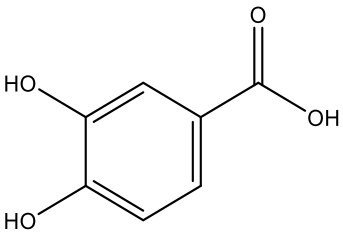
Triterpenoids and Steroids: α-amyrin, α-amyrinacetate, β-amyrin, β-amyrinacetate, pseudo taraxasterol, 18-oleanane, taraxerol, Ψ-taraxasterol, , friedelin and gluten is also found in the plant (Pattewar, 2012).

Fatty Acids, Minerals and Others: The fatty acid fraction is composed mainly of palmitic acid (89.3%), stearic acid (10.7%), and small amounts of arachidic and behenic acid. The plant also contains HCN, succinic acid, malic acid, oxalic acid, citric acid, and isocitric acid. There are plenty of vitamins and amino acids in this plant (Pattewar, 2012).
Some major compounds found in the *Genus* are shown below.

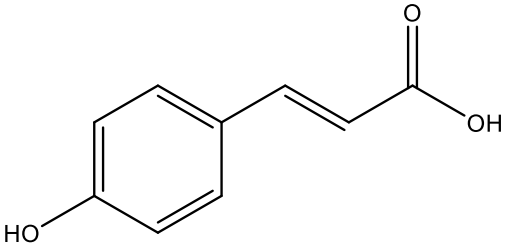
Flavonoids and polyphenols



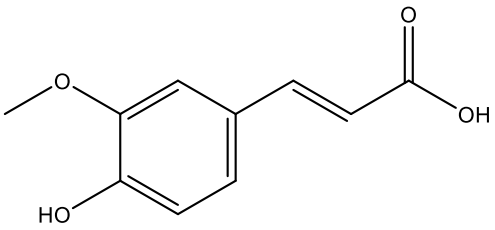
3',4'-dimethoxy quercetin



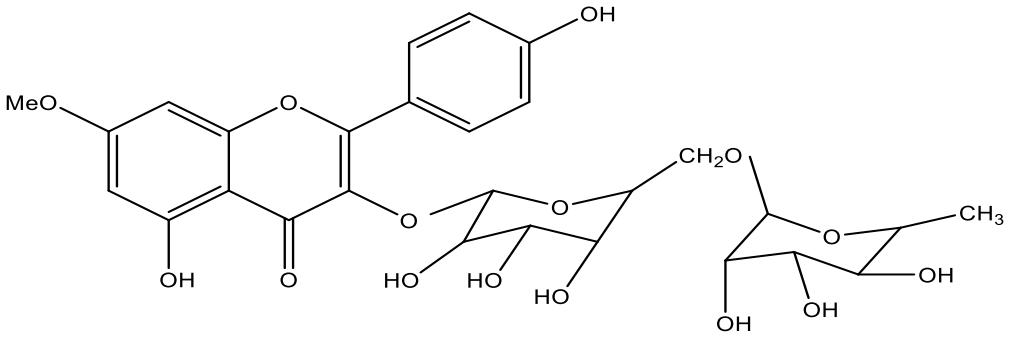
protocatechuic acid



p- coumaric acid

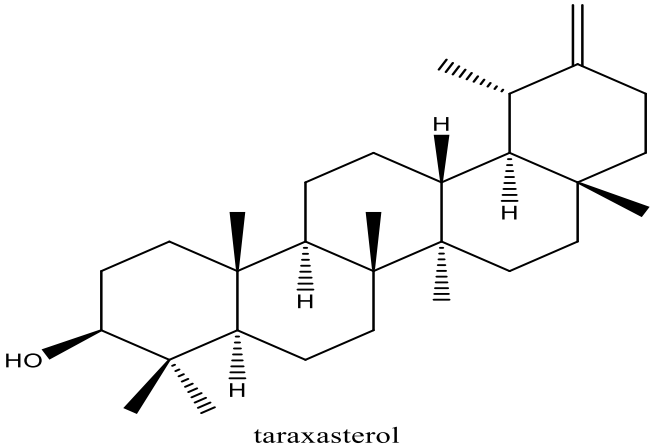


Ferulic acid

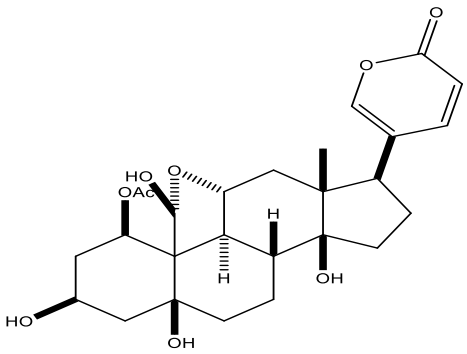


7-O-Methylkaempferol-3-O-α-L-rhamnopyranosyl-(1→6)-O-β-D-galactopyranoside

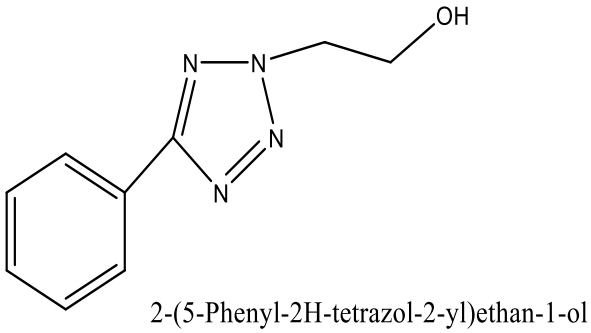
Triterpenoids and steroids



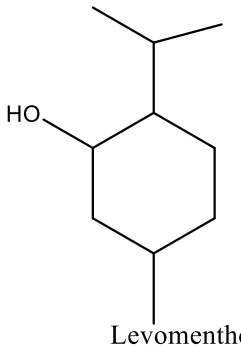
taraxasterol



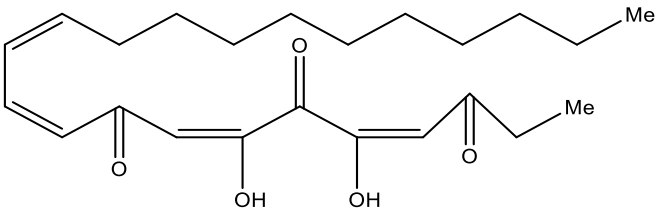
Bryophyllin



2-(5-Phenyl-2H-tetrazol-2-yl)ethan-1-ol



Levomenthol



Bryophollenone

1.3 Extraction Process

Extraction is a method of isolating organic components from mixtures. Solvent extraction is a technique for separating chemicals in two immiscible liquids based on their relative solubilities. To evaluate the biological activity of secondary metabolites, utilize natural products as herbal remedies, or isolate a known mixture of components, natural product extraction is necessary (Patel *et al.*, 2019). The following are some standard extraction techniques:

- Maceration: Maceration is a cold extraction technique and isocratic extraction technique. By submerging the plant sample in a specific solvent, this process entails the extraction of constituent elements. The extraction of thermo-moisturizing chemicals is possible with it (Patel *et al.*, 2019).

- Percolation: The tool utilized in this procedure is the percolator. It is a glass vase with apertures on both ends in the form of a narrow cone. A dried, pulverized, and finely powdered plant material was soaked with the extraction solvent in a clean container. The mixture is held for four hours after a more significant amount of solvent has been added. Then, the lower end of a percolator was sealed, and the

contents are transferred there to stand for 24 hours. The extraction solvent is then poured until the entire drug substance soaked from the top. The lower part of the percolator is then opened, letting the liquid drip gradually. The extraction is carried out by gravity force, which forces the solvent downward through the plant material, while a constant amount of solvent is introduced. The addition of solvent is stopped when it reached a volume equal to 75% of the total intended amount of the preparations. The extract is separated by using decantation and filtering methods. The marc is then express, and the last bit of solvent is added to get the needed volume (Abubakar *et al*., 2020; Patel *et al*., 2019; Poojar *et al*., 2017).

- Hot continuous extraction (Soxhlet): Using a Soxhlet extractor, a component can be continually taken out of a solid mixture. From the larger side-arm, the boiling solvent rises. An ingredient in a solid mix dissolve when a condensed drop of solvent enters the porous cup. A siphoning motion takes place as the smaller side-arm fills to the point of overflowing. The siphoning of the solvent containing the dissolved component into the boiler beneath the leftover solvent causes the cycle to repeat. The remaining solvent drains out of the porous cup as more solvent droplets enter it (Patel *et al*., 2019).

- Decoction: This continuous hot extraction technique employs a particular amount of water as a solvent. Plant material that has been dried, ground, and powdered are placed inside a clean container. Water is then added and mixed after that. Heat is then applied throughout the extraction to quicken the process. The entire process takes only a few minutes—typically, 15 minutes. The solvent ratio to crude medicine is typically 4:1 or 16:1. Plant material that is both water-soluble, and heat-stable is extracted using it (Poojar *et al*., 2017).

- Ultrasonic extraction (Sonication): Ultrasound-assisted extraction, when used as a source of natural chemicals, is an exciting method for acquiring high-value compounds that could help to make some food byproducts more valuable. The primary benefits will be increased extraction effectiveness, which will reduce energy consumption, and the use of moderate temperatures, which will benefit substances that are sensitive to heat. The applied ultrasonic power, frequency,

extraction temperature, reactor characteristics, and solvent-sample interaction are the most crucial process variables to consider. This method uses ultrasound with frequencies between 20 kHz and 200 kHz to generate cavitation by making cell barriers more permeable. Its utilization isn't widely used because of the higher prices (Esclapez *et al*., 2011).

Multiple techniques are used to pinpoint a substance taken from medicinal plant extracts. In addition to the complete structural elucidation, it comprised the identification of functional groups, the presence of several bonds and rings, the positioning of hydrogen and carbon, and the detection of multiple bonds. Mass spectroscopy (MS), ultraviolet spectroscopy (UV), nuclear magnetic resonance (NMR), and infrared spectroscopy (IR) are a few of the techniques that have been used (Abubakar *et al*., 2020).

1.4 Phytochemicals

Plants generate phytochemicals, which are non-nutritive bioactive molecules that act as stress and pathogen defense agents. These compounds are often used by plants to protect themselves from microbes, insects, and herbivores. Based on their metabolic origin, phytochemicals are divided into a wide variety of classes. Plants contain a variety of chemicals, including phenols, alkaloids, steroids, terpenes, saponins, and others. Examining medicinal plants with a folkloric reputation in great detail is essential to promote the proper use of herbal medicine and unveil their potential as a source of novel medications (Gul *et al*., 2017).

1.5 Separation of Compounds

Various separation procedures, including filtering, separating funnels, sublimation, simple distillation, and chromatography techniques can be used to separate mixtures. All these techniques are physical.

1.5.1 Thin Layer Chromatography

Adsorption chromatography using a solid-liquid interface is known as thin-layer chromatography. A solid adsorbent substance put on glass plates serves as the

stationary phase in this method. The procedure is done on a sheet of glass, plastic, or aluminum foil that has been gently coated with an adsorbent material, typically silica gel, aluminum oxide (alumina), or cellulose. A solid (stationary phase) and a liquid serve as the two phases in this solid-liquid technique (moving phase). The stationary phase allows the mobile phase to migrate upward via capillary action. If the molecules in the sample are colorless, fluorescence, radioactivity, or a specific chemical compound can be utilized to create a visible colored reactive product, such as ninhydrin or black-light visualization, to identify their positions on the chromatogram (Rehman, 2019).

TLC is one of the simplest, quickest, and least expensive chromatographic procedures. It's excellent for biochemical analysis like separating or isolating biochemical metabolites, as well as identifying natural compounds like essential oils and alkaloids.

1.5.2 Column Chromatography

Column chromatography, which is comparable to adsorption chromatography, is a typical chromatographic technique for separating the important elements from a combination. Both large-scale and small-scale component separation and purification can be done using this method (Rehman, 2019). When manufacturing or isolating new compounds, column chromatography is instrumental because very little information about the drug and its physical properties is required before the purification procedure. This technique is commonly used by the pharmaceutical industry to purify chemicals during the early stages of medication development (Silverman *et al*., 2015).

1.6 Biological Study of the Compound

Many different plant species produce secondary metabolites, which are what give the plant its natural action. Current drug development practices place a strong emphasis on natural ingredients while also adhering to accepted medical practices.

1.6.1 Antimicrobial Activity

A substance known as an antimicrobial stop or eliminates the growth of bacteria. The germs that antimicrobial medications are most effective against are used to categorize

them. Antibiotics are used to treat bacteria, whilst antifungals are used to treat fungi. In accordance with their intended uses, they can also be categorized into groups. Multiple drug resistance has made it difficult to generate new, synthetic antimicrobial drugs, prompting the search for ground-breaking antimicrobials derived from botanical sources. As a result of these reasons, current research focuses on screening natural compounds discovered in medicinally essential plants to produce new and effective medications to treat microbial diseases and infections (Hassan *et al*., 2015).

1.6.2 Antioxidant Activity

Antioxidants are " those substances which, when present in small amounts compared to the oxidizable substrate, considerably slow down or prevent oxidation." Antioxidants are significant nutraceuticals because of their numerous health advantages, and they are frequently used in the food business as lipid peroxidation inhibitors. Vegetables, fruits, seeds, forests, barks, roots, leaf spices, and herbs have all been investigated as potential antioxidant sources (Cutrim *et al*., 2019). Reactive oxygen species (ROSs) play a crucible part in degenerative diseases like ageing, cancer, cardiovascular disease, cataracts, degenerative neuron disorders, liver disease, and inflammation. When the body's ROS and antioxidant levels are out of equilibrium, free radicals form. As a result, consuming antioxidant as part of a balanced diet is vital and essential for reducing pathological disorders caused by free radicals (S. Chandra Mohan, 2012).

1.6.2.1 Principle of DPPH assay

The electron-transfer-dependent antioxidant assay known as the DPPH free-radical technique. that produces a violet solution in alcohol that transforms into a colorless solution due to the presence of an antioxidant molecule. It is a stable free radical, avoiding dimerization, like how most other free radicals operate. In the methanol solution, the delocalization results in a deep violet hue, which is identified by an absorption band centred at about 517 nm. Due to the presence of pecryl residue, when a DPPH solution and a substance that could donate a hydrogen atom are combined, the product’s shape decreases, and the violet color transitions to a pale color at the end (Molyneux, 2004).

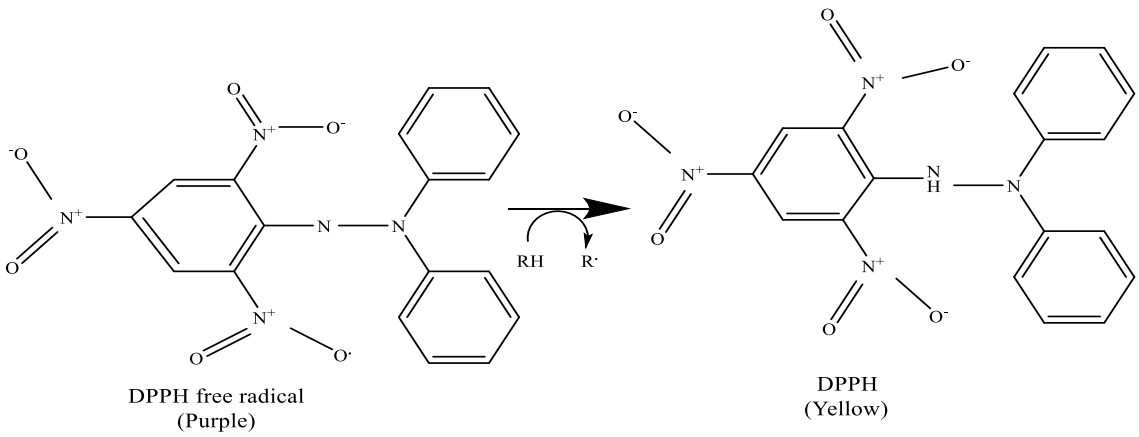


Figure 3: Mechanism of DPPH radical scavenging

Ascorbic acid, referred to as vitamin C, is a natural antioxidant with the chemical formula $C_6H_8O_6$ and a molecular mass of 176.12. It is a standard in the DPPH radical scavenging method.

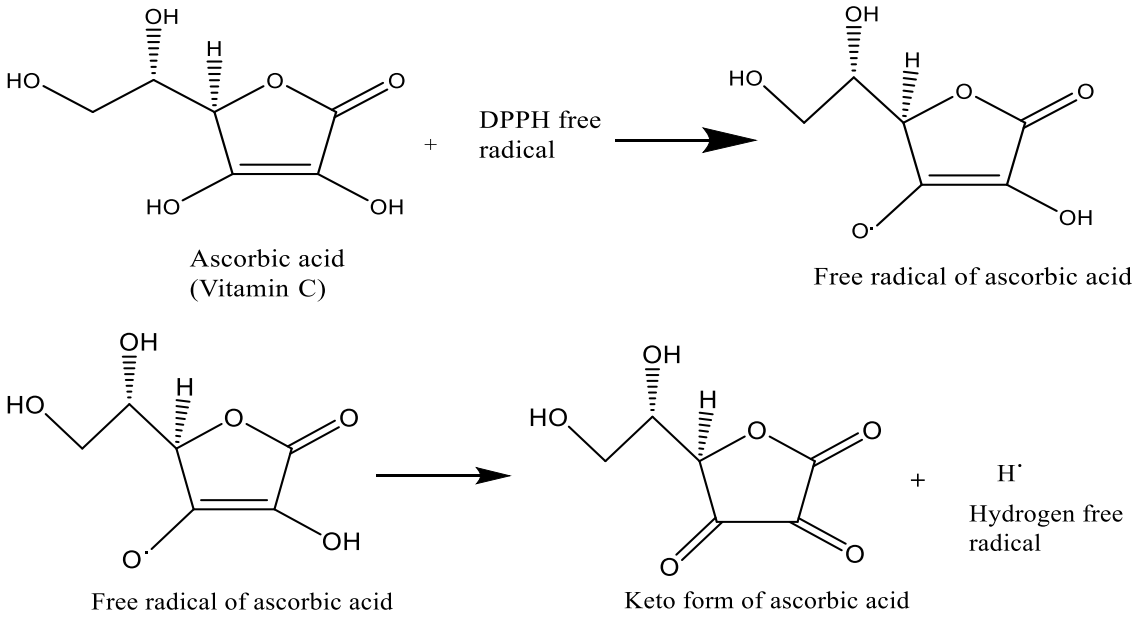


Figure 4: Mechanism of DPPH radical scavenging by ascorbic acid

1.6.3 Polyphenols and Flavonoids

Plant phenolics and flavonoids have a strong biological action, highlighting the need to identify them. Phenols are plant compounds with an aromatic ring attached to one or more hydroxyl groups. They have a variety of biological properties, including antioxidant, antimutagenic, and carcinogenic characteristics, as well as the ability to change gene expression. Similarly, flavonoids which are present in both their free state and their glycoside form in diverse plant sections, are the most prevalent class of

naturally occurring phenolic compounds. Because of their polyphenolic composition, they can remove dangerous free radicals including hydroxyl and superoxide radicals. Polyphenolic substances are more powerful antioxidants than monophenols (Balachandran *et al*., 2012).

1.6.4 Brine Shrimp Lethality Assay

The Brine Shrimp Lethality Assay is frequently used to determine whether a substance is cytotoxic or not. This test is simple, affordable, and effective. The nauplii are approximately 22 mm long, big enough to be seen without a magnifying glass, yet tiny enough to hatch in huge numbers without taking up a lot of laboratory space. This is a quick and thorough test for the bioactive component, whether it is natural or synthesized. Many organisms for statistical validation, needs no specialized equipment, and only needs a small sample size (2-20 mg or less) (Phulpoto *et al*., 2017).

1.7 Objectives

1.7.1 General Objective

- To find out the phytoconstituents and medicinal value of *Kalanchoe pinnata.*

1.7.2 Specific Objectives

- To do the extraction (cold percolation and ultrasonic extraction) with different solvents like hexane, DCM, chloroform, ethyl acetate, acetone, methanol, and water.
- To carry out phytochemical screening, GC-MS, TPC and TFC on the extracts.
- To study the bioactivity of the extracts like antimicrobial, antioxidant, antidiabetic, and cytotoxicity.
- To isolate the pure compounds using column chromatography.
- To perform UV and FTIR spectroscopy.

CHAPTER II: LITERATURE REVIEW AND RESEARCH GAP

2.1 History

The sharply divided leaf blades are called pinnatus in Latin, which means "winged," "finned," or "feathered." The discovery of *Kalanchoe* is addressed, as well as the taxonomic history of the genus in southern Africa. Regularly rained-on *kalanchoe* species do not completely lose their leaves during the dry, chilly winter months; just the bottom leaves dry up and separate. Their persistent succulent leaves offer an extra fire-fighting tactic because they are occasionally exposed to flames in their native settings throughout the winter (Pertuit, 1992).

The leaves are deadly to insects and bitter, according to Ayurveda. The bark is poisonous and bitter in addition to being tonic, alexipharmic, bowel-astringent, analgesic, and carminative. It is also helpful for inflammations, diarrhoea, vomiting, and snakebites and scorpion stings (Majaz, 2011).

The organic extract of *K. pinnata* (methanol, chloroform, petroleum ether, acetone, ethyl acetate) was prepared and antimicrobial activity was studied by agar gel diffusion method against human pathogens such *S. mutans*, *S. aureus*, and *E. coli*. Compared to other extracts, the methanol extract moved on pathogens over a wider range (Rashmi *et al.*, 2012).

2.2 Extraction and isolation process used in *K. pinnata*

K. pinnata is regarded as a panacea by the native populations of the Amazon; the creoles treat cancer and inflammation with the lightly roasted leaves, and a leaf infusion is a common treatment for fever. The root can also be infused to treat epilepsy. Alkaloids, flavonoids, sugars, saponins, triterpenes, phytosterols, tannins, glycosides, protein, amino acids, and phenolic chemicals were found in the phytochemical examination of the plant's ethanolic and aqueous extracts (Matthew *et al*., 2013).

IIT, Powai, Mumbai, did a methanolic extraction of fresh *K. pinnata* leaves and supplied GC-MS analysis results. The only three peaks in the GC-MS spectra that can be used to identify possible components from fresh leaves of *K. pinnata* are as follows: Squalene, dimethyl-5-oxotetrahydrofuran-2,3-dicarboxylic acid, and phytol. The other two phytocomponents' actions remained unknown. It has been demonstrated that squalene possesses anti-cancer, antioxidant, and other effects. Phytol and squalene are the primary bioactive components in the methanolic extract of *K. pinnata*, according to the findings (Phatak, 2015).

GC-MS analysis was used to separate bio-compounds with anti-inflammatory potential from *K. pinnata* methanolic leaf extract. Their anti-inflammatory potential was tested using assays for heat- and hypotonicity-induced hemolysis, antiproteinase activity, and inhibition of albumin denaturation. The crude contains aldehydes, ketones, and carboxylic acid, according to GC-MS analysis. The half-maximum inhibitory concentration (IC_{50}) of methanol crude leaf extract was discovered to be 100 μg/mL using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) test. For the treatment of inflammatory illnesses, phyto-compounds from *K. pinnata*'s crude leaf extract in methanol can be used because of their anti-inflammatory characteristics. Six chemicals with superior drug-relevant characteristics are available for molecular docking studies against a common inflammatory molecular target. COX-2 molecule (Agarwal *et al*., 2019).

Leaves were washed with antibacterial soap and water and dried by lyophilization (-20°C, 50 bar for 14 h). After drying, leaves were crushed using a laboratory knife mill and kept in the dark. Extracts of the dried leaf powder were produced by infusion (I) or decoction (D) at a ratio of 1:10 (w/v) using water: ethanol (70:30) as the extraction solvent. HPLC analysis of the extracts produced by decoction (LAED, lyophilized-water: ethanol decoction) and infusion was used to test the ability to inhibit the activity of α-amylase (LAEI, lyophilized-water: ethanol-infusion). By proving the acute phase hypoglycemic effect of K. pinnata extracts in rats, this study backs the use of *K. pinnata* as a natural remedy (Agüero-Hernández *et al.*, 2020).

Fresh *K. pinnata* (*Bryophyllum pinnatum*) leaves were collected from the Delhi Technological University campus, cleaned, dried in the shade, and crushed to produce 2 g of *K. pinnata* powder in 50 mL of water. The solution was filtered using Whatman filter paper and stored in a cold, dry location after boiling at 60°C for 15 minutes. *K. pinnata*, a brand-new herbal plant, was utilized in the environmentally friendly synthesis of silver nanoparticles (AgNPs). AgNPs displayed antibacterial action against the gram-negative *E. coli* bacterium as well as photocatalytic activity in the breakdown of rhodamine B dye (Aryan *et al.*, 2021).

2.3 Biological activities

7-O-methylkaempferol-3-O-L-rhamno-pyranosyl-(16)-O-D-galactopyranoside, a novel flavonol glycoside, and kaempferol-14-O-L-rhamnopyranosyl-(13)-O-D-galactopyranoside-O-α-L-rhamnopyranosyl-(1→6) are yielded by methanolic fraction of *K. pinnata* leaves. Chemical methods and 1D and 2D NMR spectroscopy are used to discover their structures (Bodakhe *et al*., 2013).

K. pinnata leaf extract in methanol was shown to have a flavonol compound. For fractionating the methanol extract, ethyl acetate is utilized. The pure ethyl acetate fraction is produced by column chromatography using ethyl acetate: n-hexane as the mobile phase (A). Compound A is identified as 3',4'-dimethoxy quercetin following additional analysis using 1D and 2D nuclear magnetic resonance (NMR) and mass spectrometry (LC-MS) (Darmawan *et al.*, 2013).

Using column chromatography, an extract from *K. pinnata* and its components, quercetin and gallic acid, can be utilized as a supplement to treat HCV infection. The isolated compounds are assessed for their anti-HCV activity and cytotoxicity using nuclear magnetic resonance spectroscopy and liquid chromatography-mass spectrometry, and their structural features are established (Aoki *et al*., 2014).

DCM and methanol extracts of leaves of *K. pinnata* have antibacterial, antifungal, and phytotoxic *in vitro*. The antibacterial activity of the plant leaves is assessed against the fungi *Epidermophyton floccosum*, *Trichophyton mentagrophytes*, *T. rubrum*, and *T. simii*, as well as the bacteria *Bacillus subtilis* (PTCC 1207), *Bacillus cereus* (PTCC

1247), *Staphylococcus epidermidis* (PTCC 1114) and *Escherichia coli* (PTCC 1047). The reference chemical was amoxicillin 10 mg/mL. DCM and methanol leaf extracts displayed smaller zone of inhibition than the usual medication. *K. pinnata* leaf extracts in DCM and methanol may contain trace amounts of antibacterial and antifungal activity (Kamal *et al*., 2014).

Silver nanoparticles have the desired physicochemical features in terms of optical, structural, thermal, and photocatalytic qualities. The use of a green approach to generate silver nanoparticles, as described here, suggests that it may be applied to the production of other metallic particles (Sharad *et al*., 2014).

Folins Ciocalteau reagent method and Aluminium chloride method are used for quantitative analysis of phenolic and flavonoids, respectively. The antioxidant activity of the leaf's ethanolic extract is assessed in vitro using traditional methods and the DPPH assay technique. A phytochemical investigation reveals the existence of phenols, flavonoids, tannins, saponins, and alkaloids (Singh *et al*., 2019).

The crude extracts are evaluated using HPLC-UV-MS. Thus, along with other flavonoids and organic acids, the extracts contain quercetin-3-O-L-arabinopyranosyl-(12)-L-rhamnopyranoside, kaempferol-3-O-L-arabinopyranosyl-(12)-L-rhamnopyranoside, quercitrin, and trans-p-coumaryl glutaric acid. These crude extracts are also evaluated in non-tumorigenic human foreskin fibroblasts, with IC_{50} values ranging from 79.5 to 90.2 g/mL (Pereira *et al*., 2018).

Investigations on the radical scavenging ability, tannin content, flavonoid and phenolic content, FTIR, and UV-visible absorption of a natural dye made from *K. pinnata* leaf powder are used to evaluate the dye. The study's findings suggest that the *K. pinnata* leaf extract obtained has high anti-oxidant qualities, including 84.92% radical scavenging activity, good coloring, a fondness for textile materials, and functional qualities, including a significant tannin, flavonoid, and phenolic component content (Rani *et al*., 2020).

In anthroposophic hospitals, *K. pinnata* has been used to treat premature labor, and more recently, as an auxiliary drug in traditional settings. In vitro, oxytocin, a hormone known to be involved in labor, causes an increase in intracellular free calcium concentration. Leaf press juice reduces this effect. Their goal is to pinpoint the elements or substances in *B. pinnatum* press juice that contribute to this inhibitory effect and analyze how they affect oxytocin-driven MAPK cascade activation. On the other hand, a bufadienolide and a flavonoid-rich fraction combined were equally effective and had a synergistic impact as *B. pinnatum* press juice. Similar effects were seen on oxytocin-induced signaling pathways by the bufadienolide-enriched portion of this pressed juice and the oxytocin-receptor antagonist and tocolytic medication atosiban (Santos *et al*., 2021).

Due to all these factors, quercetin has its pharmacological effects thoroughly researched, but its other phytoconstituents have not received the same level of attention. Determining its medicinal, pharmacological, and other helpful characteristics for humanity is crucial. The current study's main objective was to characterize various phytochemical elements and the biological activity of various *K. pinnata* preparations.

CHAPTER III: MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Chemicals Required

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

3.1.2 Instrument and Equipment

- Rotavapour (IKA, RV 10 D S96)
- Sonicator, Refrigerator
- UV lamp (UV 2510TS)
- Heating Bath, Digital weighing balance, Hot air Oven, Electric grinders
- Measuring cylinders, beakers, conical flasks, test tubes, burettes, micropipettes, pipettes, thermometers, water baths, and vial tubes.
- Double beam UV-Vis spectrophotometer (Labtronics LT-2802)
- FTIR (PerkinElmer Spectrum IR; Version 10.6.2)
- GC-MS

3.2 Methods

3.2.1 Sample Collection and Identification of Plant Materials

The Department of Botany, Amrit Campus, helped to certify the plant after collecting it from the Rupandehi district to represent the entire species.

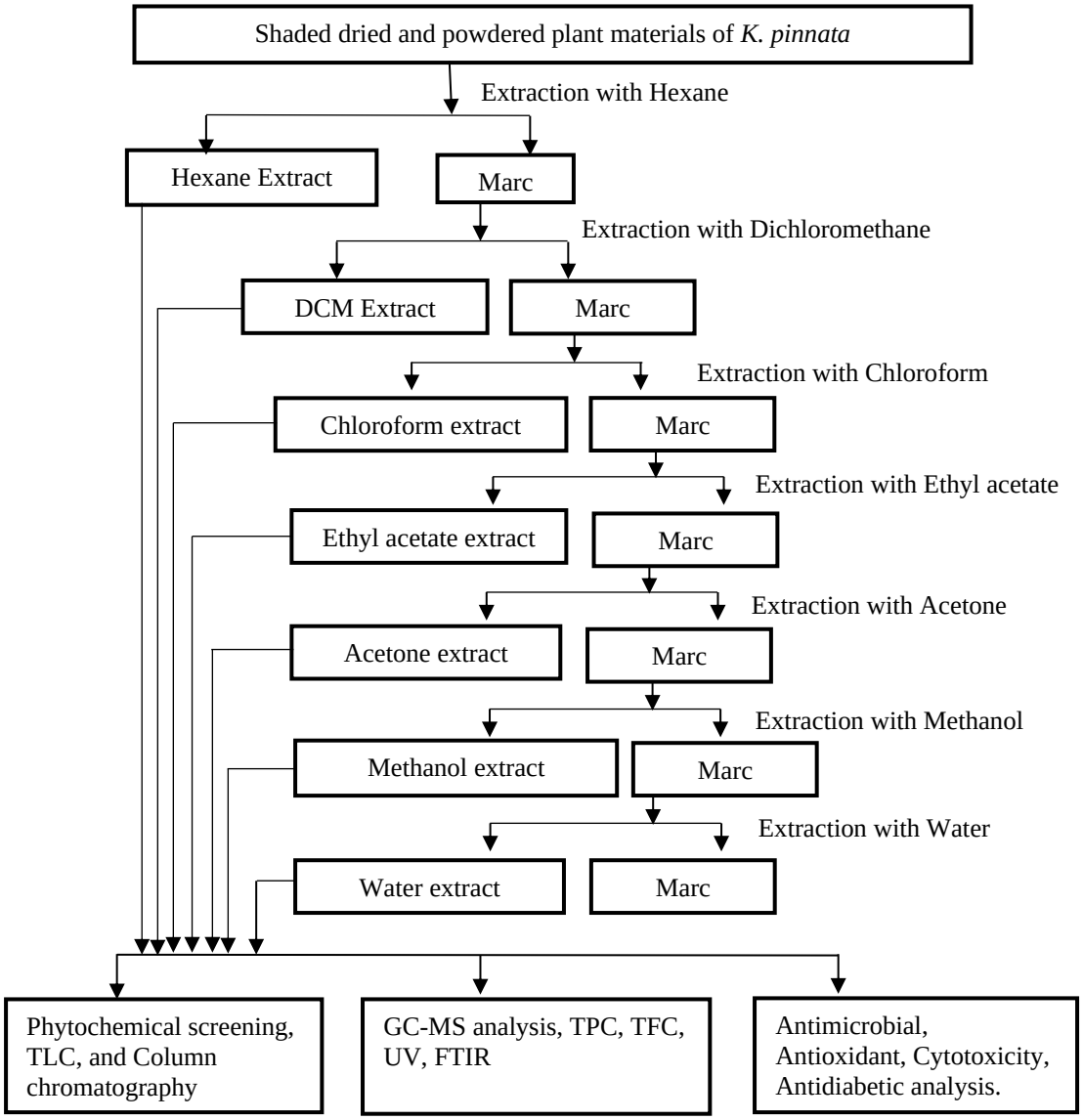
3.2.2 Sample Preparation

Locally, over 4 kg of the plant's leaves were collected, cleaned with water, and allowed to air dry under cover for a few days at room temperature. The plant leaves were shade-dried, ground into a fine powder using an electric grinder, weighed (almost 400 g), and stored at a low temperature until use.

3.2.3 Extraction Process

Among various extraction processes, the ultrasonic extraction process was performed.

In this procedure, 400 g of powdered plant was stored in a sterile, dried beaker. Hexane was placed into the beaker, well stirred, and kept in an ultrasonic cleaner tub that measured 30×15×20 cm. Distilled water was used to fill the tub to about one-third of its capacity. The total power was 350 W modules, operating at a frequency of 40 kHz. The extracted beaker was decanted, cooled to room temperature, and then filtered. A Rota evaporator was used to concentrate the filtrate. The concentrated extract was measured, dried, and stored for later use in an airtight vials tube. Like this, hexane was added to the filtered residue before it was sonicated. For three batches, the same procedure was used to obtain the crude extract. Different solvents were generated using the same procedure, including DCM, chloroform, ethyl acetate, acetone, methanol, and water extracts. The following scheme diagram illustrates the method.



Scheme 1: Research process

3.2.4 Gas Chromatography-Mass Spectroscopy

There are several potential uses for gas chromatography. The separation and analysis of multicomponent mixtures, such as solvents, hydrocarbons, and essential oils, is its major application, and it is also the one for which it is most suited *(*Al-Rubaye *et al*., 2017). Therefore, the second-most significant application areas include forensics, general trace analysis, and pollution research. Due to its ease of use, sensitivity, and effectiveness in isolating mixture components, it is a crucial tool in chemistry. The removal of impurities from compounds, the quantitative and qualitative analysis of mixtures, and the determination of thermochemical variables like solution and vaporization temperatures, vapour pressures, and activity coefficients are just a few advantages of this technique (Sermakkani *et al*., 2012).

3.2.4.1 GC-MS Analysis of Hexane and Methanol Extract

Hexane and methanol concentrated extract from the Rota evaporator was tested using GC-MS after being dissolved in chloroform. The Department of Plant Resources in Thapathali, Nepal conducted the GC-MS investigation.

3.2.5 Phytochemical Screening

The phytochemical analysis was done on various extracts. The procedure for screening phytochemicals was based on protocol (Banu *et al*., 2015). In essence, phytochemical screening aids in locating the bioactive substances that are found in plants. By observing the color reaction with different targeted reagents, it was determined that there are significant groups of natural components present in the diverse plant extracts. In Appendix A, the technique is described in detail.

3.2.6 Total Phenolic Content

Utilizing a colorimetric technique called Folin-Ciocalteu that is based on an oxidation-reduction process, the total phenolic content of the plant extract was ascertained. Gallic acid concentration is used as a benchmark (Balasundram *et al.*, 2006).

3.2.6.1 Preparation of Folin-Ciocalteau Reagent

1mL of the Folin-Ciocalteau reagent was dissolved in 10 mL distilled water, i.e. (1:10 dissolved in distilled water).

3.2.6.2 Preparation of standard gallic acid solution

A stock solution of gallic acid with a concentration of 1000 mg/mL (ppm) was made in the first step by dissolving 1 milligram of gallic acid in 1 mL of methanol. By repeatedly diluting the stock solution, several different concentrations of gallic acid were created. These concentrations included 500, 250, 150, 100, 50 and 25 μg/mL.

3.2.6.3 Construction of the calibration curve

At first, Gallic acid solution (20 μL) was poured into the test tubes. After that, put 100 μL (10%) of the Folin-Ciocalteu reagent (FCR) in a test tube and 20 μL of the extract let it sit there for 5 minutes at room temperature in complete darkness. The mixture was then mixed with 80 μL of an aqueous 7% Na_2CO_3 solution to create a final volume of 200 μL . The blue concoction was forcefully shaken and then allowed to sit in the dark for two hours. The reactant absorbance was determined at 765 nm and compared to a control (20 μL methanol instead of the extract + 100 μL F-C reagent + 80 μL sodium carbonate incubation for 2 hours).

Milligrams of gallic acid equivalent per gram of dry extract (mg GAE/g of dry extract) was used to represent the sample's total phenolic content based on the gallic acid calibration curve.

3.2.6.4 Preparation of the sample solution

First, a stock solution with an extract concentration of 1000 μg/mL (ppm) was made by dissolving 1 milligram of extract in 1 mL of methanol. Several different extract concentrations were created by repeatedly diluting the stock solution, and their absorbance values were tested using the same technique as for gallic acid.

3.2.6.5 Calculation of the total phenolic content (TPC)

The following equation was used to determine the sample's total phenolic content, which was represented as milligrams of gallic acid equivalent per gram.

$$C = cV / m \quad \text{……. (1)}$$

where,

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

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

V = Volume of the extract (mL)

m = Weight of the plant extract (μg)

For each concentration, the results were recorded as the mean of absorbance. From these figures, the linear correlation coefficient (R^2) value was determined. As shown below, the regression equation is:

$$y = mx + c \quad \text{……...(2)}$$

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's concentration. To calculate the substance's total phenolic content, equation (2) was created using the extract's concentration as an input.

3.2.7 Total Flavonoid Content

To quantify the total flavonoid content of the plant extract, an aluminum chloride colorimetric assay was performed (Chandra *et al*., 2014; Heim *et al*., 2002). Quercetin is used as the reference. The hydroxyl groups of flavonoids react with aluminum chloride in the sample created by this method to yield a chemical.

3.2.7.1 Preparation of the standard quercetin stock solution

1 mg of quercetin was dissolved in 1 mL of pure methanol to create the quercetin stock solution (stock concentration 1mg/mL). Then, serial dilution was done with the different concentration of 250 µg/mL, 150 µg/mL, 100 µg/mL, 50 µg/mL, and 25 µg/mL. A microplate well was filled with an aliquot of 100 µL of each concentration in methanol, and 100 µL of 2% aluminum chloride (in methanol) was then added for a total volume of 200 µL. The pink liquid was incubated in total darkness for 10 minutes. Finally, using a spectrophotometer and a blank solution (control) made up of 100 µL of solvent and 100 µL of 2% aluminum chloride, the absorbance was measured at 425 nm. The control and the sample are incubated simultaneously. To plot the calibration curve, the average absorbance values attained for various quercetin concentrations were employed. For the standard calibration curve, quercetin acted as a positive control. The amount of quercetin equivalent (mg QE) per gram of dried plant material was used to calculate the total flavonoid content.

3.2.7.2 Preparation of the sample solution

First, a stock solution with an extract concentration of 1000 µg/mL (ppm) was made by dissolving 1 mg of the extract in 1 mL of methanol. The stock solution was diluted multiple times to create various extract concentrations, and their absorbance values were evaluated using the same procedure as described above for quercetin.

3.2.7.3 Measurement of total flavonoid content (TFC)

It was feasible to calculate the quantity of flavonoids in the extract using the following equation:

$$C = cV/m \quad \ldots\ldots\ldots\ldots\ldots (3)$$

where,

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

c = Concentration of quercetin established from calibration curve in (µg/mL)

V = Volume of the extract (in mL)

m = Weight of the plant extract (in µg)

For each concentration, the findings were reported as the mean of absorbance. These values were used to compute the linear correlation coefficient (R^2) value. This is how the regression equation looks:

$$y = mx + c \text{ ……...(4)}$$

where,

y = Absorbance of the extract

m = Slope from the calibration curve

x = concentration of the extract

c = Intercept

Using this regression equation, the extract's concentration was estimated. As a result, equation (4) was created to calculate the substance's total flavonoid content using the extract's concentration as input.

3.2.8 Antioxidant Activity

An antioxidant assay that depends on electron transfer is the free-radical method of DPPH. It produces a violet solution in alcohol, but when an antioxidant molecule is present, the solution becomes colorless (Otohinoyi *et al*., 2014).

The following equation was used to determine the percentage of the DPPH free radical scavenging activity:

$$\text{Radical scavenging (\%)} = [(A_0 - A_S)/A_0] \times 100 \text{ ……. (7)}$$

where,

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

A_S = Absorbance of the test sample

The amount of an effective sample needed to neutralize 50% of DPPH free radicals is known as the IC_{50} (50% inhibitory concentration) value. By graphing the extract concentration vs. the corresponding scavenging action, the inhibition curve and IC_{50} values were generated.

3.2.8.1 Preparation of the 0.1 mM DPPH solution

394.32 g/mol is the molecular weight of DPPH. So, after carefully weighing 0.0019716 g of DPPH, dissolving it in methanol, and maintaining the volume at 50 mL, 0.1 mM solution of DPPH was made. Then, until use, this solution was kept in a dark place.

3.2.8.2 Preparation of (standard) ascorbic acid

10 milligrams of ascorbic acid were weighed and then diluted in 10 mL of methanol to get the stock solution of 1 mg/mL. Then, ascorbic acid solutions of 1000, 500, 250, and 125 μg/mL concentrations were successively diluted.

3.2.8.3 Preparation of sample solution

10 mg of the extracts (chloroform, ethyl acetate, and methanol) were weighed, and 10 mL of methanol were used to dilute them to make the stock solution of 1 mg/mL. Following that, extract solutions at concentrations of 1000 μg/mL, 500 μg/mL, 250 μg/mL, and 125 μg/mL were successively diluted.

3.2.8.4 Measurement of DPPH radical scavenging activity

To make a total of 200 μL, 50 μL of ascorbic acid from each concentration was pipetted into 150 μL of methanolic DPPH solution in a 96-well plate. Then, for 20 minutes, the solutions were kept in the dark. Then, using a spectrophotometer and methanol and DPPH as a blank, their absorbance value was determined at 520 nm.

The absorbance value for extracts and DPPH solution was also calculated, like how ascorbic acid was examined. Finally, the ascorbic acid and sample solution concentrations were plotted on the X-axis, the % of radical scavenging activity was plotted on the Y-axis, and IC_{50} values were computed.

3.2.9 Antimicrobial Activity

Plant extracts have the power to prevent the development of some harmful microbes. It has the capacity to prevent the growth of germs and can be assessed using antimicrobial activity (Kalemba *et al*., 2005). Using the paper disc diffusion method, microbial

growth inhibition was assessed, and the result was reported as a ZOI (Zone of Inhibition) value (*Download as PDF - Wikipedia*, 2022).

3.2.9.1 Collection of standard culture

Activated cultures of three standard strains of microbes were provided by Himalayan Research Centre, Koteshwor. The following three different types of microbes were included for the study of strain:
Gram-positive bacteria: *Bacillus subtilis* ATCC 6051
Gram-negative bacteria: *Escherichia coli* ATCC 8739
Fungi: *Candida albicans* ATCC 2091

3.2.9.2 Preparation of media

The creation of the study's media was done in compliance with the manufacturer's guidelines. These are the precise steps:

A. Nutrient agar

2.5 g of agar were dissolved in 100 mL of distilled water for this preparation, which was then autoclaved at 121°C for 45 minutes to sterilize it. It was allowed to cool to about 40°C before being distributed in the petri dish. After 20 minutes, the petri-dish was kept in the laminar flow to dry, and it became solid.

B. Nutrient broth media (Liquid Media)

It was created by dissolving 2 g of nutritious broth powder in 100 mL of distilled water. It was properly mixed to ensure proper dissolution before being sterilized in an autoclave for 45 minutes at 121°C and 15 lbs of pressure. Then it was permitted to cool.

3.2.9.3 Preparation of working solution

A sterile container was filled with 100 mL of DMSO solution and 15 mg of each crude extract. Until they were required, the vials were capped, sealed, and placed in the refrigerator (2-8 °C). The Kanamycin-based positive control was used. It was created by dissolving 5 milligrams of Kanamycin in 1000 mL of distilled water.

3.2.9.4 Screening and evaluation of antimicrobial activity

First, liquid nutrient broth media were used to cultivate the microbiological strains *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (ATCC 8739), and *Candida albicans* (ATCC 2081). Then, 100 µL of each strain's culture broth was plated onto a plate of nutritional agar and left there for 15 minutes at 37°C. After being placed on the nutrient agar plate, the bioplastic sample was incubated at 37°C for 15 minutes before being left overnight. The following day, the bioplastic's antibacterial effects on the microbiological strains *B. subtilis*, *E. coli*, and *C. albicans* were examined. The positive control used kanamycin, and the negative control used DMSO. After being observed on the plates, the ZOI created by plant extracts' antibacterial activity was evaluated using a scale.

3.2.10 α-Amylase (Anti-diabetic) Assay

Diabetes is a common and enduring illness, and the chronic hyperglycemia. It damages several body organs, tissues, and systems. It may have repercussions that have a significant negative influence on the patient's life, both physically and financially (Kasali *et al*., 2022). Numerous different substances have been found in the plant because of research into the phytochemistry. However, no any claims that these substances are effective antidiabetic substances (Nirmali Wickramaratne *et al*., 2016).

3.2.10.1 A general protocol for anti-diabetic assay

Using the 3,5-dinitrosalicylic acid (DNSA) technique, the experiment involving the inhibition of α-amylase was conducted. A minimum of 10% DMSO was used to dilute the *K. pinnata* plant extract. To create concentrations with a wide range, the sample and DMSO combination were once more mixed in buffer and NaCl at a pH of 6.9. In a 200 µL volume, the extract and α-amylase solution were combined. The mixture was then incubated at 30 °C for 10 minutes. The starch solution was then added in a 200 µL volume to each tube and set for 3 minutes. By adding 200 µL of DNSA reagent, the process was stopped. For 10 minutes, the combined sample was cooked in an 85–90°C water bath. The mixed sample was diluted with 5 mL of distilled water after reaching room temperature. A UV spectrophotometer was used to measure the absorbance at

540 nm in comparison to a blank solution. The plant extract was swapped out with 200 µL of buffer to create a blank with 100 % enzyme activity.

To calculate the % of inhibition of α-amylase inhibitory activity, the following equation was utilized. The extract concentration was plotted against the percentage of α-amylase inhibition, and from the graph, IC_{50} values were obtained.

$$\% \text{ α-amylase inhibition} = \frac{\text{Abs100\%control} - \text{AbsSample}}{\text{Abs100\%Control}} \times 100$$

3.2.11 Brine Shrimp Lethality Assay (BSLA)

The same protocols as BSLA were used during its execution (Ashfak *et al*., 2016; Abhijit, 2015). Based on a plant extract's capacity to kill lab-grown larvae, the BSLA is a useful method for determining its cytotoxicity. It is simple, inexpensive, and only requires a little quantity of test material (Sarah *et al*., 2017). For the crude extracts, it determines the $LC(\text{Lethal Concentration})_{50}$ values in terms of milligram per milliliter. It is thought that substances with LC_{50} values less than 1000 ppm (mg/mL) have the potential to have pharmacological activity (R. Hamidi *et al*., 2014).

3.2.11.1 General Procedure for Brine Shrimp Lethality Assay

Before usage, every piece of equipment utilized in the experiment was sterilized.

3.2.11.1.a Preparation of artificial sea water

The elements dissolved in one litre of distilled water to produce sea water are listed in the table below.

Table 1: Chemical composition of sea water

S.N.	Composition	Amount(g/L)
1	NaCl	23.5
2	Na_2SO_4	4
3	KCl	0.68
4	H_3BO_3	0.027
5	$Mg_2Cl_2.2H_2O$	10.68
6	$CaCl_2.2H_2O$	1.78
7	$NaHCO_3$	0.197
8	NaEDTA	0.0003

3.2.11.1.b Hatching of Brine Shrimp egg

Brine shrimp eggs weighing around 50 mg were then sprinkled over the simulated sea water that had been collected in the beaker after it had been covered with aluminum foil. A few tiny pores were made to enhance the material's ability to conduct heat and light. The beaker was then allowed to sit at room temperature for 48 hours while being exposed to the radiation from the 60-watt bulb.

3.2.11.1.c Preparation of samples

2 mg of the extract was weighed out, and 2 mL of DMSO (Dimethyl Sulfoxide) was added to it to create a stock solution with a 1000 ppm (mg/mL) concentration. By using the serial dilution procedure, we were able to produce solutions with concentrations of 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, and 0.0625 mg/mL, all derived from the initial stock solution. Three test tubes for each concentration received the 2 mL solutions from each of the 5 solutions. Like that, three test tubes each contained 2 mL of DMSO (as a blank). After they were labeled, the tubes were maintained for 24 hours.

3.2.11.1.d Calculation of assay

Calculations were made to determine the nauplii's lethality percentage for each concentration and control. To determine the percentage of mortality, count both the live and dead nauplii in each tube. The mortality percentage was then computed using the following equation.

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

3.2.12 Thin Layer Chromatography (TLC)

The foundation of chromatography is the idea that molecules in a solution can be separated from one another by depositing them on a surface or into a subsequently utilizing a mobile phase to move from a solid or liquid fixed phase (Coskun, 2016).

To identify the components, present in each extract, thin layer chromatography (TLC) is applied to all the extracts. Here, TLC was carried out on a 0.2 mm thick TLC

aluminium sheet. By changing the polarity of the solvents, the plates were produced in various solvent ratios. Table 2 displays the solvent system's concentration. With the aid of a UV fluorescent lamp, the plates were visualized.

Table 2: Concentration of solvent system of TLC

S.N.	Solvent system of TLC	% Ratio
1.	Acetone: Hexane	10%,15%,20%,25%
2.	Ethyl acetate: Hexane	10%,15%,20%,25%
3.	Methanol: Hexane	10%,15%,20%,25%
4.	Chloroform: Hexane	10%,15%,20%,25%
5.	Methanol: Chloroform	10%,15%,20%,25%
6.	DCM: Hexane	10%,15%,20%,25%

We are applying the solute as a spot allowed for the performance of a thin layer chromatographic investigation. The solvent was run after the TLC plate was placed in the beaker with enough of the solvent mentioned above. The chromatogram that has been created contains details on the various components in the mixture. The UV lamp allowed for observation of the produced chromatogram. The substance's properties, which show how it moves to the solvent in a particular chromatographic system, are determined by the constant known as the Rf value.

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

3.2.13 Column Chromatography

In the discipline of chemistry, a technique called column chromatography is employed to separate specific chemical compounds from mixtures dissolved in a liquid medium. It achieves this by using the differential adsorption of substances to the adsorbent as they move through the column at varying speeds, allowing the substances to be divided into fractions (Coskun, 2016).

The packing of the stationary phase and the employed solvent, hexane, was done in a cylindrical-shaped glass column that holds a stationary phase (silica gel). The sample, consisting of methanol extracted mixed with a little amount of silica gel, was poured from the top of the column as soon as it had been set up, after a small amount of sand had been added to the top of the silica gel. The sample was run with a solvent that had an Acetone: Hexane ratio of 1% before being collected. Following the collection of over 25mL of the mixture, the rotavapor was used to concentrate it. After collecting, TLC was completed and examined with a UV lamp. The solvent concentration increases from 1% to 2%, or close to 100%, as the sample moves across the TLC plate.

3.2.14 UV-Visible Spectroscopic Analysis

The Department of Chemistry, Amrit Campus, Kathmandu, used a Labtronics LT-2802 double beam ultraviolet-visible (UV-Vis) spectrometer to spectroscopically characterize the components recovered through column chromatography to determine their unsaturation and aromaticity. To record spectra, 1% and 5% (methanol extract) of Ac:Hex from column chromatography were used.

3.2.15 FT-IR Analysis

An effective tool for identifying the type of bonding in organic molecules, particularly the functional group, is the FTIR spectroscopic investigation. A (PerkinElmer Spectrum IR; Version 10.6.2) FTIR spectrometer was used to acquire the FTIR spectra of the single spot components produced from (1% and 5% (methanol extract) of Ac:Hex) column chromatography at the Amrit Campus in Kathmandu. FTIR spectra can be used to identify the functional groups, π-bond conjugate system, and aromatic and aliphatic structures that are present in the constituents.

CHAPTER IV: RESULTS AND DISCUSSION

4.1 Identification of Selected Plant

Prof. Dr. Bipana Devi Acharya, Department of Botany, Amrit Campus, helped to certify the plant, which was procured from the Rupandehi district of Province-5 to represent the entire species.

Table 3: Sample collection and identification

Identified plant	Common Name	Family	Place of Collection
Kalanchoe pinnata	Pattharchatta	Crassulaceae	Rupandehi district

4.2 Gram Yield

Table 4: Table showing g yield of various extract

Extract	Hexane	DCM	Chloroform	Ethyl acetate	Acetone	Methanol	Aqueous
Yield(g)	7.31	9.08	2.21	0.21	1.04	10.34	14.50

Using the ultrasonication method of extraction, the *K. pinnata* plant was extracted. Hexane, dichloromethane (DCM), chloroform, ethyl acetate, acetone, methanol, and aqueous extract each had a different % yield: 7.31 g, 9.08 g, 2.21 g, 0.21 g, 1.04 g, 10.34 g, and 14.50 g.

4.3 Qualitative Analysis of Phytochemicals

As shown in Table 5, the microchemical examination of a crude extract from the *K. pinnata* plant in several solvent systems revealed a group of phytochemicals. The phytochemical content of individual *K. pinnata* extracts was screened, and the presence of phytochemicals was identified by the appearance of colors.

Table 5: Phytochemical analysis of extracts of *K. pinnata*

S.N.	Class of phytochemicals	Hexane Extract	DCM Extract	Chloroform Extract	Ethylacetate Extract	Acetone Extract	Methanol Extract	Aqueous Extract
1	Volatile oils	+	+	+	+	+	+	-
2	Alkaloids	-	-	-	-	-	-	-
3	Carbohydrates	+	+	+	+	+	-	-
4	Phenolic compounds	-	-	-	+	+	+	-
5	Tannins	-	-	-	-	-	+	+
6	Flavonoids	-	-	+	+	+	+	+
7	Terpenoids	+	-	-	+	+	+	+
8	Quinones	-	-	-	-	+	+	+
9	Reducing sugar	-	-	+	-	-	-	-
10	Saponins	-	-	-	-	-	+	-

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

The findings indicated that the polar extracts contained most of the phytochemicals. Nearly all solvent extracts were found to include flavonoids and terpenoids. Since the extracts were prepared using an ultrasound sonicator and concentrated using a Rota-evaporator, it's possible that the absence of alkaloids may result from the decomposition of alkaloids caused by heat and molecular vibration.

The information found in the literature about this plant differs slightly from the results shown in the table above. Due to variations in plant altitude, various environmental conditions, the extraction method used, the time at which the sample was collected, lab setup and chemical grades, the result of a phytochemical screening for a given sample may differ from a screening of the same phytochemicals constituents.

4.4 GC-MS Spectra Analysis

Hexane and methanol extract of *K. pinnata* were used for the GC-MS analysis.

4.4.1 GC-MS spectra analysis of hexane extract

Seven primary components were identified by GC-MS analysis of the chemical make-up of the *K. pinnata* hexane extract. The list of significant compounds below is based on GC-MS analysis:

Table 6: Components based on GC-MS analysis of hexane extract

S.N.	Name of compound	Retention time	Molecular formula	Area%
1.	1-chloro-3-(4-phenyl-2H-1,2,3-triazol-2-yl) propan-2-ol	8.228	$C_{10}H_{11}ClN_4O$	11.13
2.	N-benzyl-2-(5-phenyl-2H-tetrazol-2-yl)acetamide	8.506	$C_{16}H_{15}N_5O$	3.90
3.	2-(5-Phenyl-2H-tetrazol-2-yl) ethan-1-ol	13.942	$C_9H_{10}N_4O$	27.44
4.	(4R,10aR, Z)-2,2-dimethyl-1,2,3,4,4a,5,6,9,10,10a-decahydro-1,4-epidiazenobenzo[8]annulene	15.677	$C_{13}H_{20}N_2$	11.98
5.	Pentadecafluorooctanoicacid, dodec-2-en-1-yl ester	24.668	$C_{20}H_{23}F_{15}O_2$	29.36
6.	Ethyl 4-methyloctanoate	26.96	$C_{11}H_{22}O_2$	10.99
7.	2-methyldodecane	28.00	$C_{13}H_{28}$	5.19

4.4.1.1 Mass spectral data of constituents identified by GC-MS

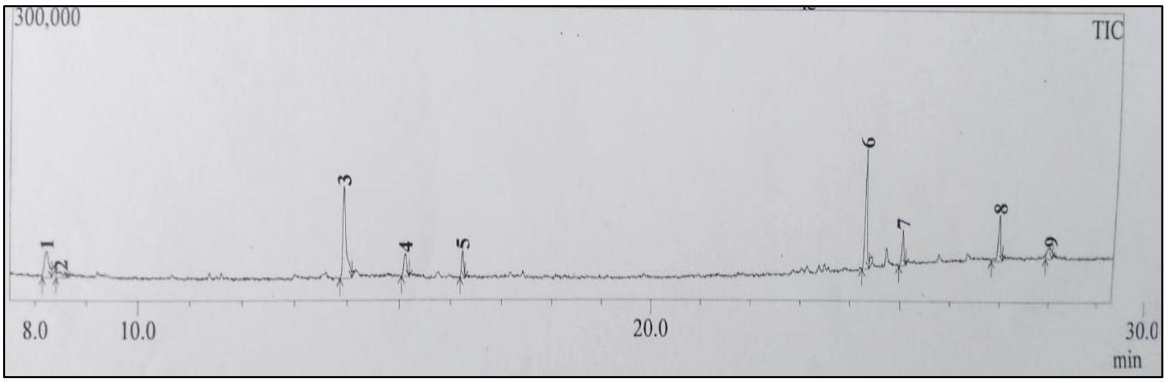


Figure 5: Chromatogram of hexane extract of *K. pinnata*

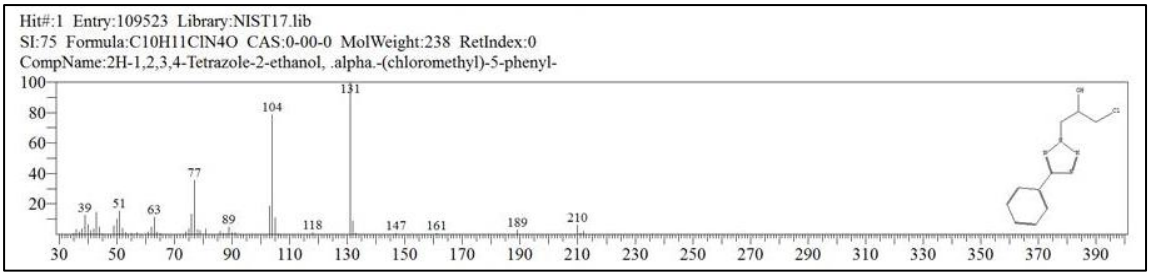


Figure 6: Chromatogram of 1-chloro-3-(4-phenyl-2H-1,2,3-triazol-2-yl) propan-2-ol

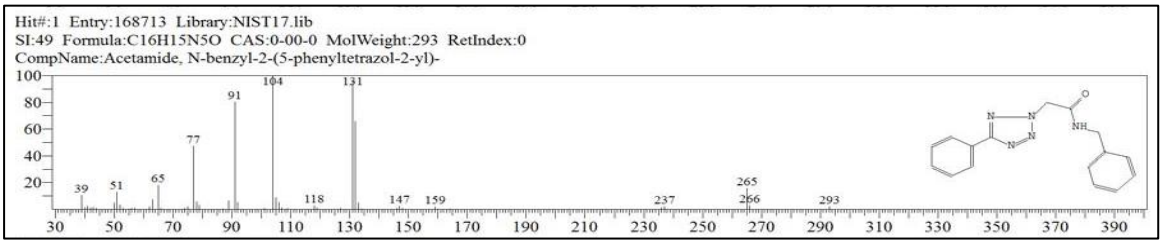


Figure 7: Chromatogram of N-benzyl-2-(5-phenyl-2H-tetrazol-2-yl)acetamide

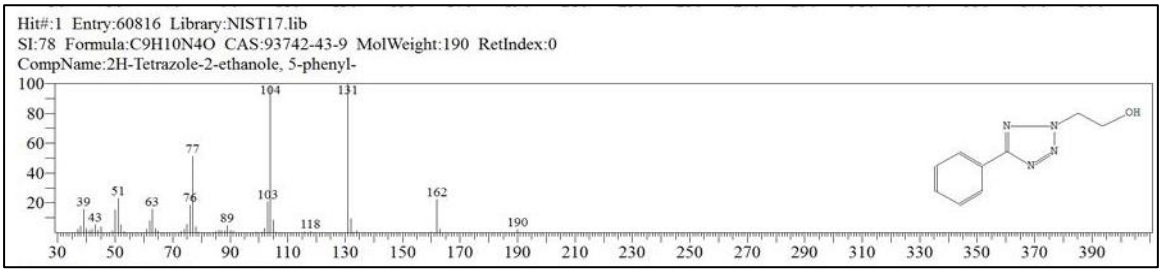


Figure 8: Chromatogram of 2-(5-Phenyl-2H-tetrazol-2-yl)ethan-1-ol

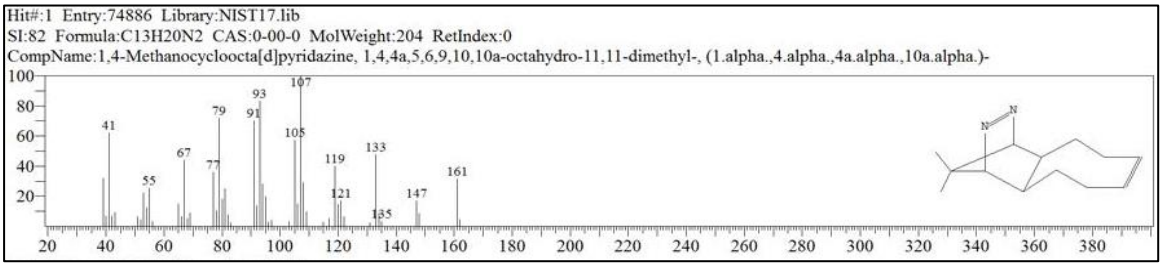


Figure 9: Chromatogram of (4R,10aR, Z)-2,2-dimethyl-1,2,3,4,4a,5,6,9,10,10a-decahydro-1,4-epidiazenobenzo[8]annulene

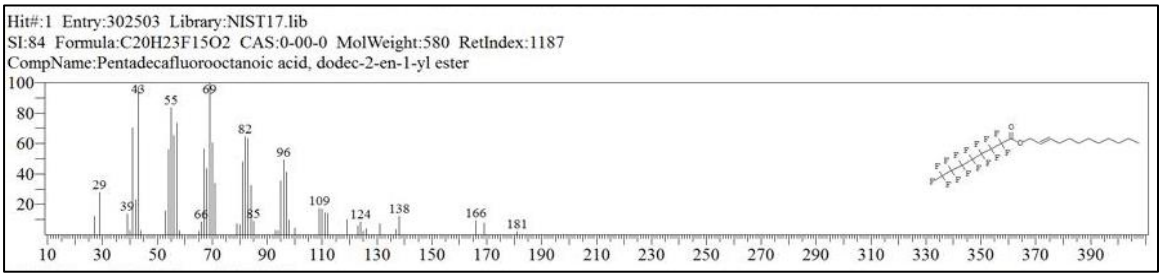


Figure 10: Chromatogram of Pentadecafluorooctanoicacid, dodec-2-en-1-yl ester

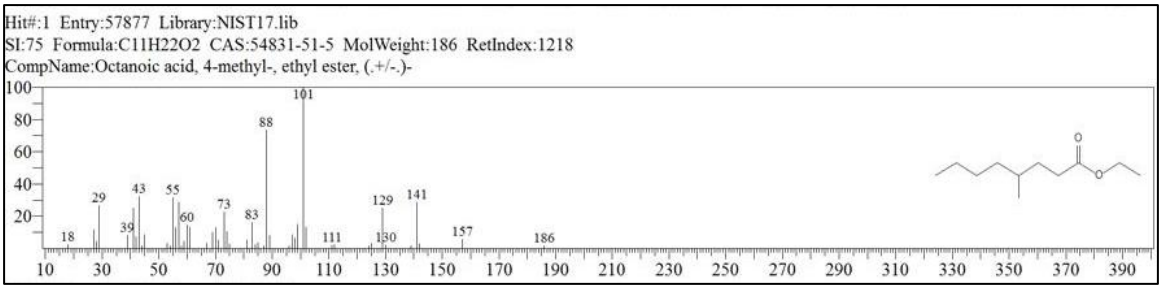


Figure 11: Chromatogram of Ethyl 4-methyloctanoate

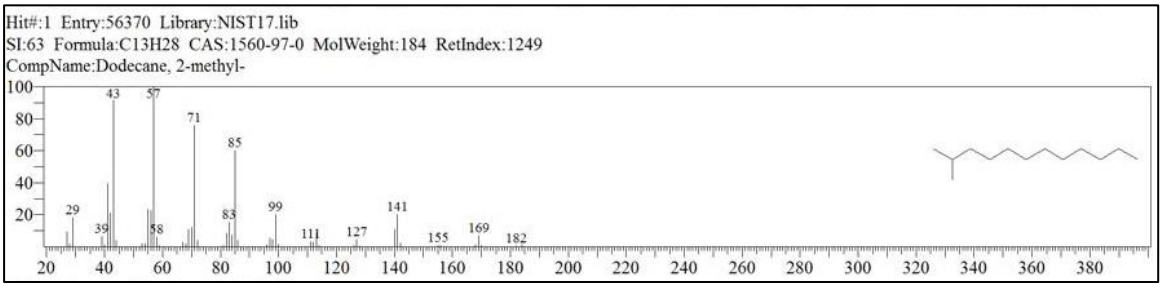


Figure 12: Chromatogram of 2-methyldodecane

4.4.2 GC-MS Spectra analysis of methanol extract

19 main components were identified by GC-MS analysis of the chemical make-up of the *K. pinnata* methanol extract. The list of significant compounds below is based on GC-MS analysis:

Table 7: Components based on GC-MS analysis of methanol extract

S.N.	Name of compound	Retention time	Molecular formula	Area%
1.	2-(5-Phenyl-2H-tetrazol-2-yl) ethan-1-ol	9.542	$C_9H_{10}N_4O$	19.61
2.	(4R,10aR, Z)-2,2-dimethyl-1,2,3,4,4a,5,6,9,10,10a-decahydro-1,4-epidiazenobenzo[8]annulene	16.229	$C_{13}H_{20}N_2$	6.29
3.	1-Bromo-2-phenyl-cyclopropanphosphonic acid, diethyl ester	15.281	$C_{13}H_{18}BrO_3P$	0.70
4.	4-Diethylaminophenyl isothiocyanate	18.255	$C_{11}H_{14}N_2S$	0.82

5.	Pentadecafluorooctanoic acid, undecyl ester	19.710	$C_{19}H_{23}F_{15}O_2$	0.79
6.	2-octyloxirane	21.876	$C_{10}H_{20}O$	0.76
7.	Methyl 6-methyloctanoate	22.244	$C_{10}H_{20}O_2$	0.89
8.	α-Methyl-α-[4-methylpentyl]oxiranmethanol	22.858	$C_{10}H_{20}O_2$	0.74
9.	Bacteriochlorophyll-c-stearyl	25.126	$C_{52}H_{72}MgN_4O_4$	1.71
10.	Levomenthol	24.284	$C_{10}H_{20}O$	1.68
11.	2-Isopropenyl-5-methyl-6-hepten-1-ol	25.028	$C_{11}H_{20}O$	0.67
12.	13-(butoxy methyl)-13-bora bicyclo [7.3.1] tridecane	25.439	$C_{16}H_{31}BO$	0.61
13.	8-Methylnonanoic acid, methyl ester	26.555	$C_{11}H_{22}O_2$	22.38
14.	Decanoic acid, silver (1+) salt	26.285	$C_{10}H_{19}AgO_2$	1.07
15.	1,2-Oxathiane, 6-dodecyl-,2,2-dioxide	27.204	$C_{16}H_{32}O_3S$	0.92
16.	Methyl-9,10-octadecadienoate	28.517	$C_{19}H_{34}O_2$	14.50
17.	Cyclododecyne	28.639	$C_{12}H_{20}$	9.65
18.	3,7-Dimethyl-6-nonen-1-ol	28.799	$C_{11}H_{22}O$	2.46
19.	Decanoic acid, methyl ester	28.930	$C_{11}H_{22}O_2$	3.73

4.4.2.1 Mass spectral data of constituents identified by GC-MS

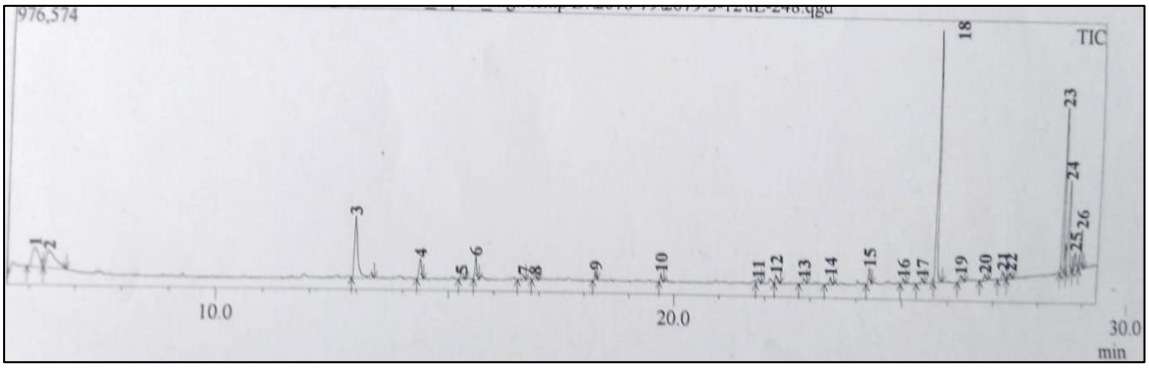


Figure 13: Chromatogram of methanol extract

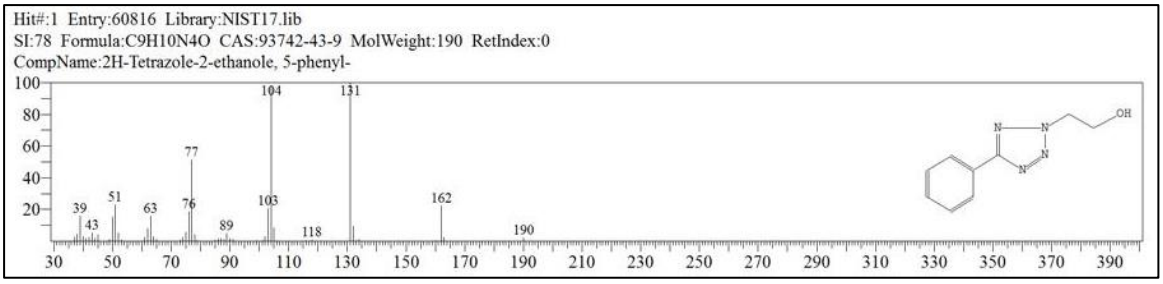


Figure 14: Chromatogram of 2-(5-Phenyl-2H-tetrazol-2-yl) ethan-1-ol

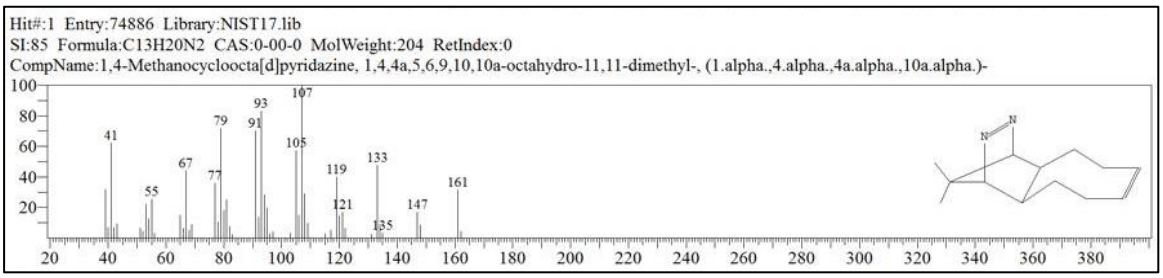


Figure 15: Chromatogram of (4R,10aR, Z)-2,2-dimethyl-1,2,3,4,4a,5,6,9,10,10a-decahydro-1,4-epidiazenobenzo[8]annulene

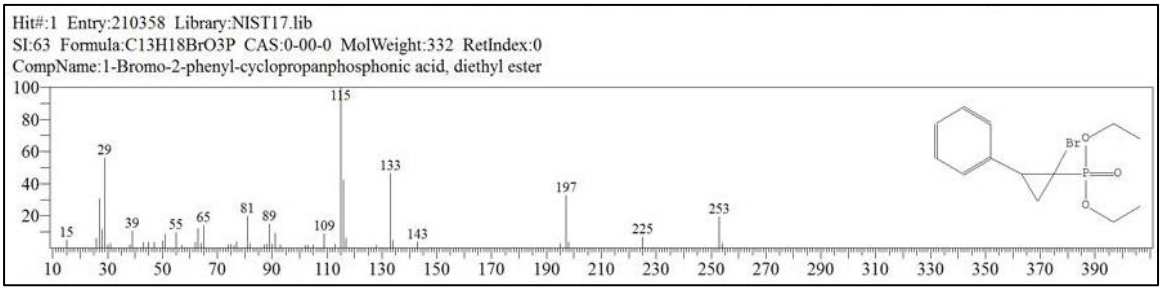


Figure 16: Chromatogram of 1-Bromo-2-phenyl-cyclopropanphosphonic acid, diethyl ester

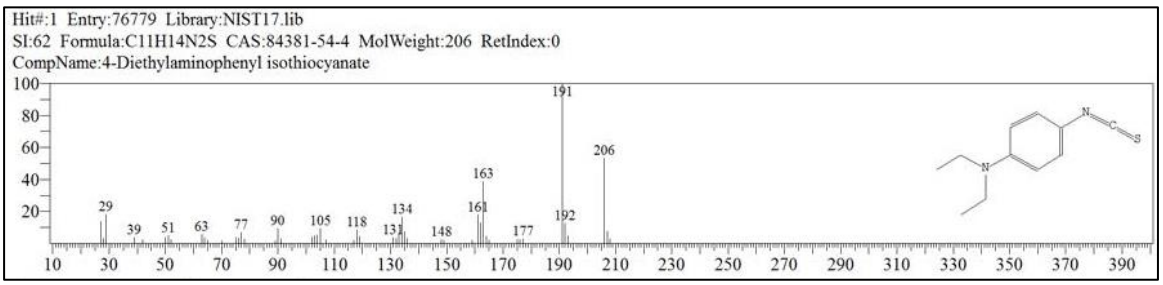


Figure 17: Chromatogram of 4-Diethylaminophenyl isothiocyanate

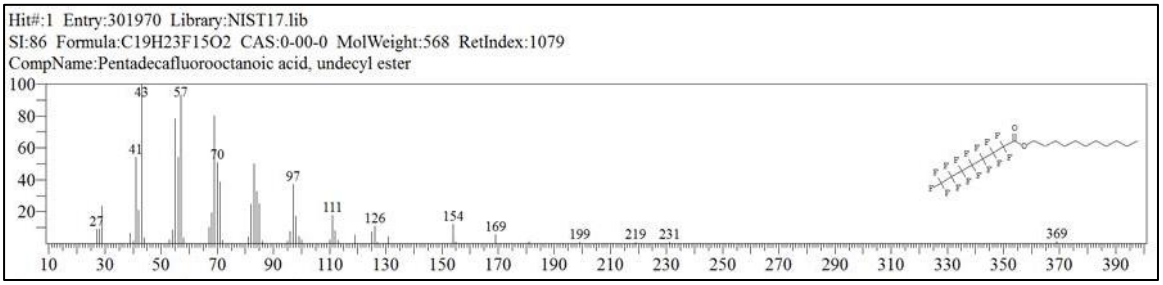


Figure 18: Chromatogram of Pentadecafluorooctanoic acid, undecyl ester

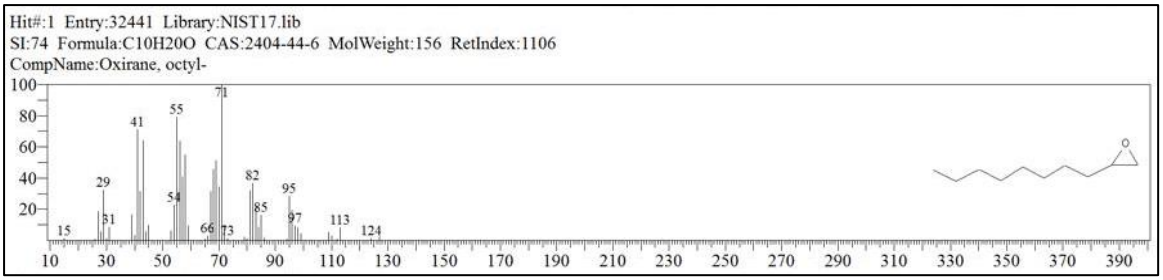


Figure 19: Chromatogram of 2- octyl oxirane

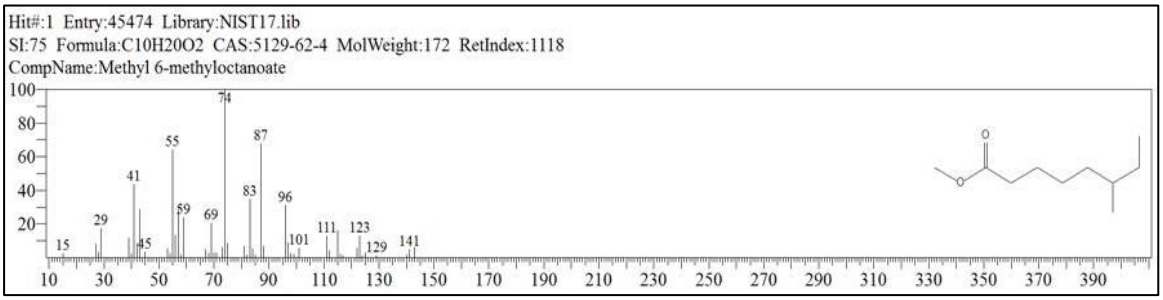


Figure 20: Chromatogram of Methyl 6-methyloctanoate

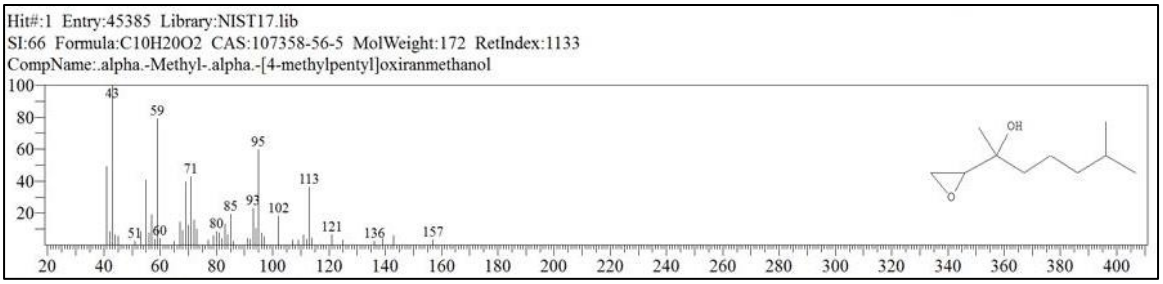


Figure 21: Chromatogram of α-Methyl-α-[4-methylpentyl]oxiranmethanol

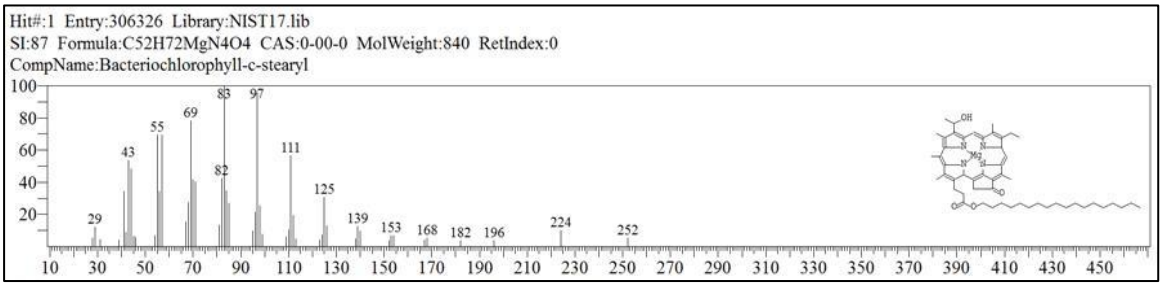


Figure 22: Chromatogram of Bacteriochlorophyll-c-stearyl

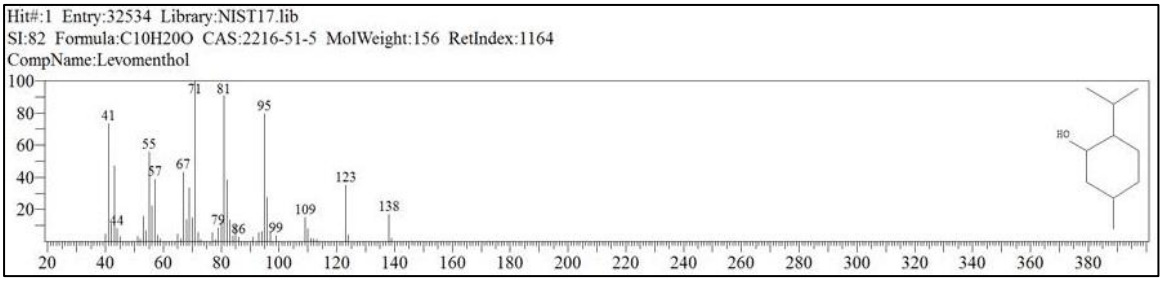


Figure 23: Chromatogram of Levomenthol

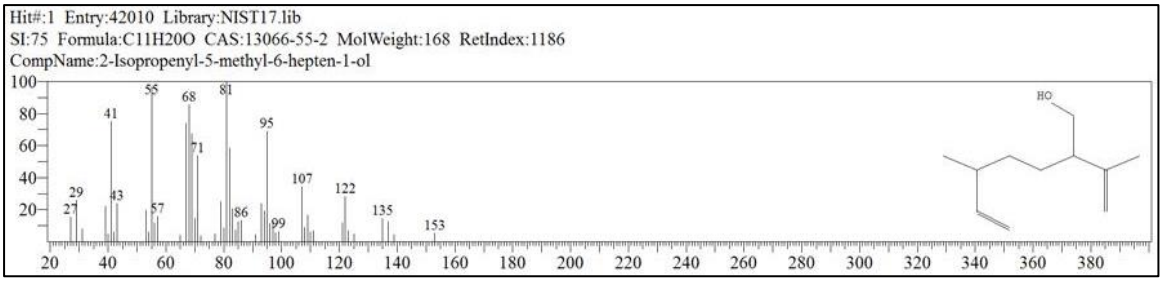


Figure 24: Chromatogram of 2-Isopropenyl-5-methyl-6-hepten-1-ol

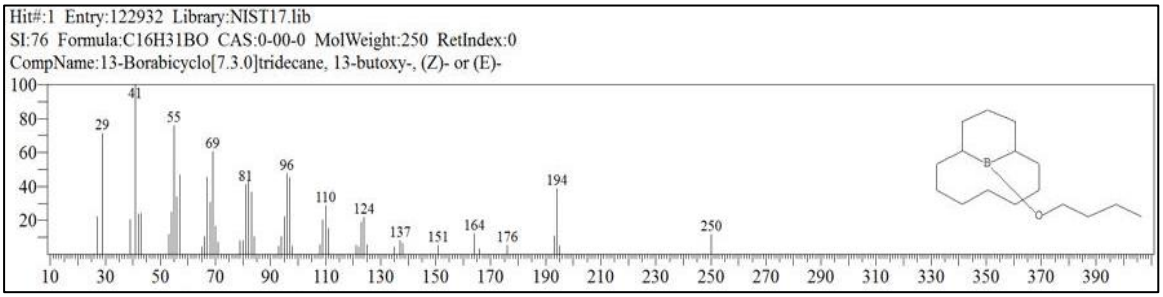


Figure 25: Chromatogram of 13-(butoxy methyl)-13-bora bicyclo [7.3.1] tridecane

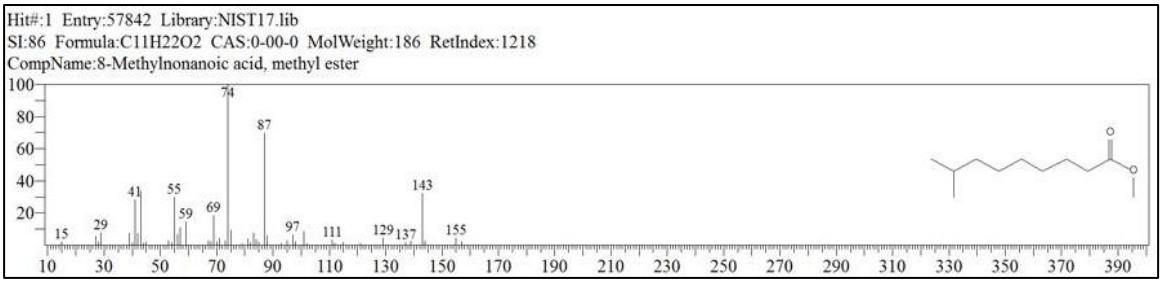


Figure 26: Chromatogram of 8-Methylnonanoic acid, methyl ester

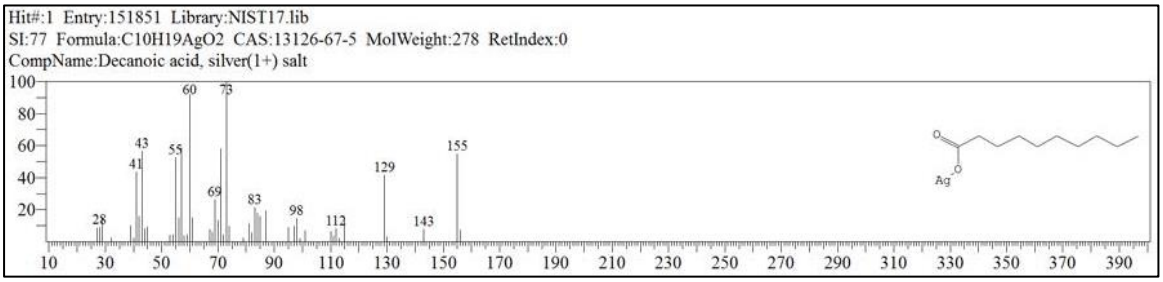


Figure 27: Chromatogram of Decanoic acid, silver (1+) salt

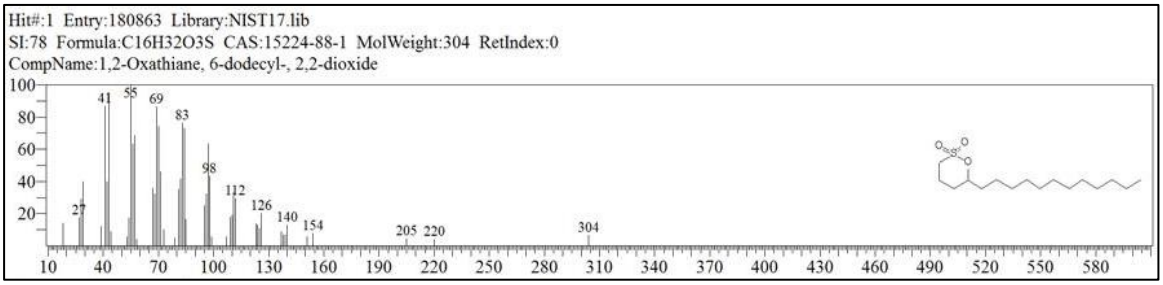


Figure 28: Chromatogram of 1,2-Oxathiane, 6-dodecyl-,2,2-dioxide

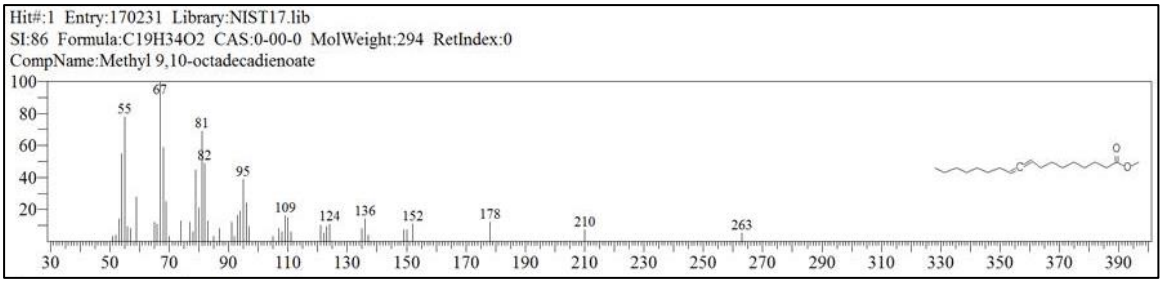


Figure 29: Chromatogram of Methyl-9,10-octadecadienoate

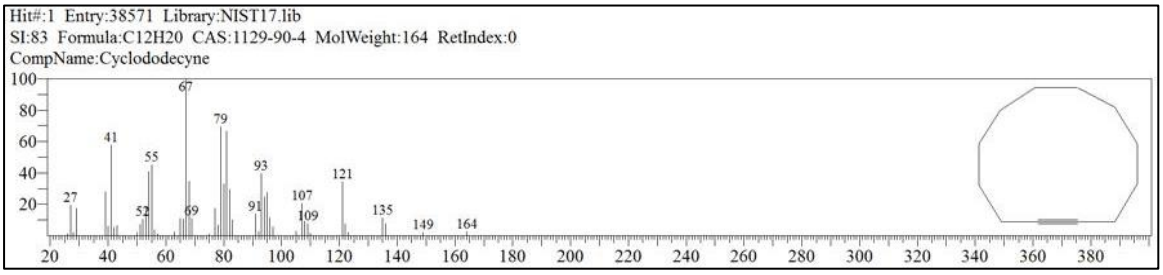


Figure 30: Chromatogram of Cyclododecyne

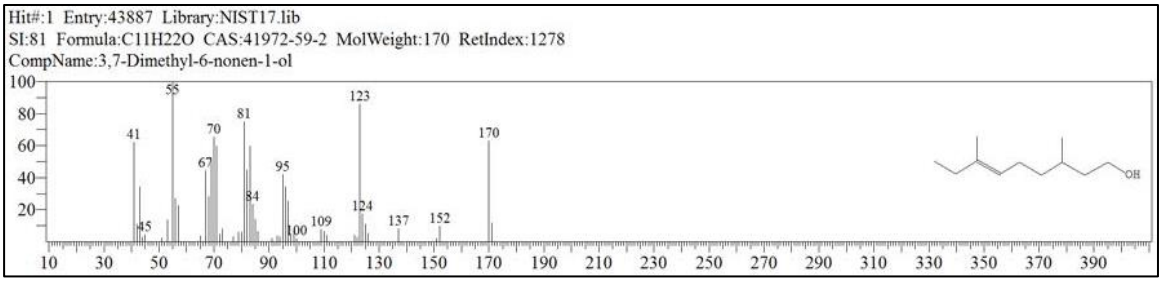


Figure 31: Chromatogram of 3,7-Dimethyl-6-nonen-1-ol

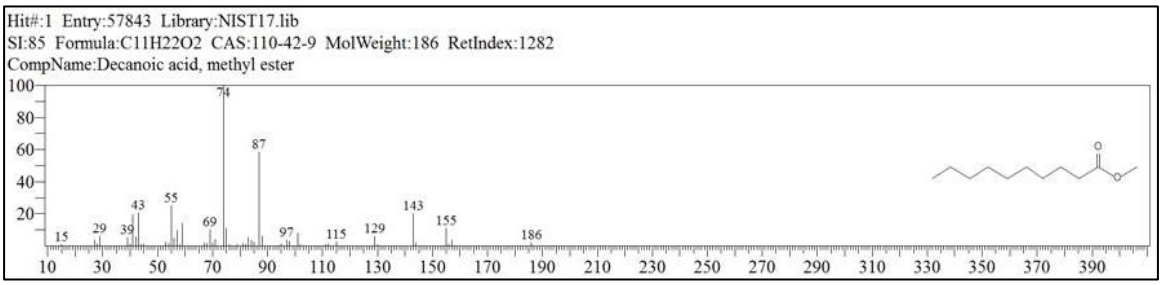


Figure 32: Chromatogram of Decanoic acid, methyl ester

4.5 Quantitative Analysis of Phytochemicals

4.5.1 Estimation of Total Phenolic Content (TPC)

4.5.1.1 Construction of the calibration curve

The quantity of polyphenols in plant extracts that, when they interact with a certain redox reagent (FCR), form a blue complex with a maximum 765 nm light absorption that can be detected using UV-visible spectrometry. The amounts of phenols are inversely correlated with the light absorption strength at that wavelength. The

observation of absorbance at various concentrations of standard gallic acid was represented graphically, with the Y-axis representing absorbance and the X-axis representing concentration. The figure depicts the absorbance curve for standard gallic acid in various concentrations such as 500, 250, 150, 100, 50 and 25 µg/ mL.

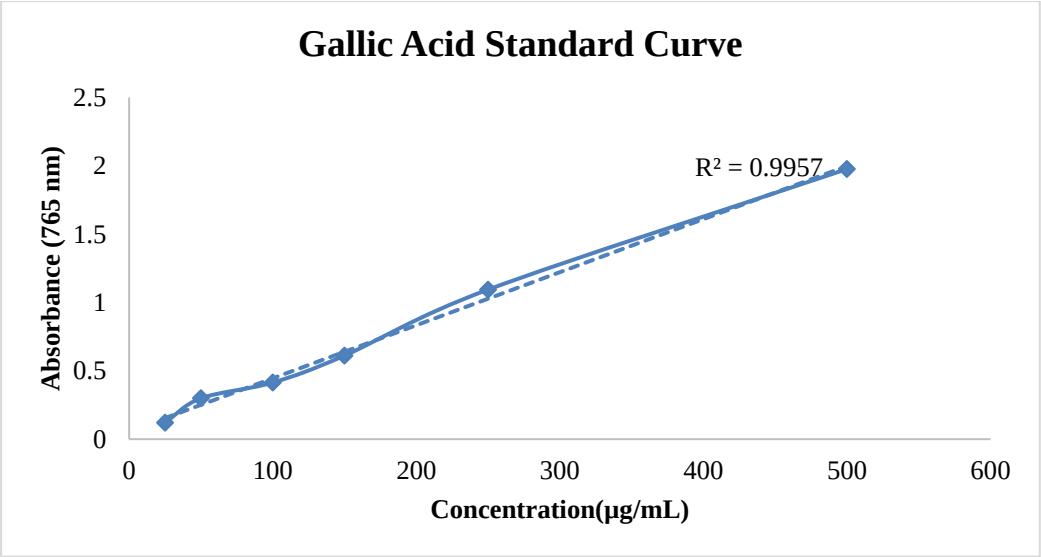


Figure 33: Calibration curve for standard Gallic acid

The equation created from the standard Gallic acid curve in figure 33 was used to determine the content of the ethyl acetate and methanol extract.

4.5.1.2 Calculation of total phenolic content in different extracts

Table 8: Total Phenolic content in Ethyl acetate and Methanol extract

	Concentration (µg/mL)	Observed Data(O.D) $_{Sample}$	O.D $_{control}$	O.D $_{value}$
Ethyl acetate	1000	2.003	0.054	1.949
Methanol	1000	0.824	0.054	0.77

Table 9: Total phenolic content of *K. pinnata* extracts

Extract	Ethyl acetate	Methanol
Total phenolic content (mg GAE /g extract)	485.17	182.87

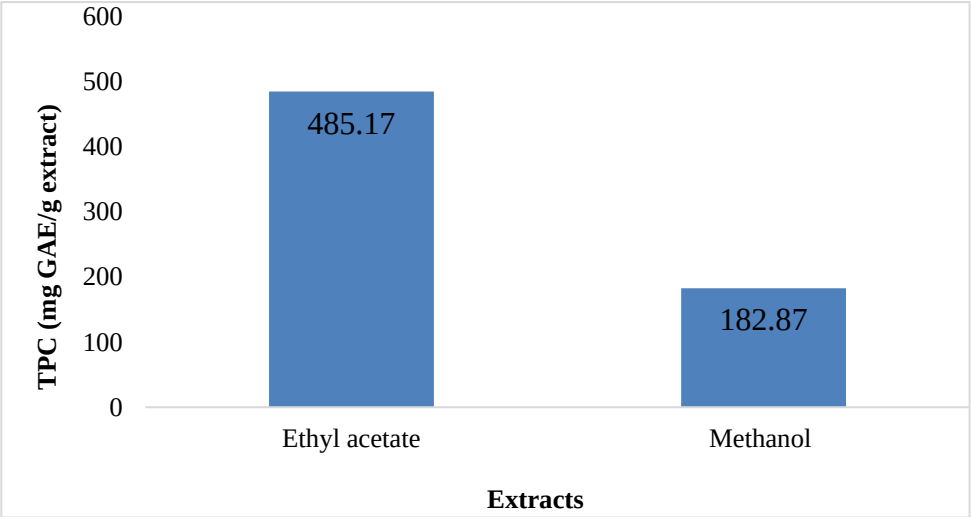


Figure 34: Total phenolic contents of *K. pinnata* extracts

The results show that *K. pinnata*'s methanol extract has 182.87 mg GAE/g whereas its ethyl acetate extract has 485.17 mg GAE/g. This study shows that phenolic compounds generally have higher solubility in polar organic solvents than in non-polar ones.

4.5.2 Estimation of Total Flavonoid Content (TFC)

4.5.2.1 Construction of calibration curve

The overall flavonoid content of plant extracts was measured using an aluminum chloride colorimetric method. The concentrations of the graph plots in the figure below range from 250 µg/mL,150 µg/mL, 100 µg/mL, 50 µg/mL, and 25 µg/mL.

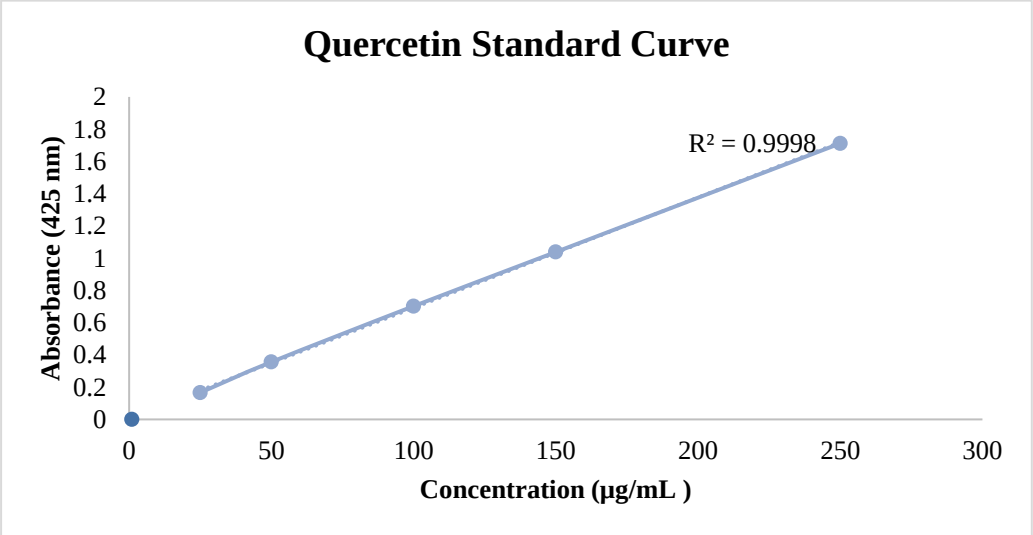


Figure 35: Calibration curve for standard Quercetin

To calculate the concentration of the ethyl acetate and methanol extract, an equation derived from the typical quercetin curve in figure 35 was used.

4.5.2.2 Calculation of total flavonoid content in different extracts

Table 10: Total flavonoid content in ethyl acetate and methanol extract

	Concentration (µg/mL)	Observed Data(O.D) Sample	O.D control	O.D value
Ethyl acetate	1000	0.3533	0.0693	0.2839
Methanol	1000	0.2073	0.0693	0.1380

Table 11: Total flavonoid content of *K. pinnata* extracts

Extract	Ethyl acetate	Methanol
Total flavonoid content (mg QE/g extract)	40.25	19.05

In comparison to methanol, ethyl acetate has a total flavonoid concentration of 40.25 mg QE/g. They were calculated using the R^2 equation and the formula C= cV/m and given as mg of quercetin equivalents (QE) per gram of extract in dry weight (mg/g).

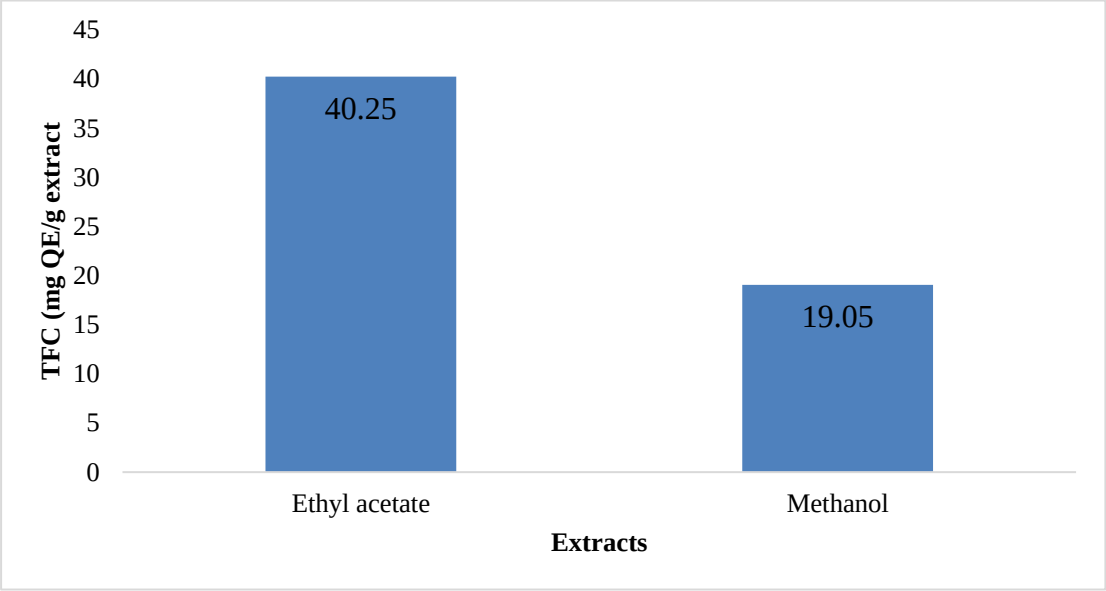


Figure 36: Total flavonoid content of *K. pinnata* extracts

4.5.3 Antioxidant Activity

The degree of antioxidant activity was determined using the DPPH free radical scavenging test. The antioxidant potential, which is inversely correlated with the IC_{50}

value, can be calculated using a linear regression of the percentage of inhibition vs. antioxidant activity.

Following measurements and records were made of each solution's absorbance:

Table 12: Antioxidant activity of Ascorbic acid

Sample	Concentration (µg/mL)	Absorbance (nm)			Average absorbance (nm)	% of scavenging
1	Control	0.48	0.524	0.47	0.491	
2	2.5	0.433	0.438	0.406	0.425	13.364
3	5	0.385	0.362	0.366	0.371	24.491
4	10	0.303	0.304	0.309	0.305	37.856
5	20	0.21	0.195	0.177	0.194	60.515
IC_{50}	14.42 µg/mL					

Multiple doses of ascorbic acid were employed to neutralize free radicals, and the observed absorbance was plotted on a graph, as seen in the figure.

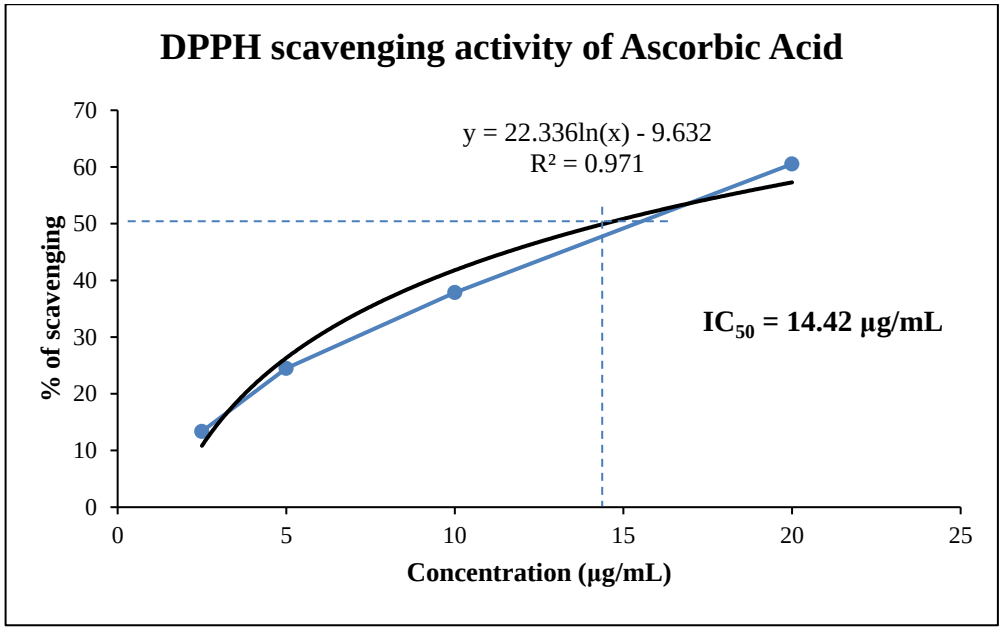


Figure 37: Antioxidant activity of Ascorbic acid

From this graph, the IC_{50} value of ascorbic acid is 14.42 µg/mL.

At 520 nm, the absorbance values of various chloroform extract concentrations were determined. The % inhibition of DPPH radicals against the sample represented in the table below was calculated using these values.

Table 13: Antioxidant activity of chloroform extract

S.N.	Concentration (µg/mL)	OD_{sample}	$OD_{control}$	% of scavenging
1	3000	0.256	0.629	54.68
2	2000	0.374	0.629	46.53
3	1000	0.484	0.629	40.54
4	500	0.545	0.629	23.05
5.	250	0.572	0.629	12.34
	IC_{50} = 2219.41 µg/mL			

The chloroform extract of *K. pinnata* was determined to have an IC_{50} value of 2219.41 µg/mL. The illustration below illustrates this:

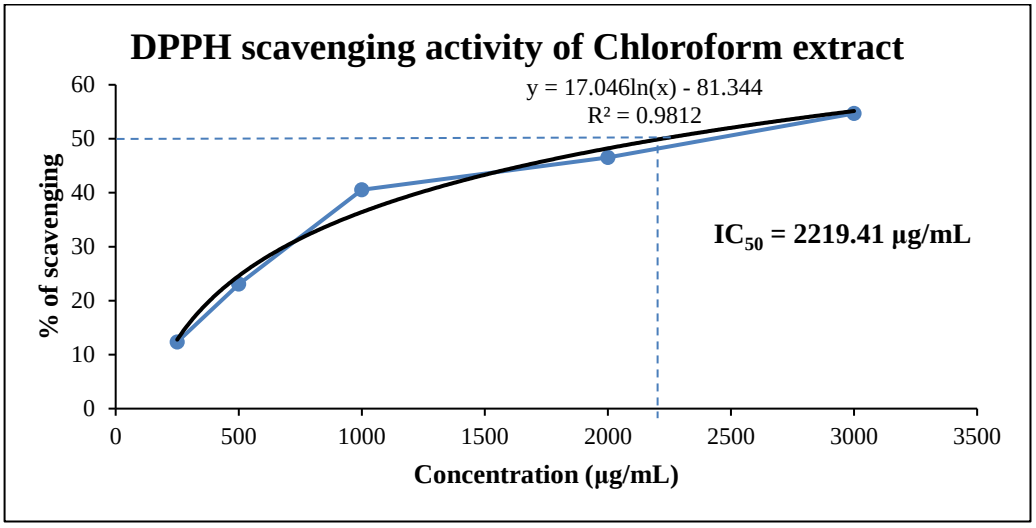


Figure 38: Antioxidant activity of chloroform extract

At 520 nm, the absorbance values of various ethyl acetate extract concentrations were determined. These numbers were used to determine the sample's percentage inhibition of DPPH radicals, which is displayed in the table below:

Table 14: Antioxidant activity of ethyl acetate extract

S.N.	Concentration (µg/mL)	OD_{sample}	$OD_{control}$	% of scavenging
1	1000	0.170	0.629	72.91
2	500	0.29	0.629	53.89
3	250	0.38	0.629	39.21
4	125	0.43	0.629	31.37
	IC_{50} = 365.22 µg/mL			

The ethyl acetate extract's IC_{50} value was determined to be 365.22 µg/mL. The illustration below illustrates this.

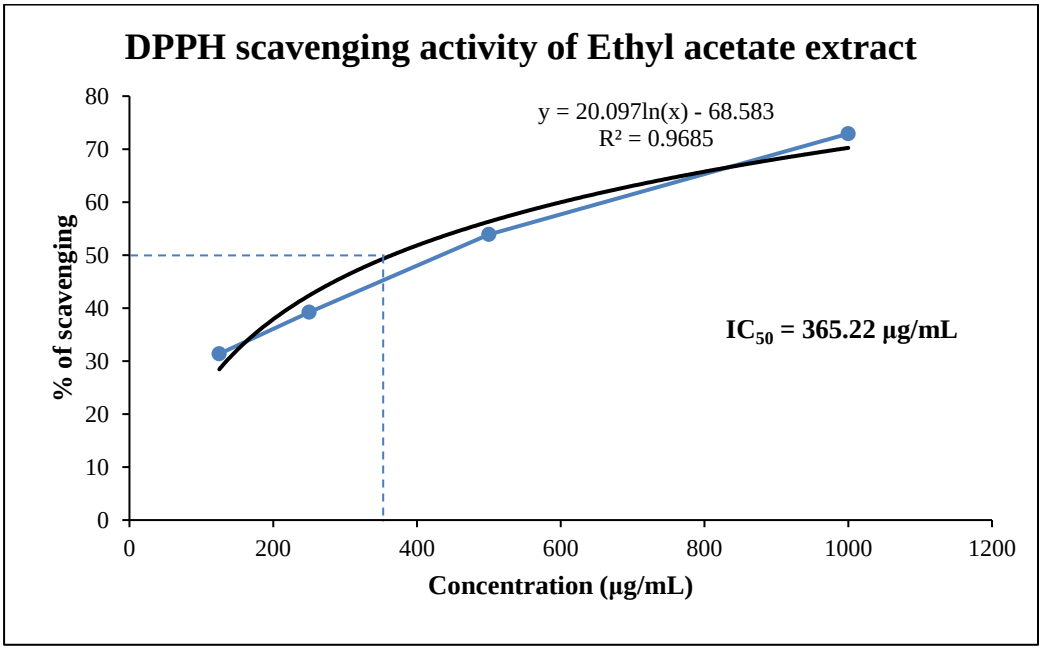


Figure 39: Antioxidant activity of ethyl acetate extract

At 520 nm, the absorbance values of various methanol extract concentrations were determined. The percentage inhibition of DPPH radicals against the sample, as given in the table below, was determined using these results.

Table 15: Antioxidant activity of Methanol extract

S.N.	Concentration (µg/mL)	OD_{sample}	$OD_{control}$	% of scavenging
1	1000	0.133	0.629	78.80
2	500	0.245	0.629	61.04
3	250	0.352	0.629	43.93
4	125	0.422	0.629	32.80
	IC_{50} = 293.53 µg/mL			

The IC_{50} methanol extract has a value of 293.53 µg/mL. The figure below displays this.

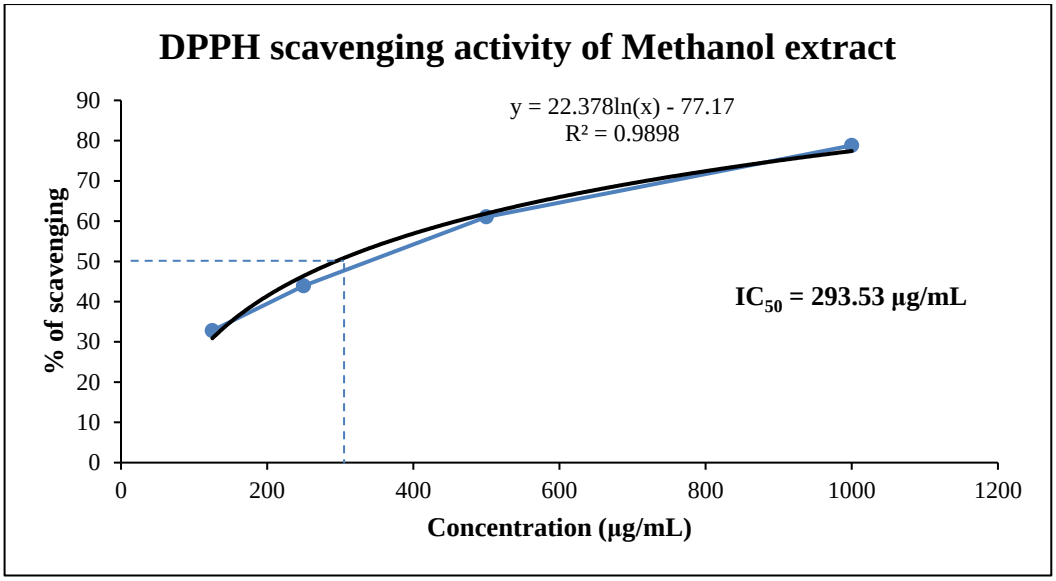


Figure 40: Antioxidant activity of methanol extract

The figure below displays the IC_{50} values for the plant extracts.

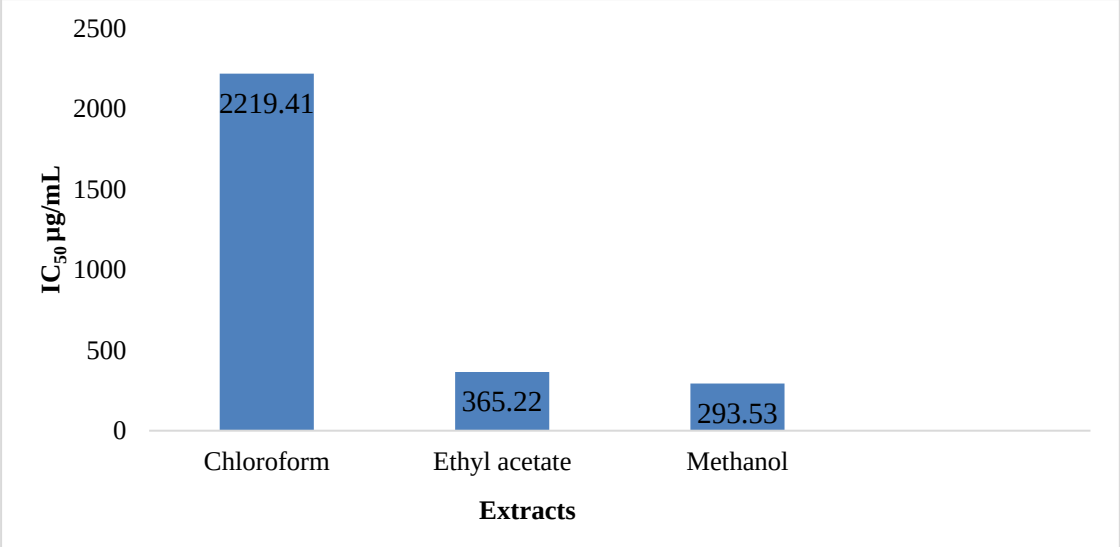


Figure 41: IC_{50} values of different extracts

Comparatively, among these three extracts, the methanol extract of *K. pinnata* shows higher antioxidant activity. Additionally, ethyl acetate extract has also powerful antioxidant properties, but chloroform extract doesn't show good antioxidant action. These values demonstrate the extraordinary antioxidant property and are comparable to the typical ascorbic acid concentration (14.42 µg/mL). The hydroxyl group in extracts with a high phenolic concentration works as a hydrogen donor and assists in radical scavenging, increasing antioxidant activity.

4.5.5 Antimicrobial Activity

To determine whether plant extracts had any antimicrobial properties, the diameter of the zone of inhibition (ZOI) that they produced on microbes was examined. Using the method described in this section, the ability of different *K. pinnata* leaf extract fractions to stop microbial growth at a fixed concentration (5 µg/mL) was assessed. The diameter of the inhibitory zone was provided as the result. A zone of inhibition (ZOI) measurement in centimeters was made. As a negative control, the DMSO solvent was used. For the positive control, Kanamycin was used. Antimicrobial action differs depending on the type of microbes.

Table 16: Antimicrobial activity shown by the different extracts in diameter(cm) of the inhibition zone (ZOI)

Microbes	Negative control DMSO	Hexane extract	DCM extract	Chloroform extract	Ethyl acetate extract	Acetone extract	Methanol extract	Aqueous extract
Bacillus subtilis (ATCC 6051)	0	0.85	0.8	0.85	0.7	0.85	0.5	0.5
Escherichia coli (ATCC 8739)	0	0.65	0.8	0.7	0.65	0.6	0.8	0.7
Candida albicans (ATCC 2091)	0	0.7	0.75	0.6	0.6	0.75	0.7	0.5

The antimicrobial property of all extracts was observed, which is shown in table 16. In this test, *B. subtilis* was found to be highly inhibited on hexane, chloroform and acetone extracts which show 0.85 cm ZOI. *E. coli* was highly inhibited on DCM and methanol extract shows 0.8 cm ZOI. Similarly, DCM and acetone extract show high inhibiting power with 0.75 cm ZOI on *C. albicans*.

According to these results, it has been suggested that gram-positive, gram-negative, and fungi can occasionally infect the body's numerous organs and have a detrimental effect. These microorganisms were successfully inhibited by the extracts of this plant species in hexane, DCM, chloroform, ethyl acetate, acetone, methanol, and aqueous. Therefore, it may be determined how effective these plant species extracts are as antimicrobial agents.

4.5.6 α-Amylase (Anti-diabetic) Assay

Using the 3,5-dinitrosalicylic acid (DNSA) technique, the experiment involving the inhibition of α-amylase was conducted. The linear regression % of inhibition vs α-amylase inhibition activity can be used to determine the antidiabetic potential, which is inversely related to the IC_{50} value. The absorbance values of different concentrations of extracts were measured at 540nm.

The table below displays the percentage of acetone extract that inhibits α-amylase.

Table 17: α-Amylase inhibition of acetone extract

Concentration (µg/mL)	% of Inhibition
2000	69.33
1000	58.16
500	53.41
250	50.44
125	43.02
IC_{50} = 285.71 µg/mL	

K. pinnata acetone extract has an IC_{50} of 285.71 µg/mL. This is shown in figure below:

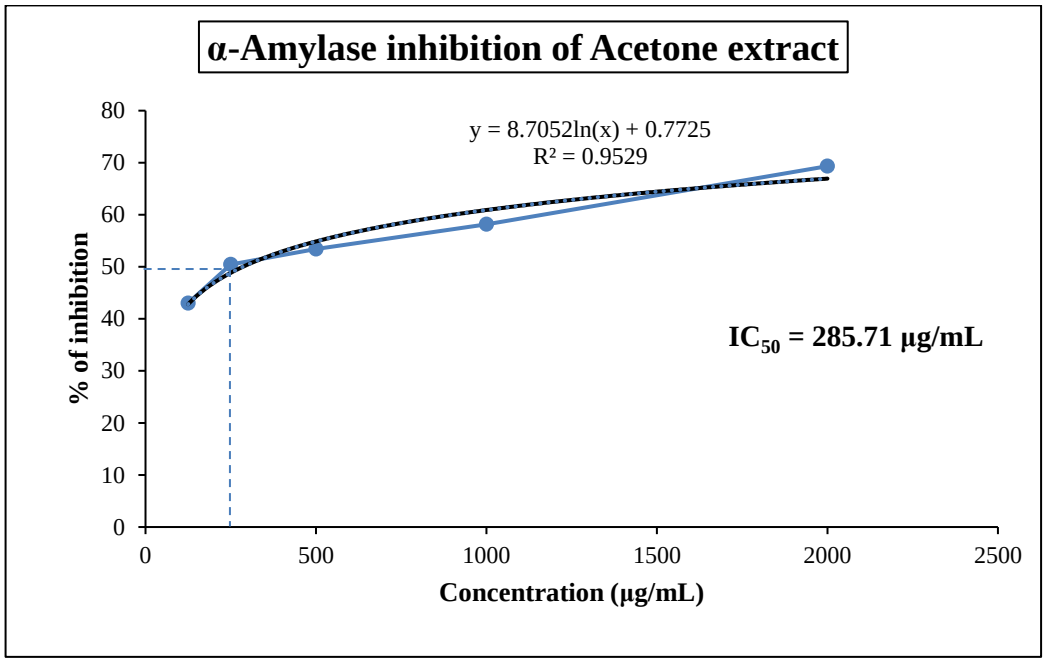


Figure 42: α-Amylase inhibition of acetone extract

The level of ethyl acetate extract-induced inhibition of α-amylase is shown in the table below.

Table 18: α-Amylase inhibition of ethyl acetate extract

Concentration (µg/mL)	% of Inhibition
2000	88.54
1000	63.43
500	47.13
250	35.24
IC_{50} = 504.21 µg/mL	

K. pinnata extract in ethyl acetate had an IC_{50} of 504.21 µg/mL. This is shown in the figure below:

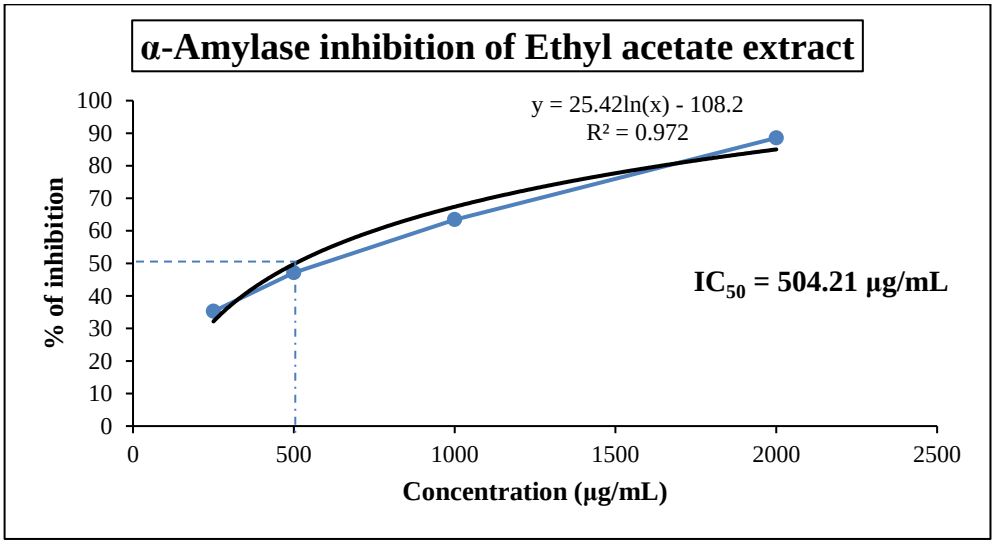


Figure 43: α-Amylase inhibition of ethyl acetate extract

The percentage of methanol extract-induced inhibition of α-amylase is shown in the table below.

Table 19: α-Amylase inhibition of methanol extract

Concentration (µg/mL)	% of Inhibition
500	60.30
250	52.00
125	43.69
62.5	40.00
IC_{50}=195.39 µg/mL	

K. pinnata methanol extract has an IC_{50} value of 195.39 µg/mL. This is shown in the figure below.

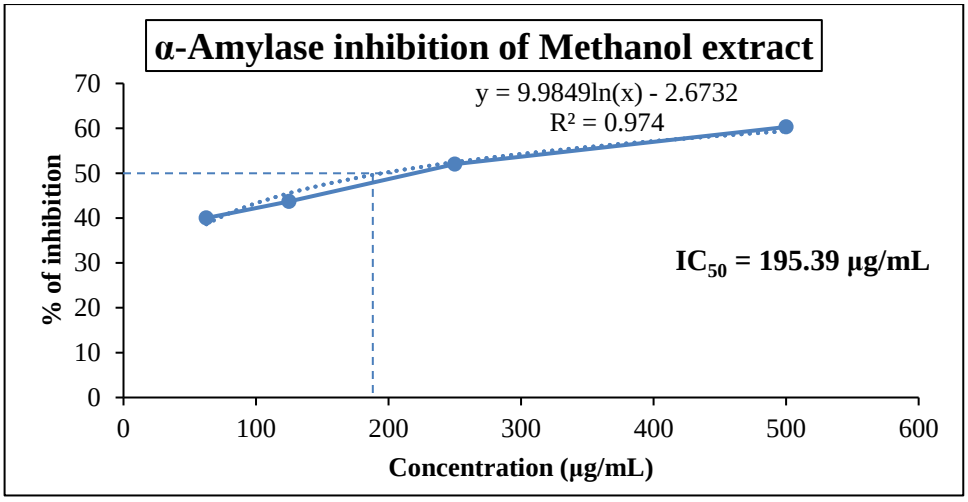


Figure 44: α-Amylase inhibition of methanol extract

The comparable IC_{50} value of the plant extracts is shown in the figure below:

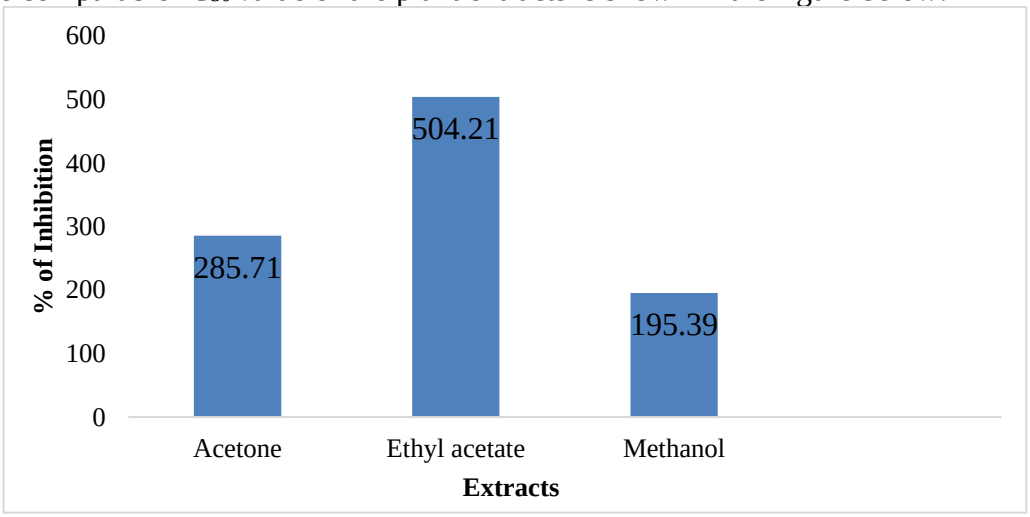


Figure 45: IC_{50} values of different extracts α-Amylase inhibition

Comparatively, among these three extracts, methanol extract of *K. pinnata* shows higher α-amylase inhibition activity. Additionally, acetone extract has more powerful antidiabetic properties than ethyl acetate extract. These three extracts collectively exhibit strong antidiabetic action.

4.5.7 Brine Shrimp Lethality Assay (BSLA)

After the freshly hatched live nauplii were exposed to concentrations of 1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, and 0.0625 mg/mL, respectively, the LC_{50} value of chloroform, acetone, and methanol extracts was computed. It was discovered that the concentration of the extract directly correlated with the level of lethality. The most

and least number of brine shrimp larvae perished at doses of 1 mg/mL and 0.0625 mg/mL, respectively.

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

Table 20: Calculation of mortality % of chloroform extract

Concentration (mg/mL)	No. of dead nauplii			% Mortality
1	10	10	10	100
0.5	9	10	10	96.667
0.25	5	6	6	56.667
0.125	4	2	4	33.333
0.0625	1	3	1	16.667
LC_{50} = 0.181 mg/mL				

The LC_{50} value of chloroform extract of *K. pinnata* was 0.181 mg/mL. This is shown in the figure below.

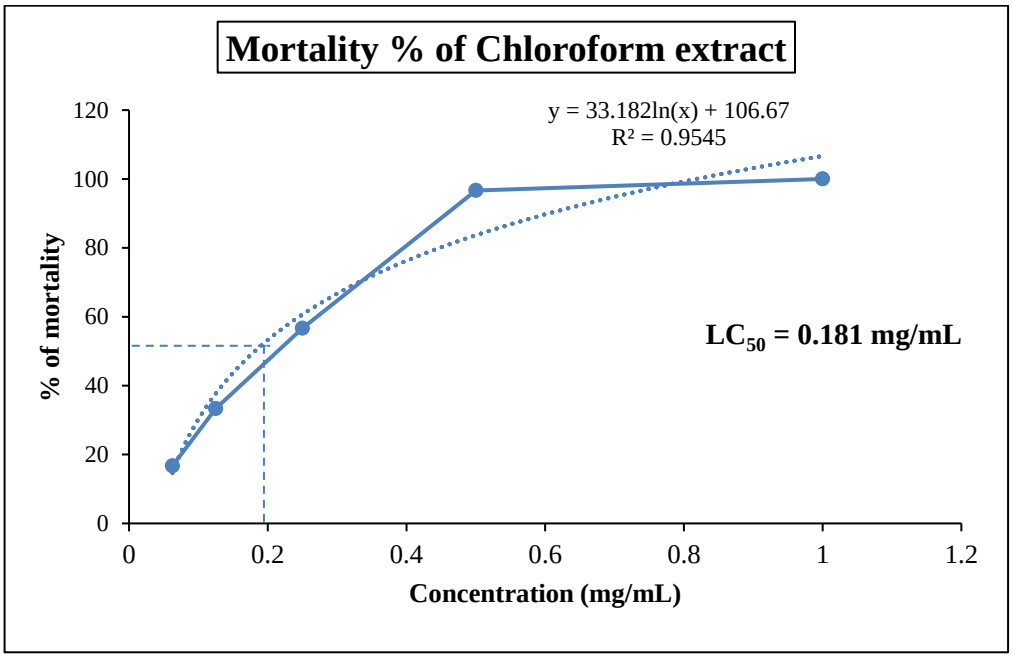


Figure 46: Cytotoxicity activity of chloroform extract

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

Table 21: Calculation of mortality % of acetone extract

Concentration (mg/mL)	No. of dead nauplii			% Mortality
1	10	10	10	100
0.5	9	9	8	86.667
0.25	6	5	6	56.667
0.125	5	4	4	43.333
0.0625	2	1	2	16.667
LC_{50} = 0.175 mg/mL				

The LC_{50} value of acetone extract of *K. pinnata* was 0.175 mg/mL. This is shown in the figure below.

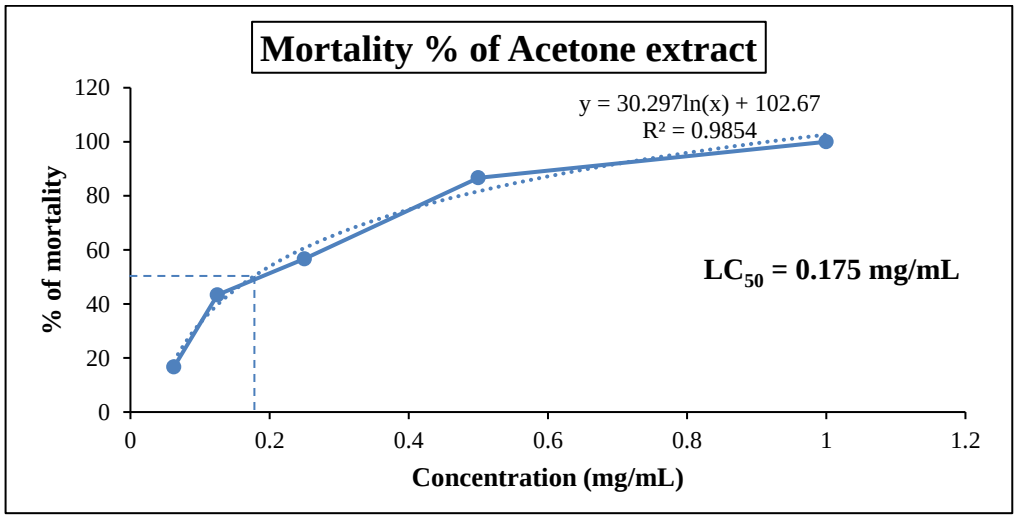


Figure 47: Cytotoxicity activity of acetone extract

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

Table 22: Calculation of mortality % of methanol extract

Concentration (mg/mL)	No. of dead nauplii			% Mortality
1	10	10	10	100
0.5	5	6	6	56.667
0.25	4	5	5	46.667
0.125	1	2	1	13.333
0.0625	0	1	1	6.667
LC_{50} = 0.290 mg/mL				

The LC_{50} value of methanol extract of *K. pinnata* was 0.29 mg/mL. This is shown in the figure below.

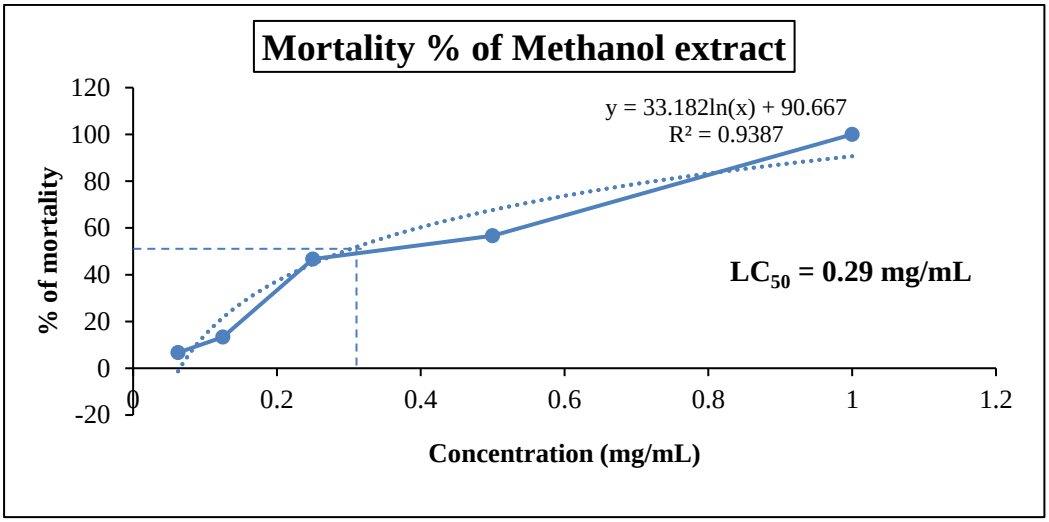


Figure 48: Cytotoxicity activity of methanol extract

As a result, extracts were discovered to be pharmacologically significant and harmful to *Artemia salina* (brine shrimp) larvae. Among these three extracts, Chloroform extract show high mortality % while methanol shows least mortality %.

4.5.8 Thin layer Chromatography

The quantitative analysis of the methanol extract of *K. pinnata* was done by TLC, during which the polarity of Acetone: Hexane was increased gradually. The plates were exposed to several solvent developments before being seen under a UV-fluorescence lamp. The figure displays the plates that have been visualized.

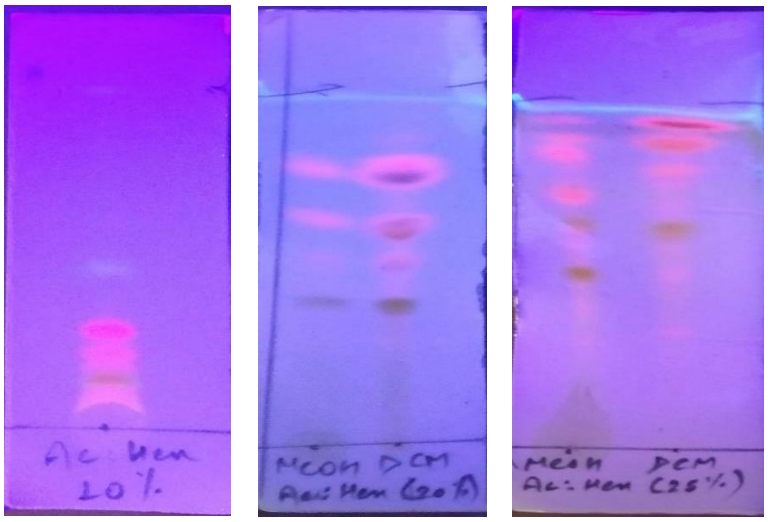


Figure 49: TLC plate at 10%, 20% and 25% Acetone: Hexane

4.5.9 Column Chromatography

The *K. pinnata* methanol extract underwent column chromatography. Acetone and hexane solvent system was created in various ratios and allowed to pass through the column. A TLC test is carried out after the fractions have been concentrated using Rota vapour and collected in a conical flask. The table below lists the solvent systems with various ratios and Rf values.

Table 23: Column chromatography of methanol extract of *K. pinnata*

S.N	Solvent System	Ratio	Fraction	Weight in gm
1	100% Hexane	100	1-5	0.003 g
2	1% Acetone: Hexane	1:99	5-42	0.23g
3	2% Acetone: Hexane	1:99	43-63	0.09g
4	3% Acetone: Hexane	1:99	64-77	0.15g
5	5% Acetone: Hexane	2:98	78-101	0.21g
6	7.5% Acetone: Hexane	2:98	102-139	0.07g

From the column chromatography, a single spot was found, and the compound will send for NMR analysis. Some visualized plates of spots are shown below:

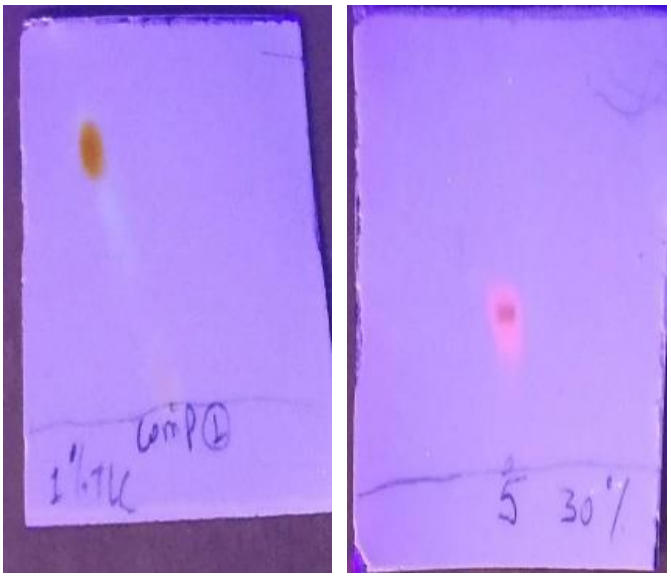


Figure 50: Single spot of 1% Ac:Hex and 5% Ac:Hex

4.5.10 UV Spectroscopic Measurements

An organic compound's unsaturation or presence of a lone pair of electrons can be determined via the UV measurement.

4.5.10.1 UV spectroscopic measurement of (methanol extract) 1% Ac: Hex

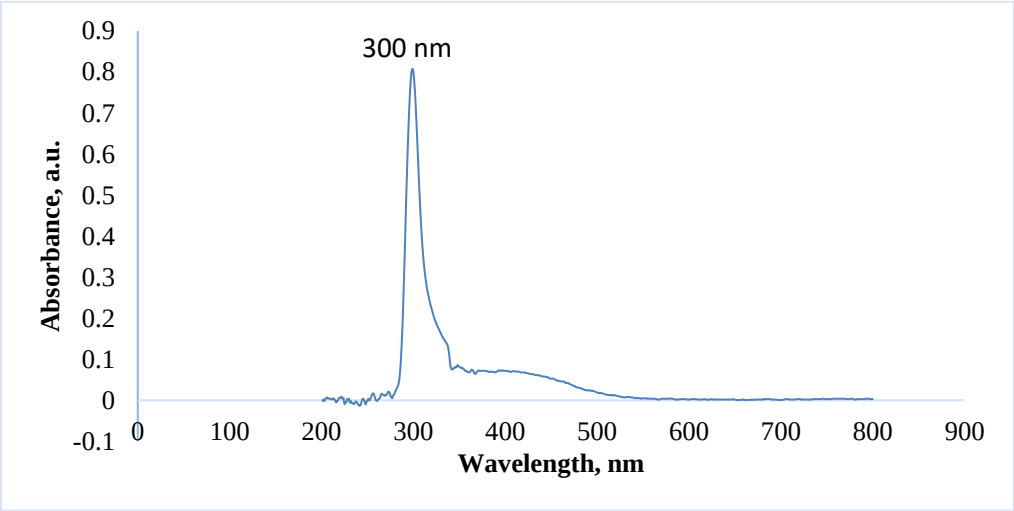


Figure 51: UV spectrum of (methanol extract) 1% Acetone: Hexane

The strong peak at 300 nm in the spectra shown in figure 51 gives the presence of compounds having unsaturation, aromaticity, and conjugation.

4.5.10.2 UV spectroscopic measurement of (methanol extract) 5% Ac: Hex

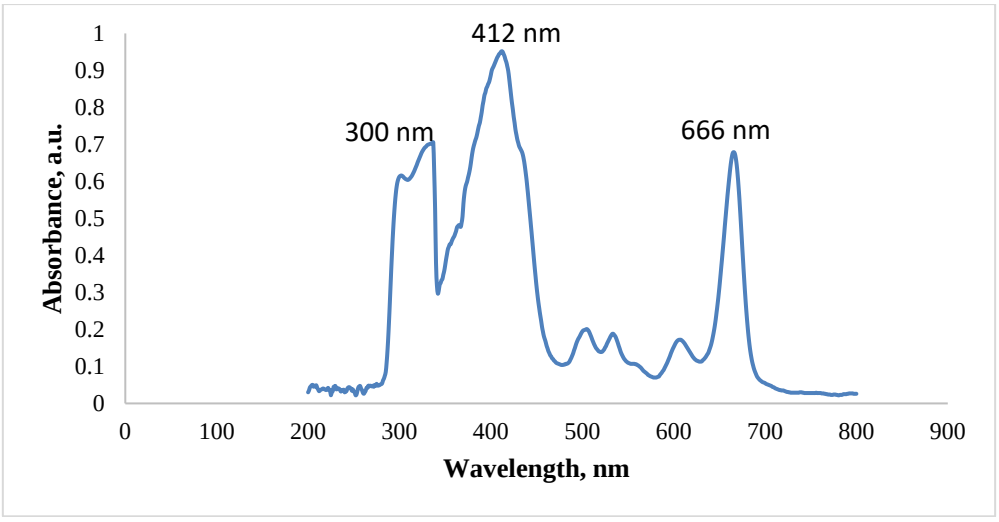


Figure 52: UV spectrum of 5% (methanol extract) Acetone: Hexane

The strong peak at 300 nm, 412 nm and 666 nm in the spectra shown in figure 52 denotes the compound contains higher aromaticity, conjugation, and unsaturation.

4.5.11 FT-IR Analysis

The type of bonding, the aromatic and aliphatic structures, and, most significantly, the functional group present can all be identified via FTIR spectroscopy analysis of organic molecules. The sample's chemical structure is revealed by the FTIR spectrum, which also verifies the presence of numerous functional groups and organic compounds. The bond between the carbon and heteroatoms can be recognized using the stretching frequency in the FTIR spectrum.

4.5.11.1 FT-IR spectrum of (methanol extract) 1% Acetone: Hexane

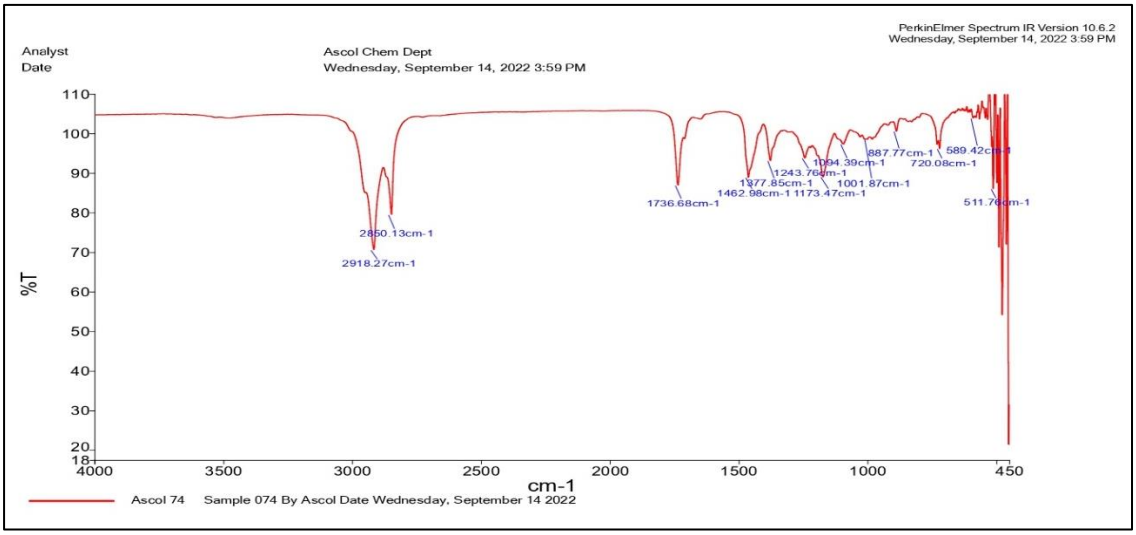


Figure 53: FT-IR spectrum of (methanol extract) 1% Acetone: Hexane

The table below shows the absorption, types of stretching and appearance of the 1% Acetone: Hexane.

Table 24: FTIR peak value and functional groups of (methanol extract) 1% Ac:Hex

Absorption (cm^{-1})	Types of stretching	Appearance
2918.27 2850.13	C-H	Strong
1736.68	C=O	Strong
1462.98	$-CH_2$	Medium
1377.85 1243.76	$-NO_2$	Medium
1094.39	C-O	Medium

The results were compared using an infrared chart of FTIR analysis to confirm the presence of functional groups such as the strong band was observed at 2918.27 and 2850.13 cm^{-1} corresponds to C-H stretching. The strong band at 1736.68 cm^{-1} indicates the presence of C=O stretching assigned to carbonyl group. The medium band at 1462.98 cm^{-1} corresponding CH_2, CH_3 and C-H bond alkanes. The medium band at 1377.85 and 1243.76 cm^{-1} corresponding to $-NO_2$ stretching. The medium band at 1173.47 cm^{-1} corresponds to C-O group (ether). In the methanol extract of *K. pinnata* leaves, these functional groups are connected to the bioactive phytochemicals.

4.5.11.2 FT-IR spectrum of (methanol extract) 5% Acetone: Hexane

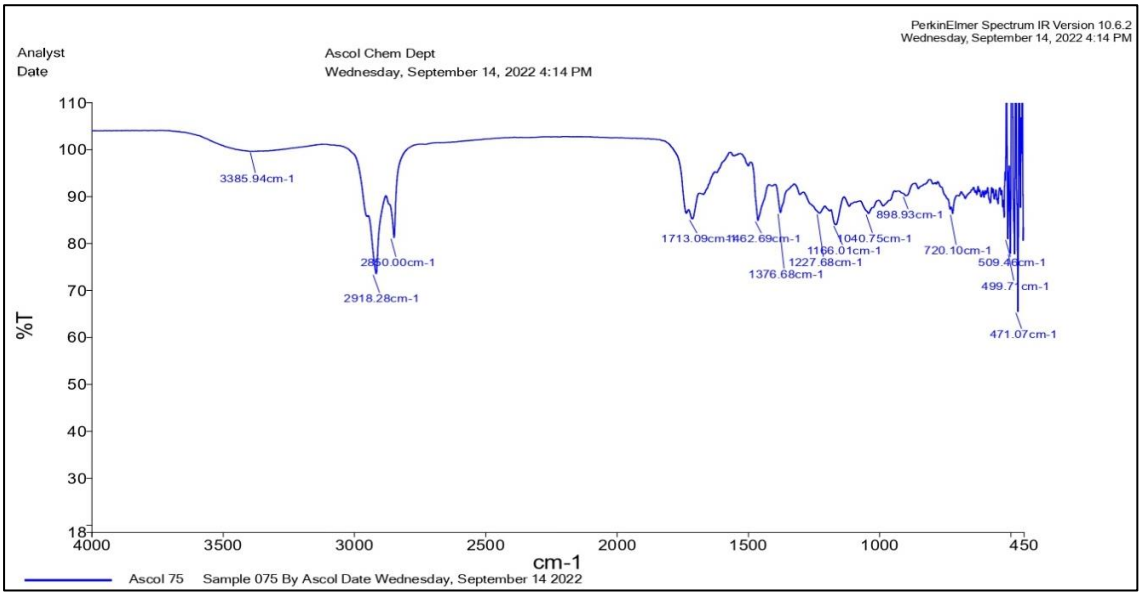


Figure 54: FT-IR spectrum of 5% (methanol extract) Acetone: Hexane

The table below shows the absorption, types of stretching and appearance of the 5% Acetone: Hexane.

Table 25: FTIR peak value and functional groups of 5% (methanol extract) Ac:Hex `

Absorption (cm^{-1})	Types of stretching	Appearance
3385.94	O-H	Weak
2918.28 2850.00	C-H	Strong
1713.09	C=O	Strong
1462.69	$-CH_2$	Medium
1376.68 1227.68	$-NO_2$	Medium
1040.75	C-O	Medium

The results were compared using an infrared chart of FTIR analysis to confirm the presence of functional groups such as the medium band was observed at 3385.94 cm^{-1} O-H stretching corresponding to hydroxy group. The strong bands were observed at 2918.28 and 2850.00 cm^{-1} corresponds to C-H stretching. The strong band at 1713.09 cm^{-1} indicates the presence of C=O stretching assigned to carbonyl group. The medium band at 1462.98 cm^{-1} corresponding $-CH_2$, $-CH_3$ and C-H bond alkanes. The medium band at 1376.68 cm^{-1} and 1227.68 cm^{-1} corresponding to $-NO_2$ group. The medium band at 1040.75cm^{-1} corresponds to C-O group (ether). The bioactive phytochemicals in the methanol extract of *K. pinnata* leaves are linked to these functional groups.

CHAPTER V: CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Following phytochemical analysis of several *K. pinnata* extracts, the findings showed that volatile oils, polyphenols, flavonoids, terpenoids, quinones, saponins, glycosides, reducing sugars, and tannins were all present. GC-MS analysis of hexane extract identified 7 main possible components while methanol extract identified 19 main possible components. Among them, 2 components were found to be similar in both extracts. The ethyl acetate extract (485.17 mg GAE/g) contained higher phenolic content than methanol extract (182.87 mg GAE/g). Additionally, the total flavonoid concentration in the ethyl acetate extract was higher (40.25 mg QE/g) than it was in the methanol extract (19.05 mg QE/g). The methanol extract showed higher antioxidant activity than ethyl acetate and chloroform extract.

The α-Amylase inhibition assay showed higher potential on methanol extract in comparison to ethyl acetate and acetone extract. All the extracts showed good antimicrobial activity. Among the three strains, *B. subtilis* showed higher zone of inhibition (ZOI), i.e., 0.85 cm. The cytotoxicity assay showed higher lethality concentration (LC_{50}) on chloroform extract (0.181 mg/mL) as comparison to acetone (0.175 mg/mL) and methanol extract (0.29 mg/mL).

The best TLC was shown by methanol extract in the Acetone: Hexane solvent system which was further run into a column. From the column chromatography, single spot is obtained at 1% Ac: Hex and 5% Ac: Hex. The methanol extract from the leaves of *K. pinnata* exhibited the existence of multiple bonds, aromaticity, conjugation, and unsaturated organic molecules in the UV-visible region, according to the UV spectrophotometer's results. The presence of O-H, C-H, C=O, $-CH_2$, $-NO_2$ and C-O were verified by FT-IR findings.

5.2 Suggestions for further work

The presence of numerous biologically active compounds in the plants, as revealed by phytochemical analysis, can be attributed to their potential biological and pharmacological activities. As a result, it is desirable to prepare plant extracts in

primary solvents using different extraction techniques than those described here, to extract a wider variety of phytochemicals in more significant quantities. Further research is required to elute novel active compounds from this plant, which may create a new way to treat various chronic diseases. Phytochemical screening, GC-MS, Antibacterial, Antioxidant, Antidiabetic, and NMR analysis are the topics of my dissertation. The interpretation of NMR spectra is still under investigation. As a result, there is still much work to be done before the interpretation is complete. The following subjects have been highlighted as possible future research areas:

- Only three microorganisms were used in the antimicrobial activity tests. More tests can also be performed on various microbes, which aids in developing new antimicrobics for the treatment of microbial disorders.
- The plant *K. pinnata* may contain a variety of significant substances. Therefore, it is possible to carry out identification, isolation, and structure elucidation.

REFERENCES

Abhijit, R. (2015). Phytochemical screening and in-vitro evaluation of antioxidant , cytotoxicity , antifungal activities of *Kalanchoe pinnata* (L). *Journal of Science and Arts*, *4*(33), 343–350.

Abubakar, A. R., & Haque, M. (2020). Preparation of medicinal plants: Basic extraction and fractionation procedures for experimental purposes. In *Journal of Pharmacy and Bioallied Sciences* (Vol. 12, Issue 1). https://doi.org/10.4103/jpbs.JPBS_175_19

Agarwal, H., & Shanmugam, V. K. (2019). Anti-inflammatory activity screening of *Kalanchoe pinnata* methanol extract and its validation using a computational simulation approach. *Informatics in Medicine Unlocked*, *14*. https://doi.org/10.1016/j.imu.2019.01.002

Agüero-Hernández, A. L., Rosales-López, C., Herrera, C., Vargas-Picado, A., Muñoz, R., & Abdelnour-Esquivel, A. (2020). Hypoglycemic effect of *Kalanchoe pinnata* (Lam) Pers. leaf extract. *Pharmacognosy Journal*, *12*(3). https://doi.org/10.5530/pj.2020.12.84

Akarsh, S., & Thippeswamy, B. (2020). Phytochemical analysis for secondary metabolites and in-vitro antimicrobial activity of *cuscuta reflexa*. *Plant Archives*, *20*(1).

Al-Rubaye, A. F., Hameed, I. H., & Kadhim, M. J. (2017). A Review: Uses of Gas Chromatography-Mass Spectrometry (GC-MS) Technique for Analysis of Bioactive Natural Compounds of Some Plants. *International Journal of Toxicological and Pharmacological Research*, *9*(01). https://doi.org/10.25258/ijtpr.v9i01.9042

Anulika, N., Ignatius, E., … E. R.-I. J. T., & 2016. (2016). The chemistry of natural product: Plant secondary metabolites. *Researchgate.Net*, *4*(August), 1.

Aoki, C., Hartati, S., Santi, M. R., Lydwina, Firdaus, R., Hanafi, M., Kardono, L. B. S., Shimizu, Y., Sudarmono, P., & Hotta, H. (2014). Isolation and identification of substances with anti-hepatitis c virus activities from *kalanchoe pinnata*. *International Journal of Pharmacy and Pharmaceutical Sciences*, *6*(2).

Aryan, Ruby, & Mehata, M. S. (2021). Green synthesis of silver nanoparticles using *Kalanchoe pinnata* leaves (life plant) and their antibacterial and photocatalytic activities. *Chemical Physics Letters*, *778*, 138760. https://doi.org/10.1016/J.CPLETT.2021.138760

Ashfak, K., Chowdhury, A., Huq, M. E., Ali, S., Huq, I., Royhan, M. J., Chy, N. U., Kabir, I., Bin, R., & Auniq, J. (2016). Antioxidant , cytotoxic and thrombolytic activity of leaves of *Kalanchoe pinnata* (LAM .) PERS . *Journal of Pharmacognosy and Phytochemistry*, *5*(4), 309–315.

Balachandran, I. & Sulaiman, C. T., (2012*).* Total Phenolics and Total Flavonoids in Selected Indian Medicinal Plants. *Indian Journal of Pharmaceutical Sciences, 74(3).* Https://Doi.Org/10.4103/0250-474X.106069.

Balasundram, N., Sundram, K., & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, *99*(1). https://doi.org/10.1016/j.foodchem.2005.07.042

Banu, K. S., & Cathrine, L. (2015). General Techniques Involved in Phytochemical Analysis. International Journal of Advanced Research in Chemical Science, 2(4).

Bodakhe, K. S., Namdeo, K. P., Bodakhe, S. H., & Pandey, D. P. (2013). A new flavonol glycoside from *kalanchoe pinnata* leaves. *Asian Journal of Chemistry*, *25*(17). https://doi.org/10.14233/ajchem.2013.15313

Braddick, H. J. J. (2015). Gas Chromatography–Mass Spectrometry (GC–MS). In *Nature* (Vol. 224, Issue 5217). https://doi.org/10.1038/224391a0

Chandra, S., Khan, S., Avula, B., Lata, H., Yang, M. H., Elsohly, M. A., & Khan, I. A.

(2014). Assessment of total phenolic and flavonoid content, antioxidant properties, and yield of aeroponically and conventionally grown leafy vegetables and fruit crops: A comparative study. *Evidence-Based Complementary and Alternative Medicine*, *2014*. https://doi.org/10.1155/2014/253875

Chaudhary, R. P., Uprety, Y., & Joshi, S. P. (2016). Plant Biodiversity in Nepal : Conservation and Legal Status. *Frontiers of Botany*, *December 2017*, 224–268.

Chintoju, N., Konduru, P., Kathula, R. L., & Remella, R. (2015). Importance of Natural Products in the Modern History. *Research & Reviews: Journal of Hospital and Clinical Pharmacy*, *1*(1), 5–10.

Coskun, O. (2016). Separation techniques: Chromatography. *North Clin Istanbul*, *3*(2), 156–160. https://doi.org/10.14744/nci.2016.32757

Cutrim, E. S. M., Teles, A. M., Mouchrek, A. N., Filho, M. V. E., & Everton, G. O. (2019). Evaluation of antimicrobial and antioxidant activity of essential oils and hydroalcoholic extracts of *zingiber officinale* (ginger) and *rosmarinus officinalis* (Rosemary). *Revista Virtual de Quimica*, *11*(1). https://doi.org/10.21577/1984-6835.20190006

da Silva, S. B., Barbosa, J. R., da Silva Martins, L. H., Rai, M., & Lopes, A. S. (2021). Traditional Uses, Phytochemicals and Pharmacological Properties of *Chenopodium ambrosioides* L. (*Dysphania ambrosioides*) *L. Mosyakin* & Clemants. *Ethnopharmacology of Wild Plants*, *February*, 234–245. https://doi.org/10.1201/9781003052814-14

Darmawan, A., Megawati, & Fajriah, S. (2013). 3',4'-Dimethoxy quercetin, a flavonol compound isolated from *Kalanchoe pinnata*. *Journal of Applied Pharmaceutical Science*, *3*(1). https://doi.org/10.7324/JAPS.2013.30116

Download as PDF - Wikipedia. Retrieved July 8, 2022, from https://en.wikipedia.org/w/index.php?title=Special:DownloadAsPdf&page=Disk_diffusion_test&action=show-download-screen

Esclapez, M. D., García-Pérez, J. V., Mulet, A., & Cárcel, J. A. (2011). Ultrasound-Assisted Extraction of Natural Products. *Food Engineering Reviews*, *3*(2), 108–120. https://doi.org/10.1007/s12393-011-9036-6

Fattorusso, E., Taglialatela-scafati, O., Eicher, L. F., Diederichsen, T., & Speicher, U. (2007). *Edited by The Way of Synthesis Medicinal Plant Biotechnology The Chemistry of Heterocycles Drug Bioavailability*.

Gul, R., Jan, S. U., Faridullah, S., Sherani, S., & Jahan, N. (2017). Preliminary Phytochemical Screening, Quantitative Analysis of Alkaloids, and Antioxidant Activity of Crude Plant Extracts from *Ephedra intermedia* Indigenous to Balochistan. *Scientific World Journal*, *2017*(Figure 1). https://doi.org/10.1155/2017/5873648

Hanson, J. R. (2003). *Natural products: The Secondary Metabolites*. Royal Society of Chemistry.

Hassan, W., & Zainab Kazmi, S. N. (2015). Antimicrobial Activity of *Cinnamomum tamala* Leaves. *Journal of Nutritional Disorders & Therapy*, *06*(02). https://doi.org/10.4172/2161-0509.1000190

Kalanchoe pinnata - *Wikipedia*. Retrieved November 23, 2021, from https://en.wikipedia.org/wiki/*Kalanchoe_pinnata*

Kalemba, D., & Kunicka, A. (2005). Antibacterial and Antifungal Properties of Essential Oils. *Current Medicinal Chemistry*, *10*(10). https://doi.org/10.2174/0929867033457719

Kamal, Y., Ch, B. A., Uzair, M., Irshad, N., Yaseen, M., & Hussain, I. (2014). Different extracts of leaves of *Kalanchoe Pinnata*. *Journal of Clinical Microbiology*, *6*(4), 446–450.

Kasali, F. M., Kadima, J. N., Tusiimire, J., Ajayi, C. O., & Ganafa Agaba, A. (2022). *Effects of the Oral Administration of Aqueous and Methanolic Leaf Extracts of*

Chenopodium ambrosioides L. (Amaranthaceae) on Blood Glucose Levels in Wistar Rats. https://doi.org/10.2147/JEP.S356564

Koyama, K., Ikeda, H., Poudel, P. R., & Goto-Yamamoto, N. (2012). Light quality affects flavonoid biosynthesis in young berries of Cabernet Sauvignon grape. *Phytochemistry*, *78*. https://doi.org/10.1016/j.phytochem.2012.02.026

Kumar, S., & Pandey, A. K. (2013). Chemistry and Biological Activities of Flavonoids. *TheScientificWorldJournal*, *2013*, 162750.

Kunwar, R. M., Mahat, L., Acharya, R. P., & Bussmann, R. W. (2013). Medicinal plants, traditional medicine, markets and management in far-west Nepal. *Journal of Ethnobiology and Ethnomedicine*, *9*(1), 1–10. https://doi.org/10.1186/1746-4269-9-24

Kurek, J. (2019). Introductory Chapter: Alkaloids - Their Importance in Nature and for Human Life. *Alkaloids - Their Importance in Nature and Human Life*, 1–7. https://doi.org/10.5772/intechopen.85400

Majaz, Q. (n.d.). THE MIRACLE PLANT (*KALANCHOE PINNATA*): A PHYTOCHEMICAL AND PHARMACOLOGICAL REVIEW. *International Journal of Research in Ayurveda & Pharmacy*, *2011*(5), 1478–1482. www.ijrap.net

Matthew, S., Khosla, K. K., Matthew, C., & Bhowmik, D. (2013). Preliminary Phytochemical Studies Of *Kalanchoe pinnata* (Lam.) Pers. *Journal of Medicinal Plants Studies Year*, *1*, 19–23. www.plantsjournal.com

Mendoza, N., & Silva, E. M. E. (2018). Introduction to Phytochemicals: Secondary Metabolites from Plants with Active Principles for Pharmacological Importance. In *Phytochemicals - Source of Antioxidants and Role in Disease Prevention*. https://doi.org/10.5772/intechopen.78226

Mera, I. F. G., Falconí, D. E. G., & Córdova, V. M. (2019). Secondary metabolites in

plants: Main classes, phytochemical analysis and pharmacological activities. In *Bionatura* (Vol. 4, Issue 4). https://doi.org/10.21931/RB/2019.04.04.11

Molyneux, P. (2004). The Use of the Stable Free Radical Diphenylpicryl-hydrazyl (DPPH) for Estimating Antioxidant Activity. *Songklanakarin Journal of Science and Technology*, *26*(December 2003). https://doi.org/10.1287/isre.6.2.144

Mule, P., Upadhye, M., Taru, P., & Dhole, S. (2020). A Review on Bryophyllum pinnatum (Lam.) Oken . *Research Journal of Pharmacognosy and Phytochemistry*, *12*(2), 111. https://doi.org/10.5958/0975-4385.2020.00019.9

Nepal Tourism Board. (2018). Geography of Nepal. *Nepal Tourism Board*, *2*(0 mi).

Nirmali Wickramaratne, M., Punchihewa, J. C., & Wickramaratne, D. B. M. (2016). *In-vitro alpha amylase inhibitory activity of the leaf extracts of Adenanthera pavonina*. https://doi.org/10.1186/s12906-016-1452-y

Otohinoyi, D., Ekpo, O., & Ibraheem, O. (2014). Effect of ambient temperature storage on 2,2-diphenyl-1-picrylhydrazyl (DPPH) as a free radical for the evaluation of antioxidant activity. *International Journal of Biological and Chemical Sciences*, *8*(3). https://doi.org/10.4314/ijbcs.v8i3.39

Padma, M., Ganesan, S., Jayaseelan, T., Azhagumadhavan, S., Sasikala, P., Senthilkumar, S., & Mani, P. (2019). Phytochemical screening and GC–MS analysis of bioactive compounds present in ethanolic leaves extract of Silybum marianum (L). *Journal of Drug Delivery and Therapeutics*, *9*(1), 85–89. https://doi.org/10.22270/jddt.v9i1.2174

Panche, A. N., Diwan, A. D., & Chandra, S. R. (2016). Flavonoids: An overview. *Journal of Nutritional Science*, *5*. https://doi.org/10.1017/jns.2016.41

Parajuli, A., Chand, D. B., Rayamajhi, B. K., & Khanal, R. (2015). *Spatial and temporal distribution of forest fires in Nepal Invasion of alien plant species and their impact on different ecosystems of Panchase Area, Nepal View project*.

September, 7–11.

Patel, K., Panchal, N., & Ingle, P. (2019). Techniques Adopted for Extraction of Natural Products Extraction Methods: Maceration, Percolation, Soxhlet Extraction, Turbo distillation, Supercritical Fluid Extraction. *International Journal of Advanced Research in Chemical Science*, *6*(4). https://doi.org/10.20431/2349-0403.0604001

Pattewar, S. V. (2012). *Kalanchoe Pinnata* : Phytochemical and Pharmacological Profile. *International Journal of Phytopharmacy*, *2*(1), 1–8. https://doi.org/10.7439/ijpp.v2i1.223

Pereira, K. M. F., Grecco, S. S., Figueiredo, C. R., Hosomi, J. K., Nakamura, M. U., Henrique, J., & Lago, G. (2018). *Chemical Composition and Cytotoxicity of Kalanchoe pinnata Leaves Extracts prepared using Accelerated System Extraction (ASE)*.

Pertuit, A. J. (1992). *Kalanchoe*. *Introduction to Floriculture*, 429–450. https://doi.org/10.1016/B978-0-12-437651-9.50022-1

Phatak, R. S. (2015). GC-MS analysis of bioactive compounds in the methanolic extract of *Kalanchoe pinnata* fresh leaves. *Journal of Chemical and Pharmaceutical Research*, *7*(3).

Phulpoto, A. H., Qazi, M. A., Mangi, S., Ahmed, S., Ihsan-Ul-Haq, Phul, A. R., & Kanhar, N. A. (2017). Bioremediation of oil-based paint from aqueous media by novel indigenous brevibacillus parabrevis strain NAP3 and its toxicity assessment. *Polish Journal of Environmental Studies*, *26*(1), 229–237. https://doi.org/10.15244/pjoes/62905

Poojar, B., Ommurugan, B., Adiga, S., Thomas, H., Sori, R. K., Poojar, B., Hodlur, N., Tilak, A., Korde, R., Gandigawad, P., In, M., Sleep, R., Albino, D., Rats, W., Article, O., Schedule, P., Injury, C. C., Sori, R. K., Poojar, B., … Gandigawad, P. (2017). Methodology Used in the Study. *Asian Journal of Pharmaceutical and Clinical Research*, *7*(10), 1–5. https://doi.org/10.4103/jpbs.JPBS

R. Hamidi, M., Jovanova, B., & Kadifkova Panovska, T. (2014). Toxicological evaluation of the plant products using Brine Shrimp (*Artemia salina* L.) model. *Macedonian Pharmaceutical Bulletin*, *60*(01), 9–18. https://doi.org/10.33320/maced.pharm.bull.2014.60.01.002

Rajendran, A., Rafiqkhan, M., Selvam, D., & Thangaraj, V. (2017). Pharmacognostic Profile and Phytochemical Analysis of *Cinnamonum Zeylanicum* Bark Extracts. *33 Research Article*, *5*(1), 33–39. http://nehrucolleges.net/jo.html

Rajsekhar, P. B., Arvind Bharani, R. S., Ramachandran, M., Jini Angel, K., & Rajsekhar, S. P. V. (2016). The "wonder plant" *Kalanchoe pinnata* (linn.) pers.: A review. *Journal of Applied Pharmaceutical Science*, *6*(3). https://doi.org/10.7324/JAPS.2016.60326

Rani, N., Jajpura, L., & Butola, B. S. (2020). Sustainable Coloration of Protein Fibers Using *Kalanchoe-Pinnata* Leaf Extract. *Journal of Natural Fibers*. https://doi.org/10.1080/15440478.2020.1731904

Rashmi, Singh, A., Jain, A., Kaushal, P., Bhatia, D., & Malik, D. K. (2012). Antimicrobial and Antioxidant Activities of *Kalanchoe pinnata* Against Pathogens. *Journal of Pharmacy Research*, *5*(10).

Rehman, A. and. (n.d.). *No Title*. Akash, M. S. H., & Rehman, K. (2019). Column Chromatography. In Essentials of %0APharmaceutical Analysis (pp. 167–174). Springer, Singapore.

S. Chandra Mohan*, V. B. , R. E. and A. S. P. (2012). Antioxidant and Phytochemical Potential of Medicinal Plant *Kalanchoe Pinnata. International Journal of Pharmaceutical Sciences and Research*, *3*(3), 881–885. www.ijpsr.com

Santos, S., Zurfluh, L., Mennet, M., Potterat, O., von Mandach, U., Hamburger, M., & Simões-Wüst, A. P. (2021). *Bryophyllum pinnatum* Compounds Inhibit Oxytocin-Induced Signaling Pathways in Human Myometrial Cells. *Frontiers in Pharmacology*, *12*. https://doi.org/10.3389/fphar.2021.632986

Saraf, A., & Saraf, A. (2014). Phytochemical and antimicrobial studies of medicinal plant *piper longum* Linn. *International Journal of Pharmacognosy and Phytochemical Research*, *6*(2), 213–222.

Sarah, Q. S., Anny, F. C., & Misbahuddin, M. (2017). Brine shrimp lethality assay. *Bangladesh Journal of Pharmacology*, *12*(2), 186–189. https://doi.org/10.3329/bjp.v12i2.32796

Sermakkani, M., & Thangapandian, V. (2012). GC-MS analysis of *Cassia italica* leaf methanol extract. *Asian Journal of Pharmaceutical and Clinical Research*, *5*(2), 90–94.

Sharadanand Phatak, R., Subhash Hendre, A., & Rohan Sharadanand Phatak, C. (2014). Total antioxidant capacity (TAC) of fresh leaves of *Kalanchoe pinnata*. *Journal of Pharmacognosy and Phytochemistry JPP*, *2*(25).

Sharma, M. (2020). *Kalanchoe Pinnata* and Its Remedial Properties To Treat Kidney Stone– a Lesser Known Plant. *International Journal of Biology, Pharmacy and Allied Sciences*, *9*(11). https://doi.org/10.31032/ijbpas/2020/9.11.5259

Silverman, R. B., & Holladay, M. W. (2015). The Organic Chemistry of Drug Design and Drug Action: Third Edition. In The Organic Chemistry of Drug Design and Drug Action: Third Edition. https://doi.org/10.1016/C2009-0-64537-2

Singh, S. K., Patel, J. R., & Dangi, A. (2019). Physicochemical, Qualitative and Quantitative Determination of Secondary Metabolites and Antioxidant Potential of *Kalanchoe Pinnata* (Lam.) Pers. Leaf Extracts. *Journal of Drug Delivery and Therapeutics*, *9*(1). https://doi.org/10.22270/jddt.v9i1.2225

APPENDICES

A. Phytochemical Screening Protocol

1. Test for Volatile Oils

To about 500 mg extract, 0.5 mL methanol was added, shaken vigorously, and filtered. A few drops of the filtrate were put on a filter paper using a capillary tube.
Yellow spot persistent even after evaporation of the solvent indicates the presence of volatile oils.

2. Test for Alkaloids

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

i. Mayer's Test

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

ii. Dragendroff's Test

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

3. Test for Terpenoids

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

4. Test of Flavonoids (Shinoda's Test)

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

5. Test for Phenolic Compounds/ $FeCl_3$ Test

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

6. Test for Glycosides

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

i. Molisch's Test

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

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

7. Test for Reducing Sugars

To about 1 mL extract, 1 mL distilled water was added followed by 1 mL Fehling's reagent (1:1 mixture of Fehling's solution A and B). Then the mixture was warmed over a water bath for 30 minutes. The appearance of a red brick precipitate indicates the presence of reducing sugars.

8. Test for Quinones

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

9. Test for Saponins

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

10. Test for Tannins

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

B. Preparation of reagents

1. Mayer's Reagent

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

2. Dragendroff's Reagent

Bismuth nitrate, $Bi(NO_3)_3$ (4.000 g) was dissolved in 5 N nitric acid (10 mL) to make solution A. Next, potassium iodide, KI (13.5 g) was dissolved in distilled water (20 mL) to make solution B. These two solutions were mixed in a 50 mL volumetric flask. Picric acid (0.25 g) was dissolved in 50 mL of distilled water to make an aqueous picric acid solution. The solution was neutralized with sodium bicarbonate ($NaHCO_3$).

3. Molisch's Reagent

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

4. Neutral Ferric Chloride ($FeCl_3$) Solution

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

Photos



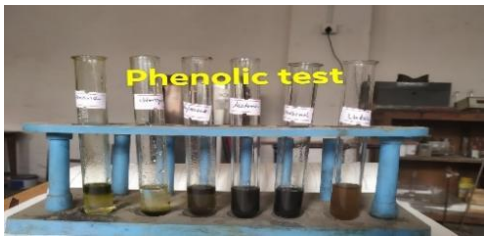
Sample collection

Ultrasonic extraction

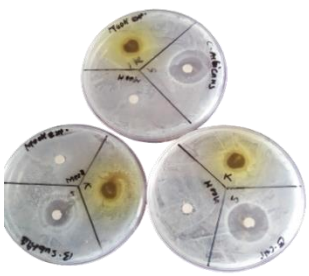
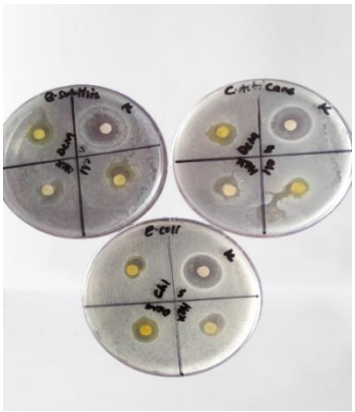

Filtration of extract


Rota evaporator

Extract collection

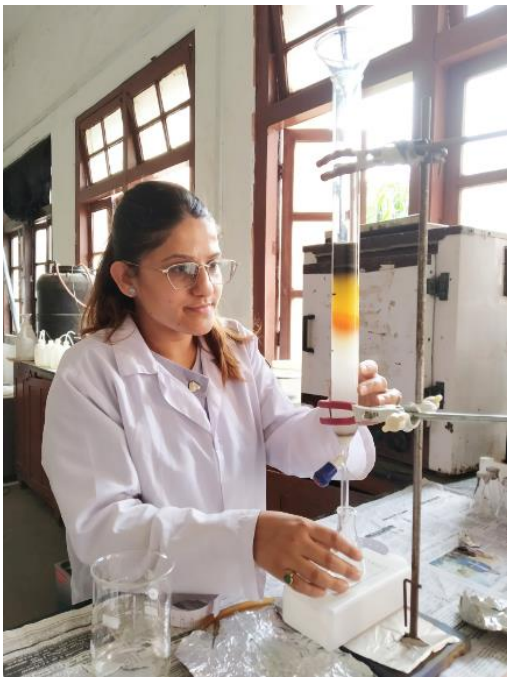


Phytochemical screening

ZOI of *K. pinnata* leaves extracts

Performing TLC



Column chromatography