CHEMICAL AND BIOLOGICAL STUDIES OF *Achyranthes aspera L.* FROM MAHOTTARI DISTRICT OF NEPAL

A DISSERTATION SUBMITTED TO THE

DEPARTMENT OF CHEMISTRY

AMRIT CAMPUS

INSTITUTE OF SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

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

By

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Symbol No.: CHE 1884/2076

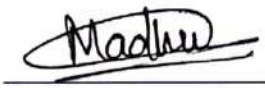
T.U. Regd. No.: 5-2-0037-0363-2015

MARCH, 2024

DECLARATION

This dissertation entitled **"CHEMICAL AND BIOLOGICAL STUDIES OF *Achyranthes aspera L.* FROM MAHOTTARI DISTRICT OF NEPAL"** is being submitted to the Department of Chemistry, Amrit Campus, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the partial fulfillment of the Master of Science (M.Sc.), degree in Chemistry is a research work carried out by me under the supervision of Assoc. Prof. Dr. Ram Lal (Swagat) Shrestha Department of Chemistry, Amrit Campus, Tribhuvan University, Nepal.

I, Madhu Kumari Chaudhary, hereby declare that this work presented herein is genuine and originally done by me and has not been published or submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.



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RECOMMENDATION LETTER

This is recommended that the dissertation work entitled **"CHEMICAL AND BIOLOGICAL STUDIES OF *Achyranthes aspera L.* FROM MAHOTTARI DISTRICT OF NEPAL"** had been carried out by Ms. Madhu Kumari Chaudhary as partial fulfillment for the Master's Degree in Chemistry under my supervision. To the best of my knowledge, this work has not been submitted for any degree to any institution.

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ACKNOWLEDGEMENTS

The successful completion of this study was possible with the support, assistance, motivation, coordination, and suggestions of various people. I would like to express my wholehearted gratitude and appreciation to my Supervisor, **Assoc. Prof. Dr. Ram Lal (Swagat) Shrestha**, and Co-supervisor **Assoc. Prof. Dr**. **Bhushan Shakya**, M.Sc. Program Coordinator, Department of Chemistry, Amrit Campus, for his supervision, inspirable guidance, invaluable suggestions, and pragmatic touch throughout this work.

I wish to express my thanks to **Prof. Dr. Daman Raj Gautam**, Head of the Department of Chemistry, for providing me with such a great opportunity.

This study could not have been prepared so far without the continuous support and encouragement of my respected professors, lecturers, seniors, and my dual classmates. I would also like to convey my thanks to all the administrative staff as well as the laboratory staff of the department 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 a significant number of thanks to all of my friends for their continuous support, and invaluable assistance in coordination till the completion of the research.

Finally, but immensely, my most sincere obligation goes to my father, Mr. **Raj Narayan Chaudhary**, and mother, **Mrs. Sundar Devi Chaudhary**, family members from the core of my heart for their never-ending love and support in every path of my efforts.

Thanks to all of you

Madhu Kumari Chaudhary

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 sulphoxide
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
QE	:	Quercetin Equivalent
TAC	:	Total Antioxidant Capacity
TFC	:	Total Flavonoid Content
TPC	:	Total Phenolic Content
TLC	:	Thin Layer Chromatography
UV	:	Ultra-Violet
ZOI	:	Zone of Inhibition

LIST OF SYMBOLS

μ	:	micro
%	:	percentage
°	:	degree
α	:	alpha
π	:	pi
β	:	beta

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ABSTRACT

The study delves into a comprehensive exploration of *Achyranthes aspera* L., a member of the Amaranthaceae family renowned for its medicinal properties, often encountered in tropical and warm climates as a prolific weed. Central to this investigation are the intricate evaluations of its antioxidant, antibacterial, and cytotoxic potentials, coupled with a meticulous analysis aimed at identifying its bioactive phytochemical constituents. Employing an ultrasonic extraction technique, five distinct solvents were utilized—namely hexane, chloroform, ethyl acetate, methanol, and distilled water—to isolate the plant's bioactive compounds. Notably, the methanol extract emerged as the most prolific, boasting a substantial yield of 16.28 g. Through comprehensive phytochemical screening, the presence of alkaloids, flavonoids, phenolic compounds, terpenoids, carbohydrates, and tannins was confirmed, indicating the rich biochemical diversity inherent in *A. aspera*. Chloroform extract demonstrated potential antimicrobial activity against Gram-positive bacteria in the antimicrobial susceptibility test conducted over various bacterial cultures. The Total Phenolic Content value for hexane (15.86 mg GAE/g) extracts was high, according to the quantitative illustration of phytochemicals. Concerning extracts of chloroform (205.039 mg QE/g), the Total Flavonoid Content value was high. In the DPPH free radical scavenging assay, the highest IC_{50} values for methanol and the least distilled water extract were determined to be 300.66 µg/mL and 1272.83 µg/mL, respectively. The α-amylase inhibition properties were assayed using the DNSA technique, and the highest and the lowest IC_{50} values for chloroform and hexane extracts were 488.334 µg/mL and 1489.208 µg/mL, respectively. In the brine shrimp lethality assay, the highest and the lowest LC_{50} concentration values of hexane (132.55 µg/mL) and distilled water (399.81) µg/mL, respectively. TLC analysis complemented these findings, revealing distinct spot patterns among various extracts, indicative of diverse phytochemical compositions. Altogether, these findings illuminate the multifaceted pharmacological potential of *A. aspera*, underscoring its significance as a reservoir of bioactive compounds with diverse therapeutic applications.

Keywords: *Achyranthes aspera*, phytochemical screening, antioxidant, cytotoxicity, α-amylase inhibition.

सोधसार

यस अध्ययनले औषधीय गुणहरूका लागि प्रख्यात अमारान्थेस परिवारको सदस्य *Achyranthes aspera* L. को विस्तृत अन्वेषणमा समावेश गर्दछ, प्रायः उष्णकटिबंधीय र न्यानो मौसममा प्रशस्त झारको रूपमा सामना गरिन्छ। यस अनुसन्धानको केन्द्रबिन्दु यसको एन्टिअक्सिडेन्ट, एन्टिब्याक्टेरियल, र साइटोटोक्सिक क्षमताहरूको जटिल मूल्याङ्कन गर्नुका साथै, यसको बायोएक्टिभ फाइटोकेमिकल घटकहरूको पहिचान गर्नु हो। अल्ट्रासोनिक निकासी प्रविधि प्रयोग गर्दै, बिरुवाको बायोएक्टिभ यौगिकहरूलाई अलग गर्नको लागि पाँचवटा भिन्न विलायकहरू - अर्थात् हेक्सेन, क्लोरोफर्म, इथाइल एसीटेट, मेथानोल र डिस्टिल्ड वाटर प्रयोग गरियो। उल्लेखनीय रूपमा, 16.28 ग्रामको साथै, मिथानोल निकासी सबैभन्दा फलदायी रूपमा देखा पऱ्यो। व्यापक फाइटोकेमिकल स्क्रिनिङ मार्फत, एल्कालोइड्स, फ्लेभोनोइड्स, फेनोलिक यौगिकहरू, टेरपेनोइड्स, कार्बोहाइड्रेट र ट्यानिन्सको उपस्थिति पुष्टि भयो, जसले ए. *एस्पेरा* मा निहित समृद्ध जैव रासायनिक विविधतालाई संकेत गर्दछ। क्लोरोफर्म एक्स्ट्र्याक्टले विभिन्न ब्याक्टेरियल संस्कृतिहरूमा आयोजित एन्टिमाइक्रोबियल संवेदनशीलता परीक्षणमा ग्राम-सकारात्मक ब्याक्टेरिया विरुद्ध सम्भावित एन्टिमाइक्रोबियल गतिविधि प्रदर्शन गऱ्यो। फाइटोकेमिकल्सको मात्रात्मक चित्रण अनुसार, हेक्सेन (15.86 mg GAE/g) को लागि TPC मान उच्च थियो। क्लोरोफर्म (205.039 mg QE/g) को अर्काको सम्बन्धमा, TFC मान उच्च थियो। DPPH फ्री रेडिकल स्क्याभेन्जिङको लागि परखमा, मेथानोलको लागि उच्चतम IC_{50} मान र सबैभन्दा कम डिस्टिल्ड वाटर एक्स्ट्र्याक्ट क्रमशः 300.66 µg/mL र 1272.83 µg/mL निर्धारण गरिएको थियो। α-amylase निषेध गुणहरू DNSA प्रविधि दुवारा गरिएको थियो, र क्लोरोफर्म र हेक्सेन एक्स्ट्र्याक्टहरूको लागि उच्चतम र सबैभन्दा कम IC_{50} मानहरू क्रमशः 488.334 µg/mL र 1489.208 µg/mL थियो । ब्राइन झिंगा घातकता परखमा, क्रमशः हेक्सेन (132.55 µg/mL) र डिस्टिल्ड वाटर (399.81) µg/mL को उच्चतम र सबैभन्दा कम LC_{50} एकाग्रता मानहरू रहेको छ। TLC विश्लेषणले यी निष्कर्षहरूलाई पूरक बनायो । समग्रमा, यी खोजहरूले ए. एस्पेराको बहुमुखी औषधीय क्षमतालाई प्रकाश पार्छ, विविध उपचारात्मक अनुप्रयोगहरूसँग बायोएक्टिभ यौगिकहरूको भण्डारको रूपमा यसको महत्त्वलाई जोड दिन्छ।

खोजशब्द: *Achyranthes aspera*, फाइटोकेमिकल स्क्रीनिंग, एंटीऑक्सिडेंट, साइटोटोक्सिसिटी, α-amylase अवरोध।

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CHAPTER I: INTRODUCTION

1.1 Background

Nepal is surrounded by China's Tibet on one side and India on three sides. By the short Siliguri Corridor in West Bengal, Nepal and Bangladesh are divided from each other. The majority of it is mountainous, and it has eight of the ten highest peaks on earth. Along with a wide variety of habitats and animals, it also has mountains, rocky gorges, rivers, valleys, and flatlands (Rai *et al*., 2001). From frigid to tropical, Nepal's climate ranges. The climate zones of mountain ranges vary. In different physiographic and climatic zones, vegetation types are the best at describing habitat associations. The use of vegetation and habitat designations interchangeably can therefore be misleading. There are ranges from a simple mosaic of just one type of vegetation to a complex mosaic made up of several diverse plant forms (Ives & Messerli, 1981).

The diverse flora and fauna of Nepal are a result of its special geographic location, which ranges from subtropical deciduous and coniferous woodlands to alpine and sub-alpine meadows and snowcapped Himalayan peaks with their chilly streams, glaciers, and lakes. Nepal is home to more than 5,000 flowering plants, 118 ecosystems, 3% pteridophytes and 6% bryophytes, 181 mammal species, 844 bird species, 185 fish species, more than 635 butterfly species, and more than 2252 moth species (Shrinet *et al*., 2021). WHO estimates that 65-80% of people in developing countries utilize medicinal and aromatic herbs as therapies. Similarly, Nepal is a country where it is common practice to use common plants to cure minor illnesses. The Nepalese people solely use plant medicines to treat a variety of major medical illnesses, including snakebites, diabetes, kidney stones, and other ailments. To stay healthy, 70% to 80% of the people in Nepal use medicinal herbs. There are 1,463 different varieties of herbal medicinal plants used by rural Nepalese to treat a range of diseases (Kahardipraja *et al*., 2020). For their phototherapeutic characteristics, as well as for the treatment of digestive and respiratory ailments; they also serve as antioxidants; their scents serve as preservatives; and local people employ herbal plants in great amounts in traditional medicine (Baskar *et al*., 2012).

All plants produce chemical compounds that give them an evolutionary advantage, such as salicylic acid, a hormone used in plant defenses. If established, the composition and

pharmacological properties of these chemicals in medicinal plants serve as the scientific foundation for their use in contemporary medicine. These plants have the potential to be used as medicines because they contain specific chemical elements, which are technically referred to as phytochemicals. Plants naturally create phytochemicals, which are non-nutritive, bioactive compounds that act as a defense against pathogenic attack and environmental stress. Plants contain a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids (Brijyog *et al*., 2019). These compounds are taxonomically varied and can be used to create new treatments depending on how they work. A range of recently found plant extracts and products have been considered to have the ability to assist address this demand since natural alternatives to pesticides are becoming more and more in demand (Ngoc *et al*., 2014). It is an act of human solidarity and life-saving to study these medicinal herbs.

1.2 Medicinal Plants

Since ancient times, medicinal plants have been utilized to cure human illnesses by restoring physiological balance and training bodily tissues; some of these treatments are thought to improve health and maintain organic resistance to infection (Srivastava, 2014) . Herbal remedies are a mainstay of Nepalese traditional medicine. Since 1995, traditional medicine in Nepal, particularly ethnomedicine, has grown in acceptance. The COVID-19 outbreak has increased the use of medicinal herbs (Rajbhandari *et al*., 2020). Many early drugs, including aspirin, digoxin, morphine, quinine, and pilocarpine, were based on the clinical, pharmacological, and chemical studies of these ancient medicines, which were mostly derived from plants (Jamshidi-Kia *et al*., 2018). As there are around 500,000 plants in the globe, the majority of which have not yet been studied in medical practice, and as present and future studies on medical activities can be effective in treating a variety of illnesses, medicinal herbs have a promising future. Global market demand for plant-derived chemicals, pharmaceuticals, perfumes, flavors, and color compounds alone exceeds several billion dollars annually. The industrial applications for medicinal plants are shown in Figure 1 (Kunwar *et al*., 2013).

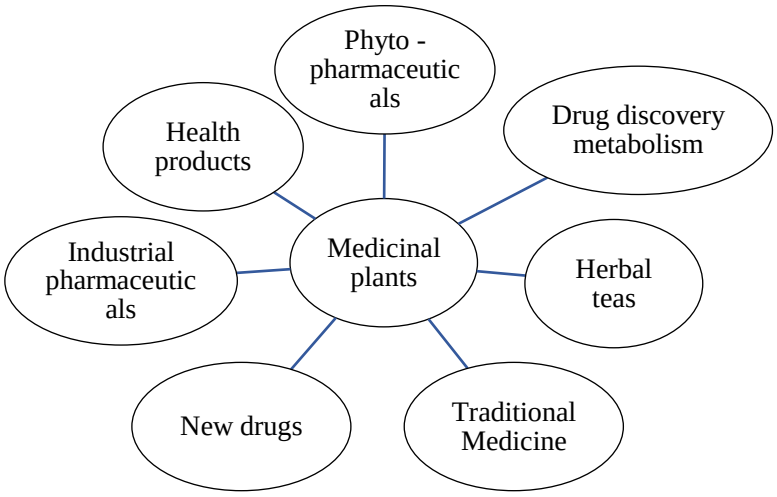


Figure 1: Industrial uses of Medicinal plant

1.3 Natural Product

"Natural products" relate to chemical substances or substances isolated from biological things. It could show up as either primary or secondary metabolites. Organic substances or phytochemicals that aren't directly engaged in the plant's typical growth, development, or reproduction are known as secondary metabolites in plants (Khandagle *et al*., 2011). Plant alkaloids, one of the most diverse classes of natural compounds, are made up of numerous unique chemical components. Secondary metabolites (SM) are categorized based on their chemical make-up (such as the presence of rings or sugars), content (such as the amount of nitrogen), solubility in organic solvents or water, and manufacturing method. The most common method used to categorize SM in plants is the biosynthetic pathway (Liu, 2022). This suggests that SM in plants can be divided into three major categories: terpenes, phenolic compounds, and alkaloids (Sharma *et al*., 2013). With over 40,000 molecules, terpenes are the largest plant SM group. Since fatty acids don't get in the way, chemically speaking, they are non-saponifiable lipids. Since isoprene serves as their primary structural component, they are referred to as isoprenoids (Yadav *et al*., 2016). Phenolic compounds have at least one hydroxyl group joined to one or more aromatic rings in their chemical structure. The majority of phenolic compounds are biosynthesized from shikimic acid and are water-soluble (Aparna & Aruna, 2014). Even though people have utilized plants containing alkaloids for at least 3000 years as medicines, teas, and potions, the molecules responsible for their effects weren't identified and defined until the nineteenth century. Alkaloids can

combine with mineral or organic acids to generate salts due to their fundamental makeup (Unissa *et al*., 2017). Alkaloid salts typically dissolve in water and diluted alcohols but, with a few rare exceptions, they are not soluble in organic solvents. Desired and unwanted chemicals are produced during extraction in a variety of different forms, some of which can be significantly different from those that have previously been removed (Huang *et al*., 2021). For extracting desired components from natural materials, some more recent or environmentally friendly extraction techniques include supercritical fluid extraction (SFC), pressurized liquid extraction (PLE), and microwave-assisted extraction (MAE). To employ natural products as herbal remedies, evaluate the biological activities of secondary metabolites, or isolate a known mixture of components, natural product extraction is necessary (Dias *et al*., 2012). The following are some common extraction techniques:

- Maceration: This extremely simple extraction technique has the drawbacks of a lengthy extraction time and poor extraction efficiency. Using this technique, a plant sample is submerged in a specific solvent while the constituent elements from the plant are extracted. It might be used to extract thermolabile components.

- Percolation: During this procedure, a percolator is used. This glass object has apertures on both sides and is shaped like a little cone. A dried, ground, and powdered plant material is soaked with the extraction solvent in a clean container. The combination is held for four hours after being given a bigger amount of solvent. After that, the mixture is poured into a percolator, its lower end sealed and allowed to stand for 24 hours. Once the drug material is completely saturated, the extraction solvent is subsequently poured from the top. The lower section of the percolator is then unlocked, enabling the liquid to drip gradually. The extraction is carried out by gravity force, which forces the solvent downward through the plant material, adding a constant amount of solvent. The amount of solvent added came to an end when it reached 75% of the preparations' total intended volume. The extract is divided by filtration and decantation. The marc is then expressed, and the last bit of solvent is added to get the needed volume. In contrast to maceration, percolation is a continuous

process in which the saturated solvent is continuously replaced by a new solvent. This makes percolation more efficient than maceration.

- ❖ Ultrasonic-assisted extraction (UAE): Ultrasonic wave energy is utilized in the extraction process in ultrasonic-assisted extraction (UAE), also known as ultrasonic extraction or sonication. The extraction efficiency is enhanced by the use of ultrasound, which causes cavitation in the solvent and speeds up the solute's dissolution and diffusion as well as the transmission of heat. The reduced energy and solvent consumption of the UAE, as well as the shortened extraction time and temperature, are further benefits. The extraction of unstable and thermolabile chemicals is applicable in the UAE. Many different kinds of natural goods are frequently extracted in the UAE.

Diverse techniques were employed to determine the composition of an extract from medicinal plant material. It comprised the identification of functional groups, the presence of multiple bonds and rings, the positioning of hydrogen and carbon, and a thorough structural explanation. A few of the methods that have been employed include mass spectroscopy (MS), ultraviolet spectroscopy (UV), nuclear magnetic resonance spectroscopy (NMR), and infrared spectroscopy (Chandrasekar *et al*., 2014).

1.4. Introduction of plant *Achyranthes aspera*

In ancient Ayurvedic and Unani medical literature, there is a wealth of knowledge, information, and advantages of herbal medicines. Many of the organic chemicals found in the plant kingdom have been employed for therapeutic and other purposes, making it a rich source of these substances (Tripathy *et al*., 2017). Because of their wide availability of chemical variety, natural products—whether they be pure chemicals or standardized plant extracts—offer more potential for new therapeutic leads (Jaisankar *et al*., 2020). Since the dawn of time, people have employed plants for their therapeutic benefits. Today, many effective and strong medications are derived from these plants (Sharma *et al*., 2013).

Achyranthes aspera L. (*A. aspera*) is an erect or procumbent, annual, or perennial herb of about 1-2 m in height, often with a woody base. Stems angular, ribbed, simple or branched from the base, often with tinged purple color, branches terete or quadrangular, striate, pubescent, leaves thick, ovate–elliptic or obovate–rounded (Pavithra *et al*.,

2021), finely and softly pubescent on both sides, entire petiolate, petiole 6 – 20 mm long, flowers greenish white, numerous in axillary or terminal spikes up to 75 cm long, seeds subcylindric, truncate at the apex, rounded at the base, reddish brown (Vijayaraj & Vidhya, 2016). The plant's therapeutic and medical properties contain antiperiodic, antiasthmatic, hepatoprotective, anti-allergic, expectorant, stomach tonic, laxative, antihelmintic, diuretic, linthontriptic, sudorific, demulcent, anti-inflammatory, anticataract, antifungal, antibacterial, hypoglycemic, antihyperlipidemic and haematinic and various other important medicinal properties (Awasthi *et al*., 2021).

The herb *A. aspera* is frequently used to treat asthmatic cough, snakebite, hydrophobia, urinary calculi, rabies, influenza, piles, bronchitis, diarrhea, renal dropsy, gonorrhea, and abdominal pain. In the early stages of asthma, a mixture of honey and dried leaf powder is helpful (Manandhar *et al*., 2021). It is astringent, anti-phlegmatic, antiperiodic, diuretic, purgative, and laxative, helpful for oedema, piles, dropsy, boils, and skin eruptions, among other conditions. Plant debris is cooked in water and used to treat pneumonia. A mild astringent is produced when the root is infused for intestinal issues. The flowering spikes or seeds are pulverized and mixed into a paste with water, which is applied externally to treat dangerous snake and reptile bites, night blindness, and cutaneous conditions (Ngoc *et al*., 2014) .

The root contains Oleanolic Acid, Amino acid, Steroids, Alkaloids, Triterpenoids, Coumarins, Ecdysterone, Ionokosterone, Rubrosterone, Oligosaccharides, Polysaccharides, Achyranthine, Glycosides, Tannins which is used in whooping cough, tonsilitis, heamorrhage, cough, and hydrophobia, as an antiasthmatic, diuretic, diaphoretic, and antisyphilitic (Singh *et al*., 2019). The seed contains Linoleic acid, Oleic acid, Palminitic acid, Stearic acid, and Behenic are employed as an emetic, purgative, and cathartic, in gonorrhea, for insect bites and in hydrophobia, cough including whooping cough, as an anti-asthmatic. The leaves are used in wounds, injuries, intermittent fever, urination, dog bites, and typhoid (Dhankhar *et al*., 2014).

(a)

(b)

(c)

(d)

Figure 2: Different parts of *A. aspera* (a) whole plant (b) leaves (c) seeds (d) roots

1.5 Traditional and medicinal use

The word Ayurveda is derived from the words au, which means life, and Veda, which means knowledge (Tripathy *et al*., 2017). Modern medicine is made up of one-fourth of plant-based components. The treatment of common ailments using conventional medicines is still used by roughly 60% to 80% of the world's population. In comparison to allopathic medication, herbal plants are more affordable and widely accepted due to their ease of accessibility, lack of adverse effects, affordable pricing, environmental friendliness, and long-lasting healing properties. Traditional medicine is described by the World Health Organization (WHO) as "the totality of all knowledge and practices, whether explicable or not, also used in diagnosis, prevention, and elimination of physical, mental, or social imbalance and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing." *A. aspera* has mostly been used for renal leprosy, cough, scrofula, fistula, skin rash, nose infection, chronic malaria, fever, asthma, piles, snake bites, diarrhea, cold,

menstrual disorders, as an astringent for wound healing, and cancer, among other conditions (Shrinet *et al*., 2021). To treat ailments, the entire plant may be useful. Medicinal characteristics can be found in leaves, stems, and bark. Also used for antifertility, forced abortions, bleeding, kidney issues, scorpion bites, boils, hemorrhoids, rheumatism, itches, toothaches, neurological difficulties, hysteria, etc. It has the potential for temple worship from ancient times. Shiva puja uses leaves during Ganesh Chathurthi, which are regarded to be lucky charms. For vomiting, use roots. Using the plant's cooked leaves, pneumonia can also be treated (Sivasankari *et al*., 2017).

1.6 Chemical Constituents of *A. aspera*

Researchers reported the isolation and identification of Saponins A and B in their chemical analysis of *A. aspera* seeds in 1970 and 1993, respectively. D-glucuronic acid, which makes up saponin A, and its β-D galactopyranosyl ester, which makes up saponin B, were both identified. Other compounds were also isolated along with these, including oleanolic acid, amino acids, and hentriacontane. Additionally, the seeds include chemical elements such as 10-tricosanone, 10-octacosanone, and 4-tritriacontanone (Kushwaha, 2019). Its roots contain a wide range of bioactive substances, such as amino acids, steroids, coumarins, alkaloids, triterpenoids, ecdysterone, ionokosterone, rubrosterone, oligosaccharides, polysaccharides, achyranthine, glycosides, and tannins. Together, these components support the pharmacological efficacy and therapeutic value of the roots (Prakash & Sagar, 2021).

On the other hand, the plant's shoots display a unique chemical profile that includes substances like Dihydroxyhenpenta Triacontanol, 27-cyclohexyl heptacosane 7-ol 17-penta-triacontanol, and 16-hydroxy-26-Methyl heptacosane-2-1. These substances highlight the special biological characteristics and possible medical uses of the shoots. They are distinguished by their complex molecular structures (Anuradha, 2017).

A. aspera, a plant of significant pharmacological interest, harbors a rich array of (Srivastava, 2014) bioactive compounds within its botanical composition. Among its constituents are essential oils, which, though present in low concentrations, warrant caution for expectant mothers due to their potential risks (Hivrale *et al*., 2011). Long-chain alcohols such as tritricontane and n-hexacos-14-enoic acid contribute to its chemical profile, alongside volatile oils like achyranthene and betane. Notably, oils

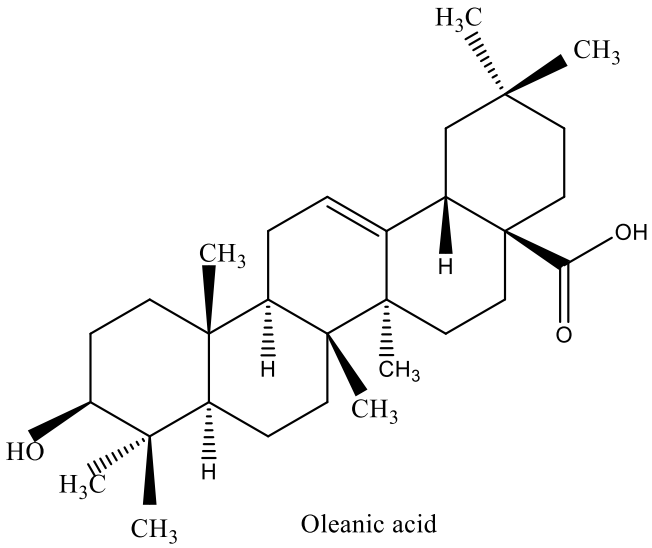
extracted from apamarga seeds suggest the presence of fatty acids, further diversifying its chemical makeup. Sterols, including trans-13-oxytocic acid, tetracontanol, and compounds like strigmasta and tricosanone, underscore the plant's pharmacological complexity. Moreover, bisdesmosidic saponins, such as β-D-glucopyranosyl esters of oleanolic acid and sapogenin, augment its medicinal potential (Barua *et al*., 2012). These compounds collectively define *A. aspera* L.'s multifaceted pharmacological profile, hinting at its promising applications in traditional medicine and modern pharmacotherapy.

1.7 Identification and classification of the plant

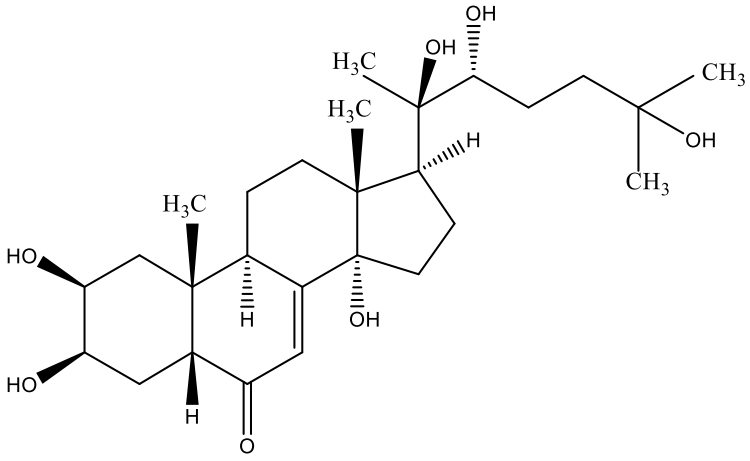
Kingdom	:	Plantae
Subkingdom	:	Tracheobinota
Super Division	:	Spermatophyta
Division	:	Mangoliophyta
Class	:	Mangoliophsida
Subclass	:	Caryophyllidae
Order	:	Caryophyllales
Family	:	Amaranthaceae
Genus	:	*Achyranthes*
Species	:	*aspera*
Binomial Name	:	***Achyranthes aspera* L.**

1.8. Names of *A. aspera* in different languages:

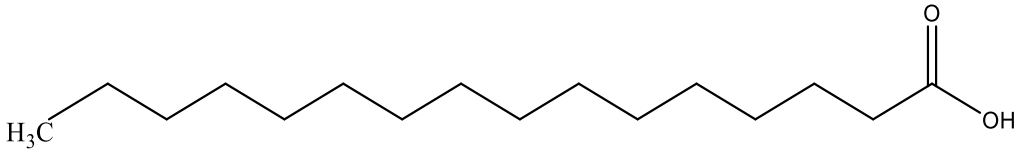
Nepali	−	Apamarga
Latin	−	*Achyranthes aspera*
Sanskrit	–	Aghata
Hindi	−	Latjira, Chirchira
Ayurvedic	−	Apaamaarga, Chirchitaa, Shikhari, Shaikharika



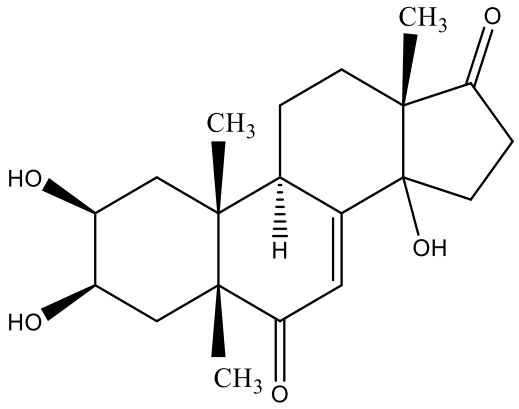
Oleanic acid



Ecdysterone



Palmitic acid



Rubrosterone

Figure 3: Structures of some chemical constituents of *A. aspera*

1.9 Phytochemicals:

Phytochemicals are naturally occurring, bioactive compounds derived from plants that do not provide any nutrients to the plant. Plants create them to act as a defense against biotic and abiotic hazards (Rusnoto *et al*., 2019). Plant matter contains a variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids. In many cases, these substances serve as a plant's defensive mechanism against herbivores, insects, and other types of microorganisms (Alam *et al*., 2009). Depending on the metabolic pathway they came from, phytochemicals can be divided into a few different categories. The chemical composition of plants includes phenolics, alkaloids, steroids, terpenes, saponins, and many more substances. To discover their potential as a source of new pharmaceuticals and to encourage the appropriate use of herbal medicine, it is critical to do in-depth research on the medicinal plants that have a folkloric reputation (Lakshmi *et al*., 2020).

1.10 Objectives of the study

The following general and specific aims have guided the selection of this plant species for this study:

1.10.1 General objectives

To find out the medicinal value of *A. aspera.*

1.10.2 Specific objectives

- To do the extraction (ultrasonic extraction) with different solvents like hexane, chloroform, ethyl acetate, methanol and distilled water.

- To conduct phytochemical screening alongside Thin Layer Chromatography (TLC), as well as determine Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) of the extract.
- To analyze Ultra-Violet spectroscopy and Fourier-transform infrared spectroscopy of extracts.
- To study the bioactivity of the extracts like antibacterial, antioxidant, antidiabetic and cytotoxicity.

CHAPTER II: LITERATURE REVIEW AND RESEARCH GAP

The research on phytochemicals reveals that variable amounts of alkaloids, cardiac glycosides, terpenoids, flavonoids, saponins, steroids, proteins, and reducing sugars are present in stems and leaves (Srivastava, 2014).

A. aspera boasts a wealth of medicinal compounds, including ecdysterone, spinasterol, and essential fatty acids. This botanical powerhouse demonstrates a diverse range of pharmacological activities, from nephroprotective and anti-inflammatory effects to antiparasitic and hepatoprotective properties. Its antimicrobial and analgesic qualities, coupled with its efficacy in treating gastric disorders, highlight its relevance as a versatile herbal remedy (Awasthi *et al*., 2021).

The methanolic extracts possess antimicrobial activity against *Vibrio alginolyticus* whereas the ethanol and chloroform extracts of the seeds of *A. aspera* show mild–to-moderate antibiotic activity against *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Raut *et al*., 2021).

The enzymes amylase and PTP 1B are both inhibited by the methanolic extract of the leaves of *A. aspera*, which has outstanding antioxidant potential (Chandrasekar *et al*., 2014). Its significant phenolic composition, whose quantitative investigation has shown the varying presence of polyphenols and flavonoids, would be the cause of these effects (Jaisankar *et al*., 2020). The use of these herbs in conventional medicine for the treatment of type 2 diabetes and its consequences may be justified in light of these findings (Sivasankari *et al*., 2017). NMR, HPLC, and other cutting-edge technologies are used to study the chemical components of these plants (Shrinet *et al*., 2021).

With the help of *A. aspera* leaf extract, an environmentally friendly synthesis of AgNPs was accomplished. This technique offered an alternate strategy for producing silver nanoparticles that could eventually take the place of more conventional techniques. The leaf extracts of *A. aspera* was able to convert silver (I) ions into *A. aspera's* AgNPs. After being produced, these final silver nanoparticle products were examined using TEM, SEM, FT-IR, UV-Vis spectroscopy, and EDXA analysis (Ngoc *et al*., 2014) .

An evaluation of *A. aspera* leaf extract's cytotoxicity and thrombolytic qualities suggests that this plant may be employed as an anticancer agent. It can also be used in

conjunction with other thrombolytic medications to treat circulatory system conditions that involve blood clots. To fully extract the medicinal properties of this herb, more in vivo and in vitro research is advised (Jakir, 2013).

An analysis of the phytochemical profile and antioxidant activity of the entire *A. aspera* plant showed the presence of carbohydrates, tannins, saponins, phenolic compounds, alkaloids, terpenoids, and flavonoids. 209.007 µg GA/mg, 17.59 µg QE/mg, and 25.12% (100 µg/mL) were the extract's total phenolic content, total flavonoid content, and antioxidant activity, respectively. Plants may contain antioxidant compounds, according to the research (Alam *et al*., 2009; Awasthi *et al*., 2021; Manandhar *et al*., 2021).

An *in vitro* study was conducted to evaluate the antioxidant and antidiabetic properties of leaf extracts from Achyranthes aspera. The inhibition activities of α-amylase and α-glucosidase enzymes were observed to be dose-dependent. In comparison to petroleum ether extract (51.87±00% for α-amylase and 46.0±0.22% for α-glucosidase inhibition at 160 µg/ml), methanolic fraction demonstrated the highest activity (55.0±0.50% for α-amylase and 53.06±0.23% for α-glucosidase inhibition at 160 µg/ml). Using the DPPH scavenging method, the antioxidant activity of the plant extracts was also investigated. Significant antioxidant activity of the separated phytochemical compounds was demonstrated by the DPPH assay. The methanolic extract demonstrated a greater capacity to scavenge DPPH radicals (68±0.44% at 250 µg/ml) (Mishra *et al.*, 2021).

The impact of applying aqueous and methanolic extracts from two different plants, *Achyranthes aspera* and *Alhagi maurorum*, on the UV protection properties of cotton fabric was examined. Results demonstrated that fabric treated with extracts from both plants exhibited excellent UV protection, as evidenced by their high ultraviolet protection factor. It was concluded that both aqueous and methanolic extracts effectively blocked UVA and UVB radiation when applied to cotton fabrics. Additionally, *Achyranthes aspera* extracts showed superior UV protection compared to *Alhagi maurorum*, with methanolic extracts performing better than aqueous extracts in terms of UV protection (Nazir *et al*., 2016).

The study focuses on the fabrication of iron oxide nanoparticles using a non-toxic, eco-friendly, and cost-effective method. Structural and morphological analyses were

conducted using X-Ray diffraction, Scanning Electron Microscope, UV-Visible spectroscopy, and IR spectroscopy, revealing crystalline nanoparticles with an average size of 20 nm. The research highlights the nanoparticles' antibacterial, antioxidant, and photocatalytic properties, demonstrating significant activity in all three areas (Ahmad *et al*., 2022).

Achyranthes aspera, commonly known as Sangketan, as a potential source of anticancer drugs. The research involves early phytochemical screening using thin layer chromatography (TLC). Sangketan powder is extracted using ethanol, methanol, and petroleum ether solvents via reflux method on a water bath. The resulting extracts are concentrated and dissolved in a suitable solvent before being eluted on Silica Gel 60 F254 stationary phase with varying mobile phase ratios (chloroform:methanol). Qualitative analysis reveals the presence of phytochemical compounds such as steroids, triterpenoids, sugars, alkaloids, phenolic compounds, and flavonoids. Thin layer chromatography with chloroform:methanol (8:2 v/v) shows gray bands after spraying with vanillin-sulfuric acid reagent. These findings indicate that *Achyranthes aspera* holds significant potential for various therapeutic and pharmacological properties (Rusnoto *et al*., 2019).

Diabetes mellitus poses a substantial health burden worldwide, with increasing incidence and associated mortality. While conventional antidiabetic medications are effective, they frequently entail undesirable side effects. Medicinal plants represent a promising avenue for alternative antidiabetic treatments. This overview explores several medicinal plants with potential antidiabetic properties, drawing insights from both preclinical and clinical studies. The therapeutic potential of these plants arises from the synergistic action of their diverse array of biologically active compounds, suggesting their relevance in diabetes management (Salehi *et al*., 2019).

This work sought to determine whether methanol extract from *Achyranthes aspera* L. leaves could promote wound healing in rats. Several quantities of the extract were topically applied to albino rats that had been given wound models. Comparing the results to control groups receiving only ointment, the experimental groups experienced higher wound contraction, a shorter epithelization period, and higher DNA content. Rats treated with the extract also showed improved neovascularization and tissue

regeneration, according to histological study. These results underline the traditional usage of leaves of *Achyranthes aspera* L. to cure wounds (Kamalakkannan & Balakrishnan, 2015).

In this study, rats with alloxan-induced diabetes were used to examine the possible effects of *Achyranthes aspera* Linn tea on lipid profiles and blood glucose levels. *Achyranthes aspera* Linn's stem, leaves, and flowers were processed into a herbal tea, and its proximate and phytochemical contents were examined. Over a twenty-one-day period, five groups of rats were used to assess the hypolipidemic and antidiabetic qualities. The herbal tea contained alkaloids, tannins, phenolics, saponins, flavonoids, phytosterols, and cardiac glycosides, according to the findings. Rats given varying amounts of the herbal tea showed a significant drop in serum triglyceride levels, but a decrease in fasting blood glucose levels. Nevertheless, there were no discernible alterations found in the levels of total, HDL, or LDL cholesterol (Njideka *et al*., 2019).

Two medicinal plants, *Achyranthes aspera* and *Cassia alata*, were investigated for their antibacterial activity against *Salmonella typhi*, *Bacillus subtilis*, *Vibrio cholerae*, *Escherichia coli*, and *Staphylococcus aureus* using organic solvent extracts (methanol, ethanol, ethyl acetate, and chloroform). Mueller-Hinton agar medium was used to assess extracts from the plant's leaves and stems at a concentration of 5 mg/ml using the disc-diffusion method. Methanolic extracts from the leaf and stem sections of C. alata shown action against *B. subtilis* and *S. typhi*, with minimum inhibitory concentrations (MIC) of 1.25 and 1.5 mg/ml, respectively, but extracts from *A. aspera* demonstrated no antibacterial activity. Only *S. aureus* was susceptible to the effects of both sections' ethanol extracts (MIC= 1.25 mg/ml) (Alam *et al*., 2009).

CHAPTER III: MATERIALS AND METHODOLOGY

3.1 Material:

3.1.1 Chemicals Required:

- Solvents hexane, chloroform, ethyl acetate, methanol and distilled water are used for an analytical grade.
- Chemical and reagents like ascorbic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), KOH, Conc. H_2SO_4, Conc. HCl, $AlCl_3$, and Phenol are used for laboratory grade.
- Mayer's reagent, Dragendroff's reagent, Fehling's reagent etc, are prepared in the laboratory with the chemical available in the laboratory.

3.1.2 Instrument and Equipment

- Rota-vapour (IKA, RV 10 D S96)
- UV lamp (UV 2510TS)
- Heating Bath, Digital weighing balance
- Oven, Electric grinders
- Measuring cylinders, conical flask, test tube, water bath

3.2 Methods:

3.2.1 Collection of plant parts:

The plant of *A. aspera* was collected from the Mahottari district of Nepal. The plant was identified by the National Herbarium and Plant Laboratory, Lalitpur.

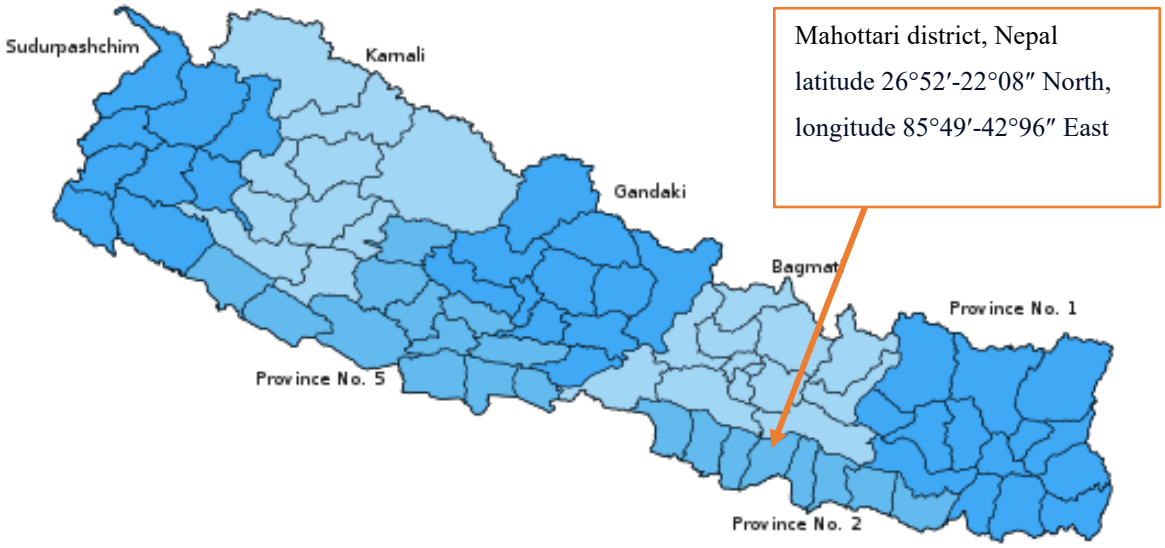


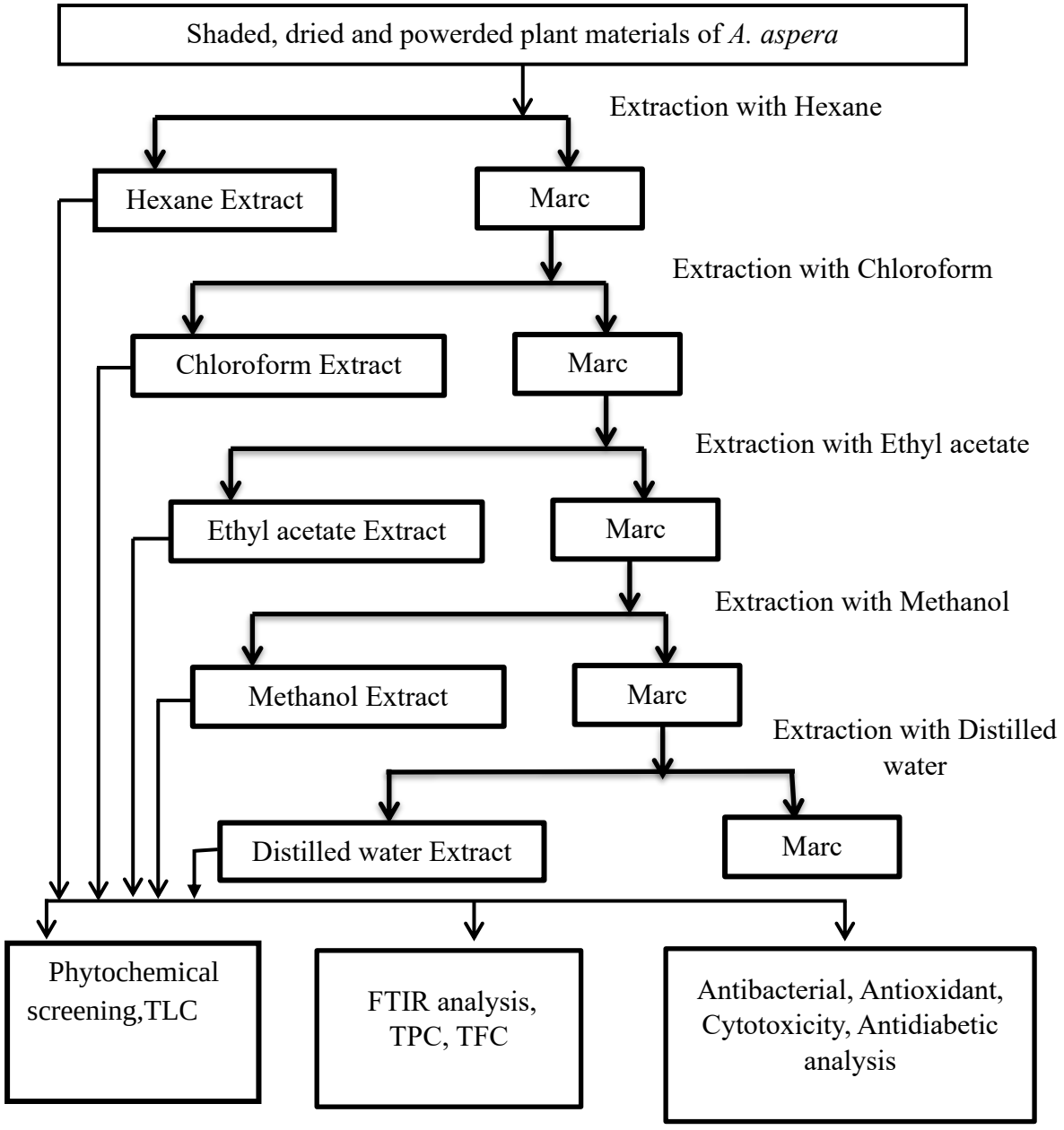
Figure 4: Collection site of *A. aspera*

3.2.2 Drying and grinding:

The plant (8 kg) was collected locally, and the leaves, roots and stem were detached, cleaned with water, and then air-dried. 8 kg of the plant was air-dried for approximately 1-5 months. The dried plants were grinded to powdered.

3.3 Extraction of Plant:

The process utilized to prepare the plant extract involved an ultrasonic-mediated extraction method. In this technique, a clean, dried beaker was used containing 0.8 kg of finely powdered plants. Approximately 1500 mL of hexane was added to the beaker, which was then appropriately swirled and placed in an ultrasonic cleaner bath. The beaker was submerged in water up to one-third of the water bath's total capacity. The power output consisted of three 50-watt modules running at a frequency of 40 kHz. After the extraction procedure was completed, the beaker was allowed to settle before filtration. The filtrate could be concentrated using a rotary evaporator. Once dried and weighed, the concentrated extract was transferred to an airtight vial tube for future use. Similarly, the residue collected after filtration underwent hexane treatment followed by sonication. This process was repeated three times to produce the crude extract. Using the same method, several different solvent extracts were obtained, including those from hexane, chloroform, ethyl acetate, methanol, and distilled water. The following schematic diagram illustrates each of these processes.



Scheme 1: Research process for extraction, screening, analysis, and biological activities.

3.4 Phytochemical Screening:

The phytochemical screening process involved a thorough assessment aimed at identifying and quantifying the presence of diverse bioactive compounds within each extract. This comprehensive analysis covered a spectrum of compound classes, including alkaloids, terpenoids, carbohydrates, phenolic compounds, tannins, flavonoids, quinones, steroids, proteins, and saponins. For screening phytochemicals, the procedure was used. The bioactive components of the plant are found by phytochemical screening. To identify the presence of diverse plant extracts' principal natural components, various reagents were used for the color reaction.

3.5 Total Phenolic Content:

The Folin-Ciocalteu colorimetric method, which is based on the oxidation-reduction reaction, was used to calculate the total phenolic content of the plant extract. As a standard, the gallic acid concentration is employed (Upadhya *et al*., 2015).

3.5.1 Preparation of Standard Gallic Acid Solution:

In the first step, a stock solution of gallic acid with a concentration of 1000 µg/mL was made by dissolving 10 mg of gallic acid in 10 mL of methanol. By repeatedly diluting the stock solution, several different concentrations of gallic acid were created. These concentrations included 100, 80, 60, 40 and 20 µg/mL.

3.5.2 Construction of the Calibration Curve:

One milliliter of the gallic acid solution at each concentration was put into test tubes. The test tubes were then given a total capacity of 10 mL by adding 5 mL of 10% Folin-Ciocalteu reagent (FCR) and 4 mL of 7% sodium carbonate solution (Na_2CO_3). The blue liquid was thoroughly stirred before being incubated for 30 minutes at 40°C in a water bath. At 760 nm, a spectrophotometer was used to evaluate the solution's absorbance in comparison to a blank solution that contained all of the chemicals except gallic acid. The experiments were all run in triplicate. The calibration curve was created using the average absorbance values that were obtained at various gallic acid concentrations.

3.5.3 Preparation of the Sample Solution

The triplicate of concentrations of the extract 1000 µg/L was prepared by serial dilution, and their absorbance values were measured using the same technique as described above for gallic acid. A stock solution of 10,000 µg/mL of the extract was made by dissolving 50 mg extract in 5 mL methanol.

3.5.4 Measurement of Total Phenolic Content:

The amount of total phenolic content in the given sample was calculated as milligrams of gallic acid equivalent using the following equation.

$$\text{The total content of the phenolic (C)} = \frac{C \times V}{m} \ldots\ldots\ldots (1)$$

Where ,

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

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

V = Volume of extract (mL)

m = Weight of the plant extract (mg)

The data were recorded as the mean of three separate determinations of absorbance for each concentration. From these values, the linear correlation coefficient (R^2) value was determined. The equation for the regression can be written as:

$y = mx + c$ …….. (2)

Where,

y = the absorbance of the extract

m = the slope of the calibration curve

x = the concentration of the extract.

c = Intercept

This regression equation was used to calculate the extract's concentration. As a result, an equation was created to calculate the substance's total phenolic content using the extract's concentration as input (2).

3.5.5 A General Protocol for Total Phenolic Content:

Folin-Ciocalteu phenol reagent (1 mL, diluted 1:10 with water) and 0.8 mL of aqueous 1 M Na_2CO_3 solution were added to 0.1 mL of the sample (1 mg/mL in methanol). The reaction mixture was left to stand for approximately 15 minutes in complete darkness before reactant absorbance was measured at 765 nm in comparison to a control. The standard was gallic acid.

Per gram of dried plant extract, the total phenolic content is represented as mg of gallic acid equivalent.

3.6 Total Flavonoid Content Assay:

Using a colorimetric assay with aluminum chloride, the total flavonoid content of the plant extract was assessed. As a reference, quercetin is utilized.

3.6.1 Principle of Total Flavonoid Content Assay:

Using the common aluminium chloride colorimetric method, the total flavonoid content

of the plant extract was determined. In the sample of this process, aluminum chloride forms a product with the hydroxyl groups of flavonoids. This substance has a 420 nm maximum absorption.

3.6.2 Preparation of the Standard Quercetin Stock Solution:

By combining 20 mg of quercetin with 20 mL of methanol, a quercetin stock solution with a 1000 µg/mL (ppm) concentration was created. Quercetin concentrations of 250 µg/mL, 150 µg/mL, 100 µg/mL, 50 µg/mL, and 25 µg/mL were created by serially diluting the stock solution. In a 20 mL test tube with 4 mL distilled water, an aliquot of 1 mL of each concentration of quercetin in methanol was added. At zero time, 0.3 mL of 5% $NaNO_2$ was added to the test tube. 0.3 mL of 10% $AlCl_3$ was added to the mixture after 5 minutes, and 2 mL of 1 M NaOH was added after 6 minutes. 2.4 mL of thoroughly distilled water was added to the mixture right away to bring its total volume up to 10 mL. The pink color mixture's absorbance was then measured using a spectrophotometer at 510 nm in comparison to a blank solution that contained all of the reagents except for quercetin. The average absorbance values discovered for various quercetin concentrations were used to plot the calibration curve.

3.6.3 Preparation of the Sample Solution:

50 mg of the extract was dissolved in 5 mL of methanol to create an extract stock solution with a concentration of 10,000 µg/mL. After this, the extract was diluted three times to a concentration of 1000 µg/mL, and the absorbance values were calculated using the same method as for quercetin.

3.6.4 Measurement of Total Flavonoid Content (TFC):

The total flavonoid content of the extract was determined using the following formula:

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

where,

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

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

V = Volume of the extract (in mL)

m = Weight of the plant extract (in g)

3.6.5 Statistical Analysis:

To calculate the linear correlation coefficient (R^2) value, the data were recorded as the mean of three absorbance measurements for each concentration. It is stated that the regression equation is,

$$y = mx + c \text{ ……..} (4)$$

where,

y = Absorbance of the extract

m = Slope from the calibration curve

x = Concentration of the extract,

c = Intercept

The extract concentration was determined using this regression equation. Equation (4) was used to determine the flavonoid content using the calculated extract concentration value.

3.6.6 A General Protocol for Total flavonoid Content:

Quercetin was used as a reference and 1mL of the sample (0.1 mg/mL in methanol) was mixed with 1mL of 2% $AlCl_3$ (dissolved in methanol) and maintained for 1hr. After that, the absorbance was measured at 415 nm against the blank.

3.7 Antioxidant Activity:

Antioxidants are often described as "molecules with reducing power" or "molecules capable of reacting with radicals" in the study of natural substances to prevent the oxidative stress induced by radicals. Chemicals known as antioxidants can delay or suppress the oxidation processes that occur as a result of the action of ambient oxygen or relative oxygen species (Raut *et al*., 2021). The oxidation processes that occur when air oxygen or other relative oxygen species are present can be delayed or stopped by substances known as antioxidants. There are several methods for achieving this. They are employed in the stability of polymeric goods, meals, cosmetics, and medications. Selecting the right method to utilize for a particular application is one of the more difficult aspects of testing antioxidants, which can be difficult at times. Since antioxidants have a wide range of functions, including quenching active pro-oxidants, scavenging free radicals, sequestering transition metal ions, decomposing hydrogen peroxide or hydroperoxides, repairing biological damage, and more, it is crucial to identify the antioxidant assay method that best suits the function being measured (Abi

Beaulah *et al*., 2011). Due to the wide range of results on natural antioxidants in food systems, there is a pressing requirement for stricter standards and stringent testing protocols to bring some order and agreement to this important area of research. Naturally, it is crucial to remember that total antioxidant capacity (TAC) and antioxidant activity (AOA) differ from the parameters for elemental analysis, for which the analyst is required to obtain results that are essentially equal from a range of approaches (Umamaheswari *et al*., 2012).

3.7.1 Principle of DPPH Assay:

The DPPH free-radical method is an antioxidant assay that is based on electron transfer. In alcohol, it creates a violet solution; however, in the presence of an antioxidant molecule, the solution turns colorless. Due to the distribution of its lone pair of electrons throughout the entire molecule, DPPH is regarded as a stable free radical (Vijayaraj & Sri Kumaran, 2018). In contrast to the great majority of other free radicals, this inhibits the molecule from dimerizing. The dark violet color of the methanol solution is a result of the delocalization process and may be recognized by an absorption band with a center wavelength of about 517 nm. When a DPPH solution is combined with a substance that can donate a hydrogen atom and still contains picryl residue, the consequence is a reduction in shape and a change in hue from violet to pale. This is owing to the continued presence of the picryl residue. A quick, simple, and affordable method for assessing antioxidant capacity uses free radicals (DPPH).

The effective sample concentration required to scavenge 50% of the DPPH free radicals is known as the 50% inhibitory concentration or IC_{50} value. Plotting extract concentration v_s the relevant scavenging effect led to the inhibition curve, which was then utilized to determine IC_{50} values.

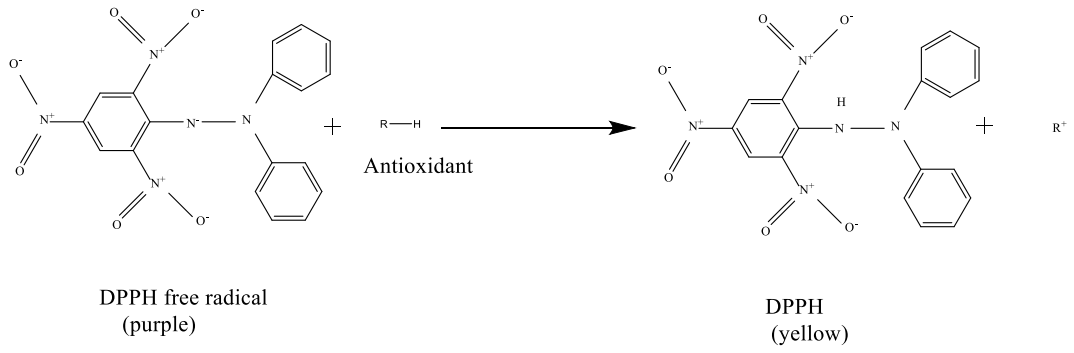


Figure 5: Mechanism of DPPH radical scavenging

3.7.2 Preparation of the 0.2 mM DPPH Solution:

Since 2,2-Diphenyl-1-picrylhydrazyl-hydrate (DPPH) has a molecular weight of 394.32 g/mol, a 100 mL solution containing 0.2 mM of DPPH was made by precisely weighing 0.0078 g of DPPH, dissolving it in methanol, and then keeping the volume constant at 100 mL. This solution was then stored in darkness until use.

3.7.3 Preparation of Ascorbic Acid Solution (Standard):

The stock solution of 1000 µg/mL was made by weighing 10 milligrams of ascorbic acid and dissolving it in 10 mL of methanol. Then, ascorbic acid solutions with concentrations of 30 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL, and 2.5 µg/mL were made by serial dilution.

3.7.4 Preparation of Sample Solution:

After weighing and dissolving 50 mg of extracts in 50 mL of methanol to produce a stock solution with a concentration of 1000 µg/mL, extract solutions with concentrations of 30 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL and 2.5 µg/mL were prepared.

3.7.5 Measurement of DPPH Radical Scavenging Activity:

Two milliliters of the 0.2 mM DPPH solution were combined in triplicate with two milliliters of the ascorbic acid solution from each concentration and left in the dark for thirty minutes. In the dark for 30 minutes, 2 mL of methanol and 2 mL of a 0.2 mM DPPH solution were combined. Their absorbance value was then measured at 517 nm using a spectrophotometer in comparison to methanol and DPPH as a blank.

Using the following equation, the percentage of the DPPH free radical scavenging activity was determined:

$$\text{DPPH Scavenging Activity (\%)} = [(A_0 - A_S)/A_0] \times 100$$

where,

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

A_S = Absorbance of the test sample

The ascorbic acid calculation method was also used to determine the absorbance values of the extract and DPPH solution.

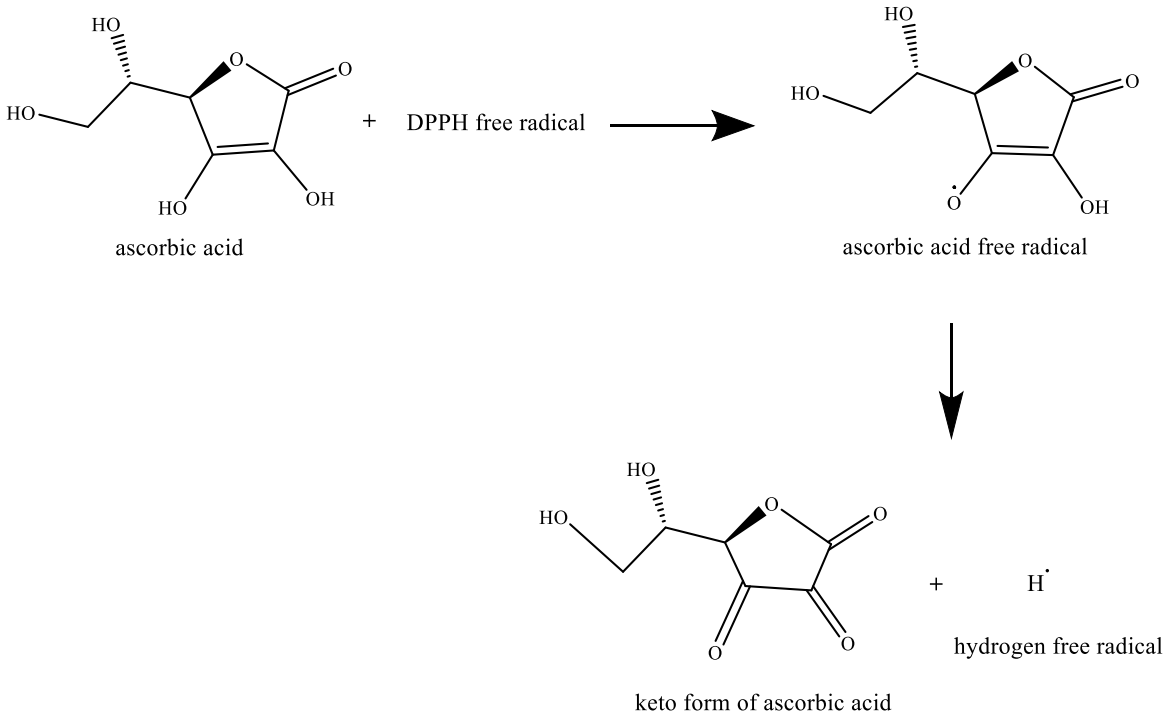


Figure 6: Mechanism of DPPH radical scavenging by ascorbic acid

Finally, the IC_{50} value was calculated using the calibration curve with sample concentration as the X-axis and percent radical scavenging activity as the Y-axis for ascorbic acid and sample solution. IC_{50} for ascorbic acid (IC_{50} average) is 18.33 µg/mL.

3.8 Antibacterial Activity

In biological screening, the impact of a crude plant extract or fraction at a specific dosage level on the species of the organism was investigated (Lakshmi *et al*., 2020). In this investigation, antibacterial assays were conducted. The agar well diffusion technique was used to screen for and analyze the antibacterial activity of crude plant extracts. The ZOI value was computed after using the agar well diffusion technique to test for bacterial growth inhibition (Ahmad *et al*., 2022).

3.8.1 Collection of Test Organism

The identified microbial strains used were obtained from the Research Institute for Bioscience and Biotechnology, Chyasal Road, Lalitpur, Nepal. Two separate bacterial species were present in the strains that were being investigated.

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

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

3.8.2 Preparation of Working Solution:

In 1 mL of DMSO, 50 mg of crude plant extract was dissolved. The tube was sealed, capped, and kept in a cool location until use.

3.8.3 Preparation of Media

A. Nutrient Agar

It was made by mixing 28 g of nutritional agar with the appropriate amount of distilled water to make a final volume of 1000 mL (28 g/L). After being boiled for 30 minutes while being constantly shaken, it was autoclaved at 121 °C. It allowed for the sterilized material to cool to about 50 °C. They were appropriately labeled and dispensed aseptically on sterilized 90 mm diameter petri plates with a 25 mL per plate ratio. The plates were then given time to set up.

B. Muller Hinton Agar (MHA)

250 mL of distilled water was used to dissolve the 9.5 g of media before sterilizing it in an autoclave for 15 minutes at 121°C and 15 lb of pressure. After allowing it to cool to roughly 50 °C, 20 mL/plate of it was then placed into petri dishes. The plates were left in their original state to solidify.

3.8.4 Screening and Evaluation of Antibacterial Activity

Gram-positive and gram-negative test organisms were used in the Agar Well Diffusion Assay to measure the antibacterial activity. Four different general lab pathogenic test organisms, including *Bacillus subtilis, Escherichia coli, Klebsiella pneumonia, and Staphylococcus aureus,* were used for the antibacterial testing. First, sterile NB media was used to dilute overnight incubated broth cultures of complete test organisms to keep the turbidity at 0.5 McFarland standards (106−8 CFU/mL). The inoculum was then poured onto MHA agar plates in an amount of around 100µL. Following that, five wells measuring 7 mm in diameter were drilled using a gel puncture, and each one received 20 L of samples at a concentration of 50 mg/mL. The plates were then incubated for 24 hours at 37°C, and the zone of inhibition was assessed using a ruler. A positive control consisting of ampicillin (1 mg/mL) and a negative control consisting of DMSO were utilized.

3.9 Antidiabetic Activity:

High blood glucose levels are a common and persistent feature of diabetes. This chronic hyperglycemia can harm the body's many organs, tissues, and systems (Hivrale *et al*., 2011). Diabetes complications can significantly harm a patient's life, both in terms of physical health and financial strain. Numerous distinct compounds within plants have been found as a result of extensive phytochemical research. It is important to note that not all of the compounds discovered through phytochemical studies in plants are useful as antidiabetic medicines (Sinan *et al*., 2020).

3.9.1 A General Protocol for Anti-Diabetic Assay:

3,5-Dinitro salicylic acid (DNSA) was used to conduct the experiment in which α−amylase was inhibited (Salehi *et al*., 2019). The *A. aspera* extract was diluted in DMSO at a minimum of 10%. The material was combined with buffer, buffer-DMSO, and NaCl at a pH of 6.9 to provide a wide range of concentrations. Afterward, a 200 µL volume of this mixture was added to the α-amylase solution. The mixture was next allowed to sit for 10 minutes at 30 °C. The starch solution was then poured 200 µL at a time into each tube, and the tubes were left to stand for 3 minutes. DNSA (Dinitro salicylic acid) reagent in the amount of 200 µL was added to terminate the reaction. An 85−90°C water bath was used to heat the combined sample for 10 minutes. 5 mL of distilled water was added to the mixture once it had warmed to room temperature. Using a UV spectrophotometer, the sample's absorbance at 540 nm was determined and contrasted with a blank solution. The plant extract was swapped out with 200 µL of buffer to produce a blank with 100% enzyme activity.

Utilizing the following equation, the proportion of α−amylase inhibition was determined:

$$\text{α-amylase inhibition} = \frac{Abs.\ control - Abs.\ Sample}{Abs.\ Control} \times 100 \quad \text{.....(5)}$$

An illustration can be created by graphing the extract concentration vs the degree of α−amylase inhibition. This graph can be used to determine the IC_{50} values or the concentrations at which α−amylase is 50% inhibited.

3.10 Cytotoxicity Activity

The cytotoxicity of plant extracts and other compounds can be assessed using the brine shrimp lethality assay. It involves determining whether they can cause death in brine

shrimp larvae, or nauplii, raised in a lab (Prakash & Sagar, 2021). To assess the potential toxicity of different substances, this assay is frequently employed as a preliminary screening method. The brine shrimp nauplii were treated during the experiment with varied concentrations of plant extract and left for 24 hours. Then, to determine whether the plant extract was successful, the motility of the nauplii was noted. The simplicity, cost-effectiveness, and low requirements for test materials make the brine shrimp lethality assay useful.

The lethality of the brine shrimp assay determines the LC_{50} values of crude extracts in terms of micrograms per milliliter (µg/mL). The level of concentration (LC_{50}) at which the tested extract kills 50% of brine shrimp nauplii. Potential pharmacological activity is considered to exist in substances having LC_{50} values less than 1000 ppm (µg/mL).

3.10.1 General Procedure for Brine Shrimp Assay:

All of the equipment used in this experiment has first undergone sterilization.

3.10.1.1 Preparation of Artificial Seawater

Rock salt was crushed, weighed, and then dissolved in 100 mL of distilled water to produce a clear solution, which was then filtered out.

3.10.1.2. Hatching of Brine Shrimp Egg

Around 50 mg of brine shrimp eggs were then dispersed over the simulated seawater that had gathered within the beaker after it had been covered with aluminum foil. A few tiny pores were made in the covering material to improve the flow of heat and light. After that, the beaker was kept at ambient temperature for 48 hours while being illuminated by a 60-watt light source. The artificial seawater environment created by this arrangement is ideal for the development of brine shrimp eggs.

3.10.1.3 Preparation of Samples

By combining 20 mg of the extract with 2 mL of DMSO (dimethyl sulfoxide), a stock solution with a 10,000 ppm concentration was prepared. It was successful to create solutions with concentrations of 2000 ppm, 1000 ppm, 500 ppm, 250 ppm and 125 ppm using the serial dilution technique. By methodically diluting it with an appropriate solvent or buffer, several solutions were created from the initial stock solution. Five test tubes were allotted for each concentration, and the 2 mL solutions from the five concentrations (2000 ppm, 1000 ppm, 500 ppm, 250 ppm, and 125 ppm) were

distributed among the twenty-five test tubes. Likewise, 2 mL of DMSO (Dimethyl Sulfoxide) was added as a blank in five different test tubes. The test tubes were marked with the appropriate concentrations and then left alone for 24 hours.

3.10.1.4 Calculation of Assay:

In this bioassay, the absence of regulated forward movement of the brine shrimp nauplii during a 30−second observation period constitutes the mortality endpoint. The number of dead and live nauplii in each test tube is counted to calculate the lethality percentage for each concentration and control. By dividing the total number of nauplii by the number of dead nauplii, the mortality rate is then determined. As a result, the death rate may be evaluated, and the impacts of various concentrations and controls on the nauplii can be compared.

$$\% \text{ Mortality} = \frac{\text{No. of dead shrimps}}{\text{Total No. of shrimps}} \times 100 \quad \ldots\ldots\ldots\ldots\ldots(6)$$

3.11 UV-Visible Spectroscopic Analysis:

Characteristic absorption bands can be seen in the UV area of the electromagnetic spectrum in organic compounds with unsaturation, such as molecules with double or triple bonds. Electrons are excited to greater energy levels, which causes the absorption to happen. Insights regarding the type and degree of unsaturation can be gained from knowing the wavelength of maximum absorption (max) and the strength of the absorption bands. The Department of Chemistry, Amrit Campus, Kathmandu employed a Labtronics LT-2802 double beam ultraviolet-visible (UV−Vis) spectrometer to spectroscopically assess and evaluate the unsaturation and aromaticity of five distinct extracts. The extracts were combined with the appropriate solvents to create a 5 ppm solution for each sample solution.

3.12 FT-IR Analysis

For identifying the type of bonding and functional groups in organic compounds, FTIR spectroscopic analysis is a useful tool. In this investigation, which was carried out at the Amrit Campus in Kathmandu, a PerkinElmer Spectrum IR spectrometer was used to produce the FTIR spectra of five distinct extracts (hexane, chloroform, ethyl acetate, methanol and distilled water). The presence of π−bond conjugate systems, aromatic and aliphatic structures, as well as functional groups, could all be detected using these spectra, which also revealed the constituents' structural makeup. The chemical makeup

and structural traits of the investigated substances are significantly illuminated by this method.

CHAPTER IV: RESULTS AND DISCUSSION

4.1 Yield

Extracts of the *A. aspera* plant were made using the ultrasonic-mediated extraction technique. The results showed that the yields of the extracts in hexane, chloroform, ethyl acetate, methanol and distilled water were 2.22 g, 5.52 g, 2.26 g, 16.28 g and 10.68 g, respectively.

Table 1: Table showing the yield of various extracts

Extract	Hexane	Chloroform	Ethyl-acetate	Methanol	Distilled water
Yield (g)	2.22	5.52	2.26	16.28	10.68
% yield	0.27	0.69	0.28	2.04	1.34

4.2 Qualitative Analysis of Phytochemicals

The results of the microchemical examination of a crude extract of *A. aspera* leaves in various solvent systems revealed a group of phytochemicals, as indicated in Table 3. The phytochemical screens of the separate *A. aspera* extract extracts were carried out, and the appearance of certain colors served as evidence of the presence of phytochemicals. In the appendix section, the test results are included.

Table 2: Phytochemical analysis of extracts of *A. aspera*

S.N.	**Class of phytochemicals**	**Hexane Extract**	**Chloroform Extract**	**Ethyl-Acetate Extract**	**Methanol Extract**	**Distilled water Extract**
1	Alkaloids	+	+	+	+	-
2	Phenolic Compounds	+	+	+	+	+
3	Flavonoids	+	+	+	+	+
4	Terpenoids	+	+	+	-	-
5	Saponins	-	-	-	-	-
6	Carbohydrates	-	+	+	+	-
7	Tannins	-	+	+	+	-

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

The findings indicated that the semi-polar and polar extracts contained the majority of the phytochemicals. Almost all solvent extracts included flavonoids.

The information in this plant's literature differed slightly from the outcomes shown in the table above. Due to changes in growth parameters, varying climatic circumstances, the extraction method and time of sample collection, as well as lab setup and chemical grades, the results of phytochemical screening for the same sample may differ from screening for the same phytochemical contents.

4.3 TLC Analysis

The qualitative analysis of different extracts of *A. aspera* was done by TLC. TLC was performed on a 20% ethyl-acetate: hexane having 1 drop ammonia solvent system. The plates were developed and visualized in UV light. The TLC spots are illustrated below.

- Hexane: No discernible spots were observed, suggesting minimal compound diversity.
- Chloroform: Three distinct spots were detected, indicating a relatively diverse chemical composition.
- Ethyl acetate: Similar to chloroform, three spots were identified, indicating a comparable level of chemical complexity.
- Methanol: Only one spot was observed, implying a less diverse array of compounds compared to the other solvents.
- Distilled water: No spots were detected, suggesting either minimal or no soluble compounds in this solvent.

These findings provide valuable insights into the chemical diversity and composition of the extracts obtained using different solvents.

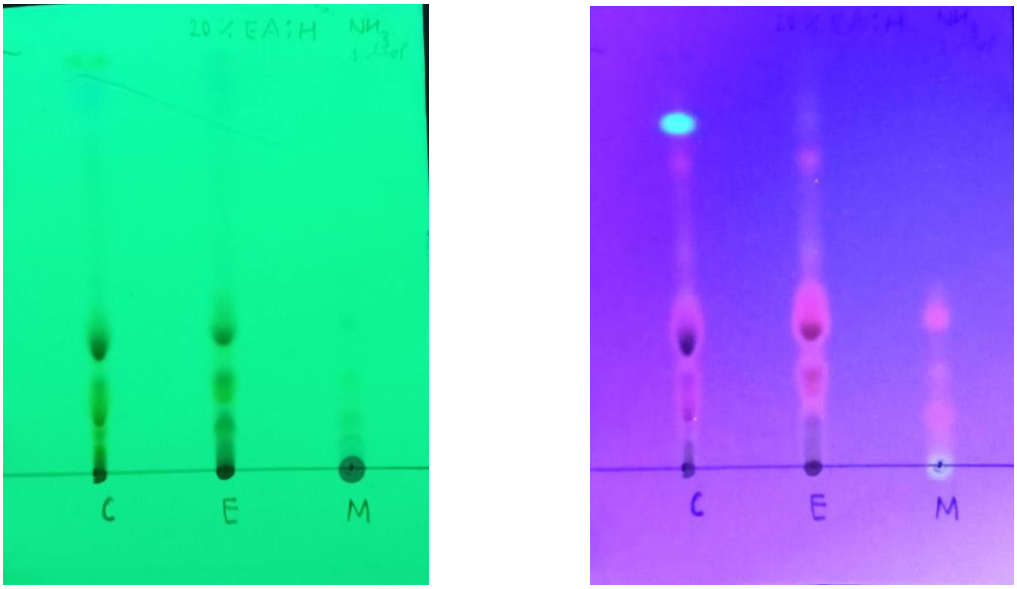


Figure 7: TLC showing spots of extracts of *A. aspera* under UV fluorescence in a 20% ethyl-acetate hexane having 1 drop ammonia solvent system

4.4 Estimation of Total Phenolic Content (TPC)

4.4.1 Construction of Calibration Curve

The Folin Ciocalteu (combination of phosphomolybdic acid and phosphotungstic acid) reagent calorimetric (FCR) method, which is based on the oxidation-reduction method utilizing gallic acid as a standard, was used to calculate the total phenolic content of plant extracts. In this process, the calibration curve's reference compound was gallic acid. A UV visible spectrometer was used to measure the absorbance of gallic acid at 765 nm wavelength at different concentrations, including 500 µg/mL, 250 µg/mL, 150 µg/mL, 100 µg/mL, 50 µg/mL and 25 µg/mL.

Plant extract polyphenols react with a specific redox reagent (FCR) to create a blue complex that absorbs light at a maximum wavelength of 765 nm. This complex can be detected by UV−Visible spectrometry. The levels of phenols are inversely correlated with the intensity of that wavelength's light absorption. With the concentration plotted on the X-axis and the absorbance plotted on the Y-axis, the observation of absorbance at various concentrations of standard gallic acid was graphically depicted. The standard gallic acid absorbance curve is shown in Figure 8.

Table 3: Gallic acid standard

Conc. (µg/mL)	Triplicates Absorbance data			Average Absorbance
500	2.332	2.121	2.208	2.22
250	1.3	1.344	1.369	1.338
150	0.891	0.854	0.821	0.855
100	0.675	0.635	0.664	0.658
50	0.544	0.554	0.53	0.543
25	0.374	0.366	0.353	0.364

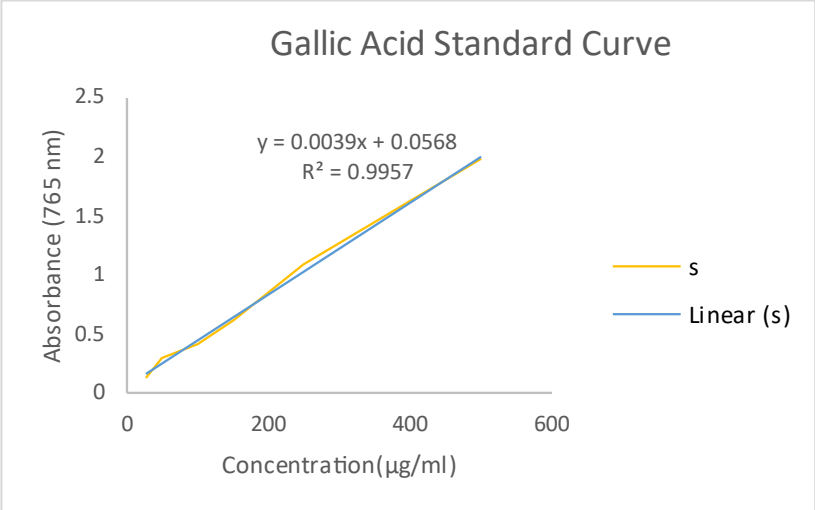


Figure 8: Calibration curve for standard gallic acid

4.4.2 Calculation of Total Phenolic Content in Different Extracts

The total phenolic content of various extracts was estimated using a calibration curve and absorbance values (triplicate of 1000 µg/mL), and the results are displayed in Table 6.

Table 4: Total phenolic content in different extracts

Extracts	Optical Density (OD) of samples			Optical Density average	Control	Optical Density value	TPC (mg GAE/ g)
Hexane	0.251	0.298	0.293	0.280	0.162	0.118	15.863
Chloroform	0.242	0.283	0.26	0.261	0.162	0.099	10.991
EtOAc	0.232	0.282	0.272	0.262	0.162	0.1	11.076
Methanol	0.199	0.234	0.251	0.228	0.162	0.066	2.358
Distilled Water	0.196	0.236	0.228	0.22	0.162	0.058	0.307

The total phenolic contents of hexane, chloroform, ethyl acetate, methanol and distilled water extracts were calculated as 15.863 mg GAE/g, 10.991 mg GAE/g, 11.076 mg GAE/g, 2.358 mg GAE/g and 0.307 mg GAE/g respectively.

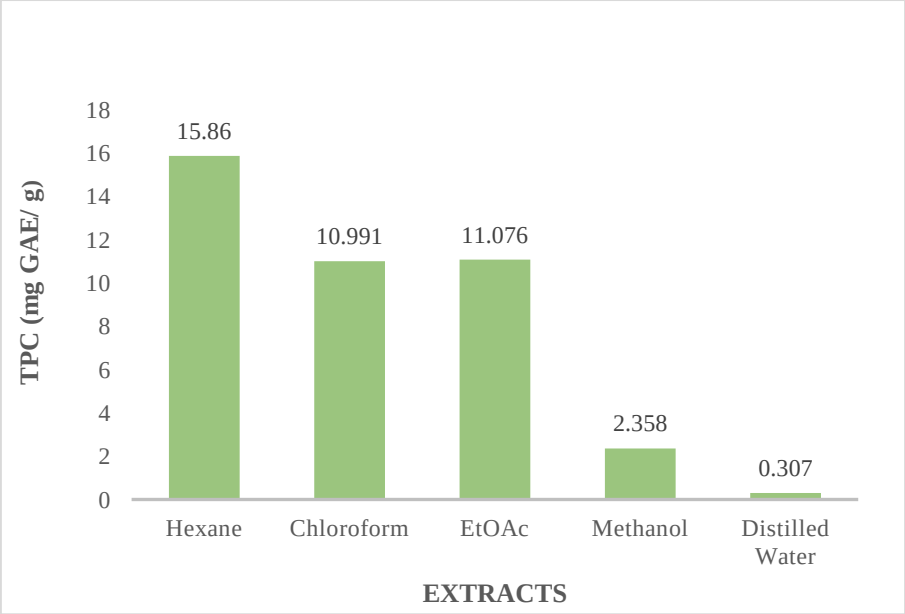


Figure 9: Total phenolic content in different extracts

4.5 Estimation of Total Flavonoid Content (TFC)

4.5.1 Construction of Calibration Curve

The total flavonoid content of plant extracts was determined using a colorimetric test with aluminium chloride. For the calibration curve construction in this procedure, quercetin was used as the reference substance. The flavonoids in plant extracts create an acid-liable compound with a bright yellow fluorescence in the presence of aluminium chloride, which was seen using a UV spectrophotometer at 510 nm. The flavonoid concentration is inversely correlated with the strength of light absorption at that wavelength. The presence of flavonoids exhibits a wide range of biological and pharmacological properties. The calibration curve for quercetin at several concentrations, including 500 µg/mL, 250 µg/mL, 200 µg/mL, 150 µg/mL, 100 µg/mL and 50 µg/mL, is shown in Figure 11.

Table 5: Quercetin standard

Conc (µg/mL)	**Triplicates Absorbance data**			**Average Absorbance**
250	1.731	1.76	1.829	1.773
150	1.117	1.089	1.093	1.100
100	0.762	0.771	0.76	0.764
50	0.416	0.416	0.422	0.418
25	0.225	0.228	0.23	0.228

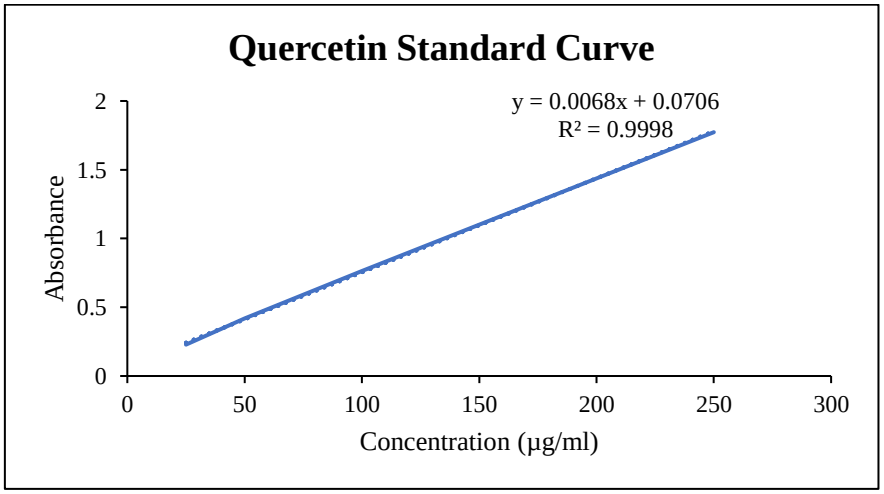


Figure 10: Calibration curve for standard quercetin

4.5.2 Calculation of Total Flavonoid Content in Different Extracts

The total flavonoid content of various extracts was determined using the calibration curve and absorbance values (triplicate of 1000 µg/mL), and the results are reported in Table 8.

Table 6: Total flavonoid content in different extracts of *A. aspera*

S.N.	Extracts	OD Samples			OD average	Control	OD value	TFC (mg QE/ g)
1	Hexane	1.371	1.31	1.208	1.296	0.107	1.190	173.715
2	Chloroform	1.446	1.471	1.611	1.509	0.107	1.403	205.039
3	EtOAc	1.315	1.401	0.907	1.207	0.107	1.101	160.676
5	Methanol	0.143	0.201	0.132	0.158	0.107	0.052	6.411
6	Distilled Water	0.158	0.177	0.186	0.173	0.107	0.067	8.617

The total flavonoid contents of hexane, chloroform, ethyl acetate, methanol, and distilled water extracts were 173.715 mg QE/g, 205.039 mg QE/g, 160.676 mg QE/g, 6.411 mg QE/g, and 8.617 mg QE/g respectively. The bar graph presentation of TFC in various extracts of *A. aspera* is displayed below.

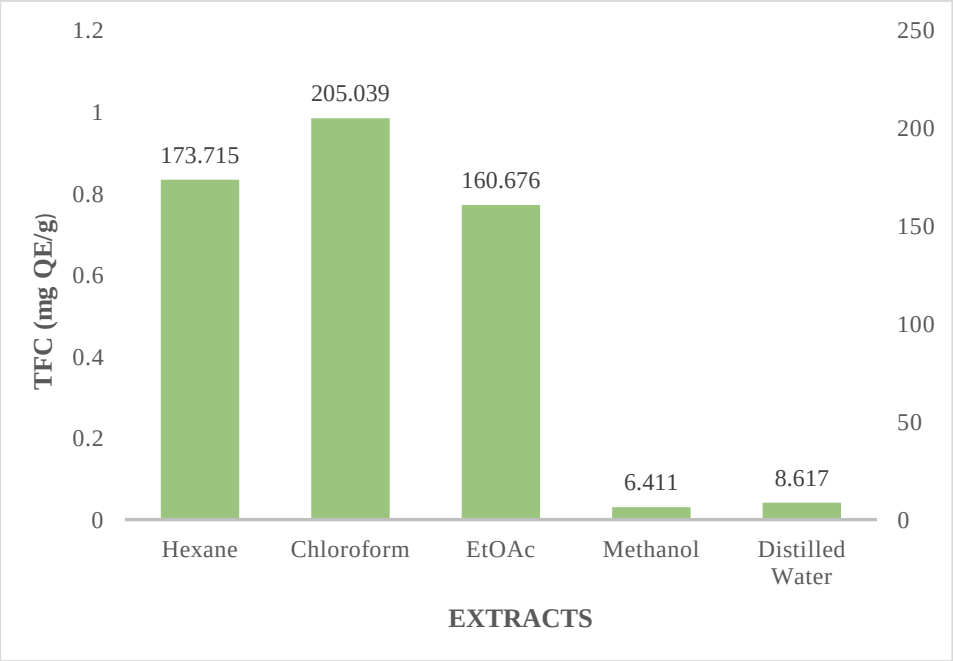


Figure 11: Total flavonoid content in different extracts of *A. aspera*

4.6 Antioxidant Activity

Superoxide dismutase, catalase, peroxidase, and other "free radical scavenging enzymes" are examples of "free radical scavenging enzymes," and antioxidant chemicals, primarily phenolic compounds, carotenoids, tocopherol, and ascorbic acid, are the main antioxidant substances found in plants (Baskar *et al*., 2012). Utilizing linear regression of percent inhibition versus antioxidant activity, the IC_{50} value and antioxidant potential have an adverse connection. The antioxidant activity increases as the IC_{50} value decreases. By graphing % free radical scavenging vs. concentration, the antioxidant activity of various extracts was ascertained. The percentage of DPPH radicals inhibited against the sample shown in the table below was determined using the absorbance values measured at 520 nm, and the IC_{50} value of the corresponding extracts was determined.

The sample's percentage inhibition of DPPH radicals was estimated using the absorbance values measured at 520 nm for various concentrations of the hexane extract. The following table shows the findings:

Table 7: Antioxidant activity of hexane extract of *A. aspera*

Sample	**Concentration (µg/mL)**	**% of scavenging**
Hexane extract of *A. aspera*	2000	83.73
	1000	65.27
	500	45.32
	125	25.48
	IC_{50}	**469.186 µg/mL**

The IC_{50} value for the hexane extract of *A. aspera* was 469.186 µg/mL, as shown in the graph below.

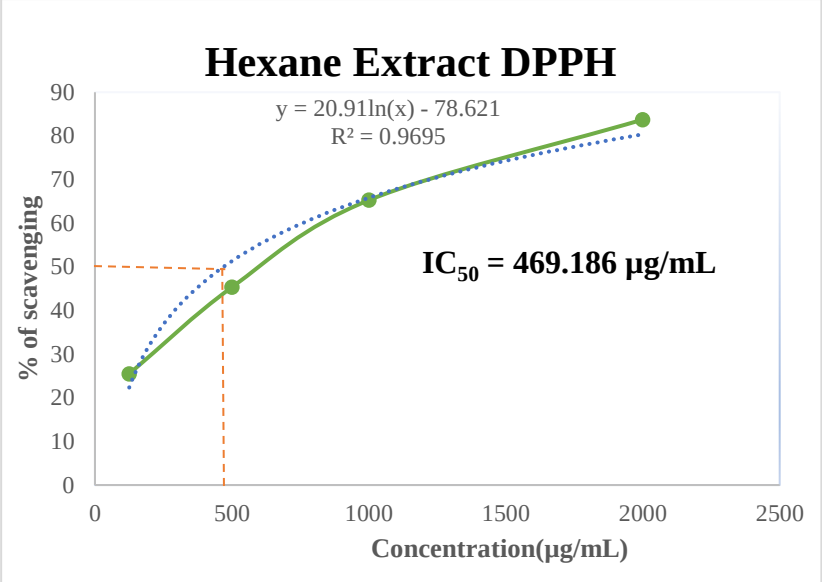


Figure 12: Antioxidant activity of hexane extract of *A. aspera*

The % inhibition of DPPH radicals for the sample was estimated using absorbance values recorded at 520 nm for various concentrations of the chloroform extract. The following table shows the results:

Table 8: Antioxidant activity of chloroform extract of *A. aspera*

Sample	Concentration (µg/mL)	% of scavenging
Chloroform extract of *A. aspera*	1000	82.87
	500	53.12
	250	41.64
	125	28.54
	IC_{50}	**332.28 µg/mL**

The chloroform extract of *A. aspera* had an IC_{50} value of 332.28 µg/mL. Figure 13 illustrates this.

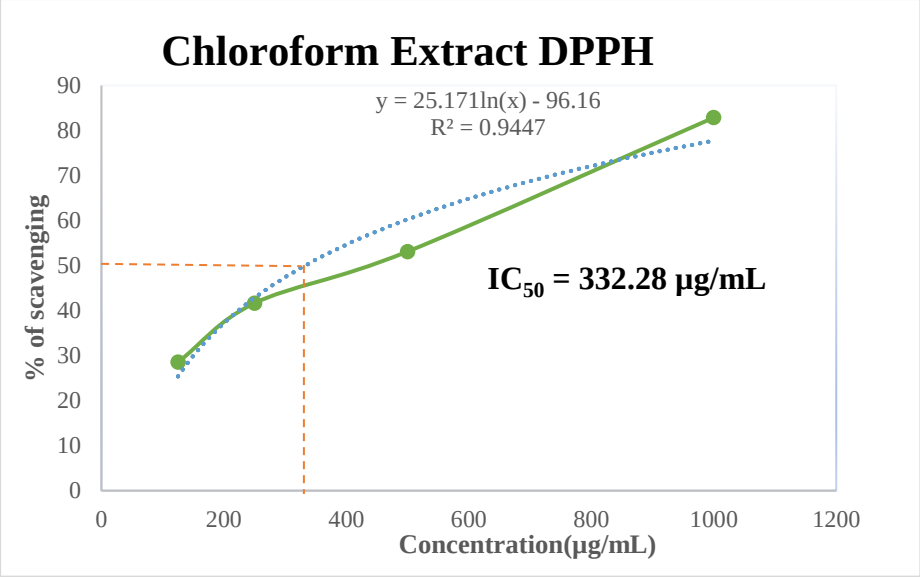


Figure 13: Antioxidant activity of chloroform extract of *A. aspera*

The % inhibition of DPPH radicals for the sample was estimated using the absorbance values measured at 520 nm for various concentrations of the ethyl acetate extract. The outcomes are shown in the following table:

Table 9: Antioxidant activity of ethyl acetate extract of *A. aspera*

Sample	**Concentration (µg/mL)**	**% of scavenging**
Ethyl Acetate Extract of *A. aspera*	2000	65.34
	1000	49.8
	500	43.12
	250	34.64
	IC_{50}	**800.31 µg/mL**

The ethyl acetate extract of *A. aspera* had an IC_{50} value of 800.31 µg/mL. Figure 14 illustrates this.

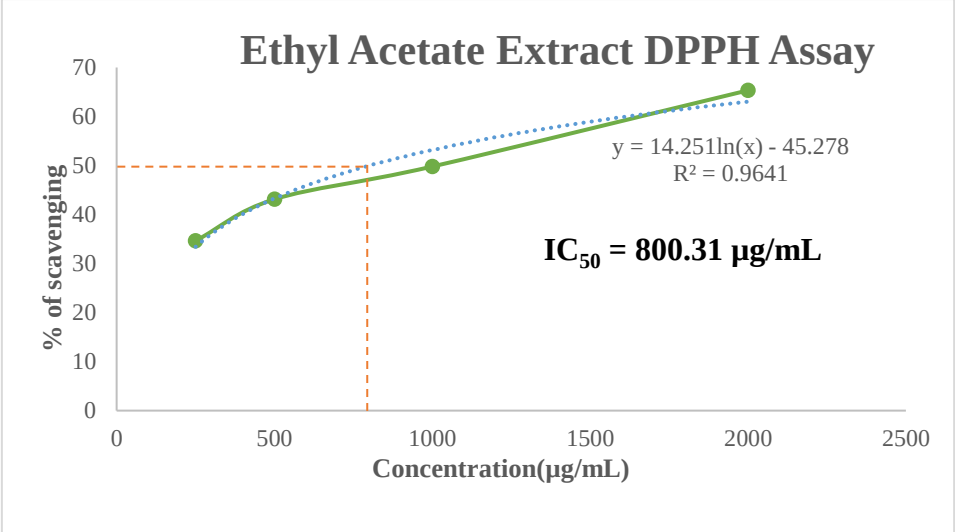


Figure 14: Antioxidant activity of ethyl acetate extract of *A. aspera*

The % inhibition of DPPH radicals for the sample was estimated using the absorbance values measured at 520 nm for various concentrations of the methanol extract. The outcomes are shown in the following table:

Table 12: Antioxidant activity of methanol extract of *A. aspera*

Sample	**Concentration (µg/mL)**	**% of scavenging**
Methanol Extract of *A. aspera*	1000	80.43
	500	66.24
	250	41.64
	125	28.54
	IC_{50}	**300.66 µg/mL**

The Methanol extract of *A. aspera* had an IC_{50} value of 300.66 µg/mL. Figure 15 illustrates this.

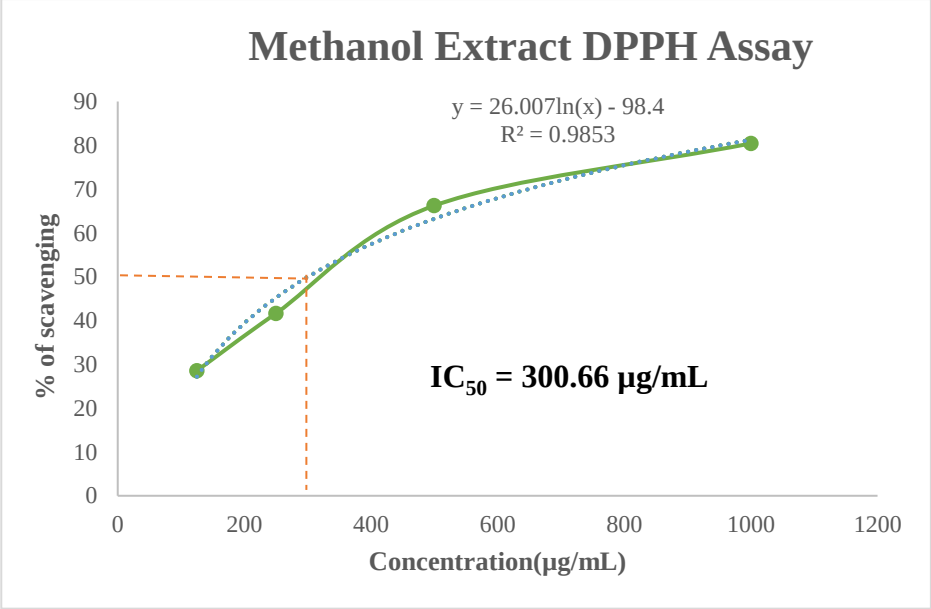


Figure 15: Antioxidant activity of methanol extract of *A. aspera*

The % inhibition of DPPH radicals for the sample was estimated using the absorbance values measured at 520 nm for various concentrations of the distilled water extract. The outcomes are shown in the following table:

Table 13: Antioxidant activity of Distilled Water extract of *A. aspera*

Sample	Concentration (µg/mL)	% of scavenging
Distilled Water Extract of *A. aspera*	2000	59.84
	1000	46.85
	500	25.44
	250	18.33
	IC_{50}	**1272.83 µg/mL**

The Distilled Water extract of *A. aspera* had an IC_{50} value of 1272.83 µg/mL. Figure 16 illustrates this.

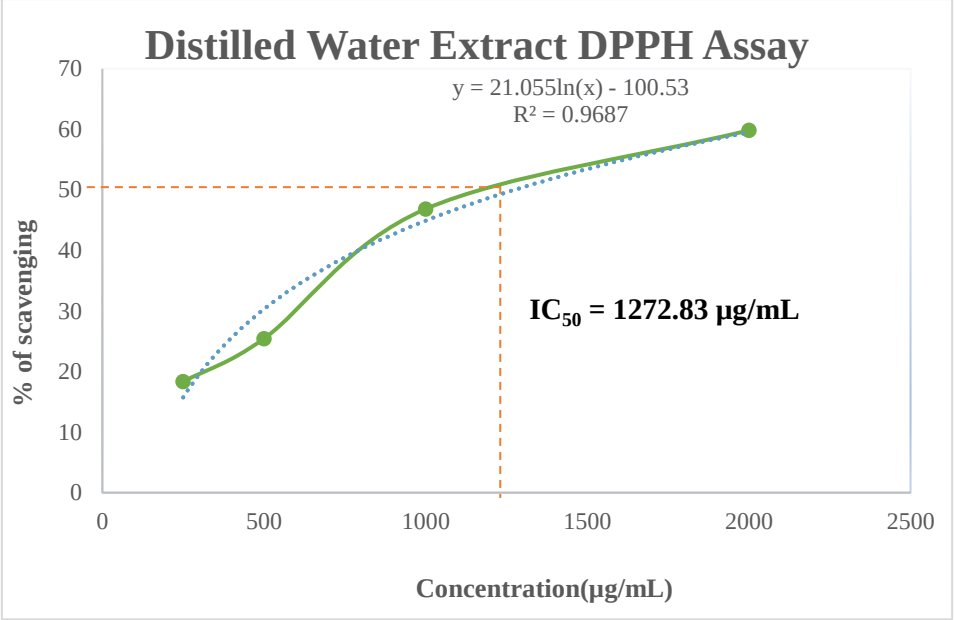


Figure 16: Antioxidant activity of Distilled Water extract of *A. aspera*

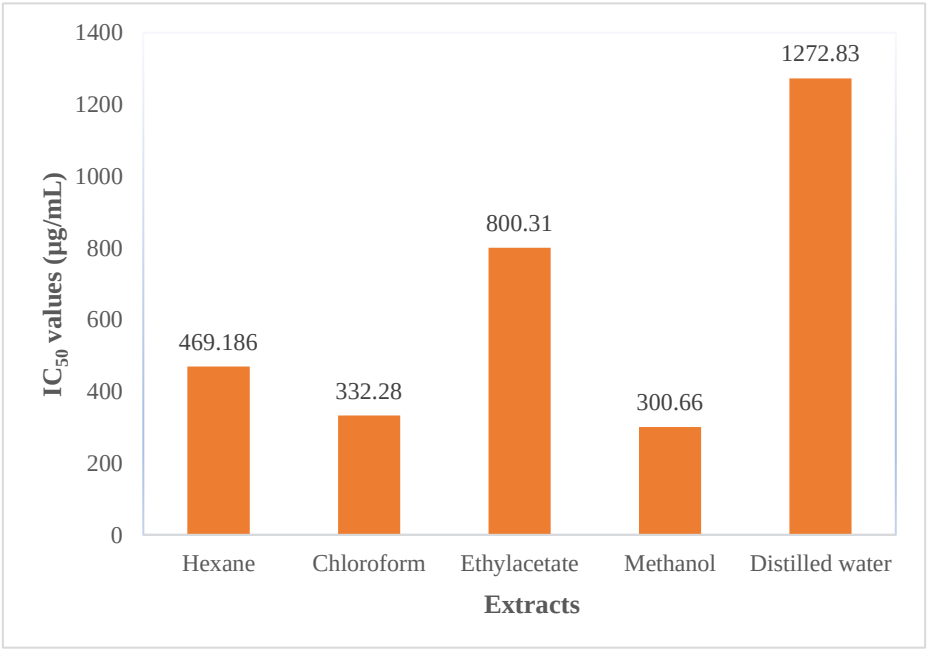


Figure 17: Comparative DPPH IC_{50} values of different extracts

The 50% inhibition of DPPH free radicals induced by a plant extract, commonly known as the IC_{50}, is calculated from the inhibition of DPPH radicals caused by the various doses of the extract. This quantitative indicator shows how much of a specific medicine or other substance (inhibitor) is required to completely inhibit an enzyme, cell, cell receptor, or microbe. The relationship between antioxidant activity and IC_{50} values

suggests that extracts with low IC_{50} values have greater antioxidant capacity than extracts with high IC_{50} values.

Comparatively, *A. aspera* plant extracts proved to be powerful antioxidants in several solvents. Among all extracts, the methanol extract was shown to be the most effective antioxidant (IC_{50}, 300.66 µg/mL) in comparison to chloroform, hexane, methanol, and distilled water were more potent antioxidants (IC_{50} for standard ascorbic acid, 18.33 µg/mL). The least potent antioxidant was distilled water extract (IC_{50}, 1273.83 µg/mL).

The abundance of phenolic compounds underscores their pivotal contribution to the robust antioxidant prowess exhibited by this particular plant species. Moving forward, it is imperative to shift focus towards conducting sophisticated in vivo investigations targeting the medicinal constituents. Such endeavors are essential for unlocking the full therapeutic potential of these compounds, paving the path towards the development of advanced natural pharmaceutical formulations of heightened efficacy and value. (Brijyog *et al*., 2019).

4.7 Antibacterial Activity

To determine the antibacterial activity of different plant extracts, the diameter of the zone of inhibition (ZOI) generated on the bacterium was measured. Using the method outlined in the preceding section, the ability of various *A. aspera* plant extract fractions to inhibit bacterial growth at a fixed concentration (50 mg/mL) was evaluated. The results were represented in terms of the diameter of the zone of inhibition. The area surrounding the antimicrobial disk in which bacteria are inhibited from growing is known as the zone of inhibition. The term "minimum inhibitory concentration" refers to the smallest amount of plant extract required to prevent microbe growth (Venkadassalapathy *et al*., 2023).

The antibacterial activity of *A. aspera* extracts in hexane, chloroform, ethyl acetate, methanol and distilled water was assessed against two Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, and two Gram-negative bacteria, *Escherichia coli* and *Klebsiella pneumonia*, using the zone of inhibition (ZOI) in which Ampicillin (1 mg/mL) was taken as a positive control and DMSO as negative control, respectively.

Table 14: Antibacterial activity of different extracts of *A. aspera*

S.N.	Bacteria	Zone of inhibition (in cm)						
		Positive control	**Negative control**	**Hexane extract**	**Chloroform extract**	**Ethyl acetate extract**	**Methanol extract**	**Distilled water extract**
1	*B. subtilis* (ATCC 6633)	3.4	0	No activity	No activity	No activity	No activity	No activity
2	*S. aureus* (ATCC 33591)	3	0	No activity	1.3, 1.2, 1.1 Concentration at 100, 50 and 25 mg/mL	No activity	No activity	No activity
3	*K. pneumonia* (ATCC 10034)	3.6	0	No activity	No activity	No activity	No activity	No activity
4	*E. coli* (ATCC 25922)	3.5	0	No activity	No activity	No activity	No activity	No activity

Table 14 displays the results of the observation of the antibacterial properties of extracts from hexane, chloroform, ethyl acetate, methanol, and distilled water at a concentration of 50 mg/mL. In contrast to other extracts, chloroform extract only demonstrated 1.3, 1.2 and 1.1 cm triplicate ZOI against *Staphylococcus aureus*.

4.8 α-Amylase Assay

Using the 3,5−dinitro salicylic acid (DNSA) method, the experiment for α−amylase inhibition was carried out. It is possible to evaluate the antidiabetic potential, which is inversely related to the IC_{50} value, using the linear regression equation of percent inhibition against α−amylase inhibition activity (Kamalakkannan & Balakrishnan, 2015). At 540 nm, the extracts' absorbance values were calculated for various concentrations.

The % α−amylase inhibition abilities of several *A. aspera* extracts are displayed in the tables and figures.

Table 15: α−Amylase inhibition of hexane extract of A. aspera

Sample	Concentration (µg/mL)	% Inhibition
Hexane extract of *A. aspera*	5000	60.22
	2500	54.06
	1250	46.88
	625	44.28
	IC_{50}	**1489.208 µg/mL**

Figure 18 shows a graphical representation of the inhibition of α−amylase by *A. aspera* hexane extract, with an IC_{50} of 1489.208 µg/mL.

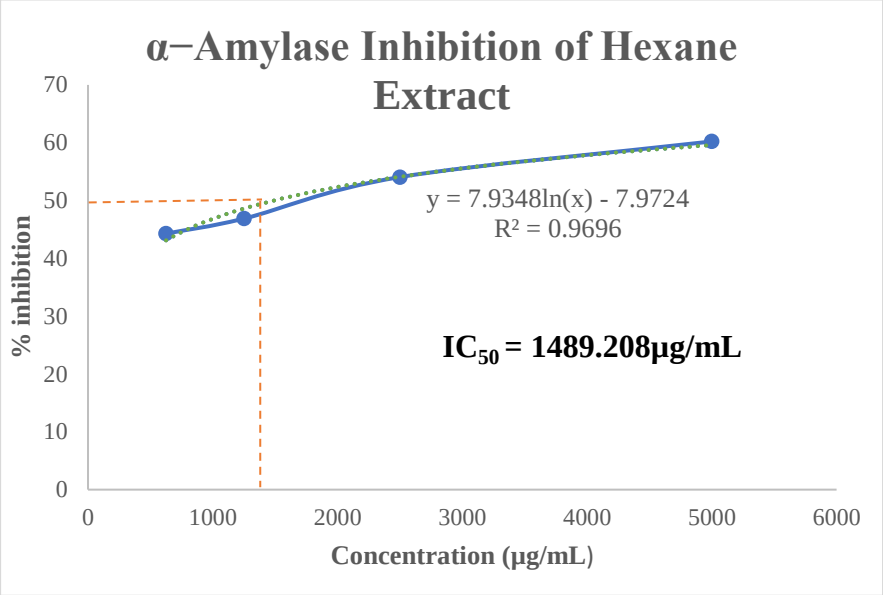


Figure 18: α−Amylase inhibition of hexane extract of *A. aspera*

The percentage of inhibition of α−amylase caused by the chloroform extract is shown in the table below.

Table 16: α−Amylase inhibition of chloroform extract of *A. aspera*

Sample	Concentration (µg/mL)	% Inhibition
Chloroform extract of *A. aspera*	5000	97.1
	2500	78.62
	625	52.89
	312.5	43.47
	IC_{50}	**488.334 µg/mL**

Figure 19 shows a graphical representation of the inhibition of α−amylase by *A. aspera* chloroform extract, with an IC_{50} of 488.334 µg/mL.

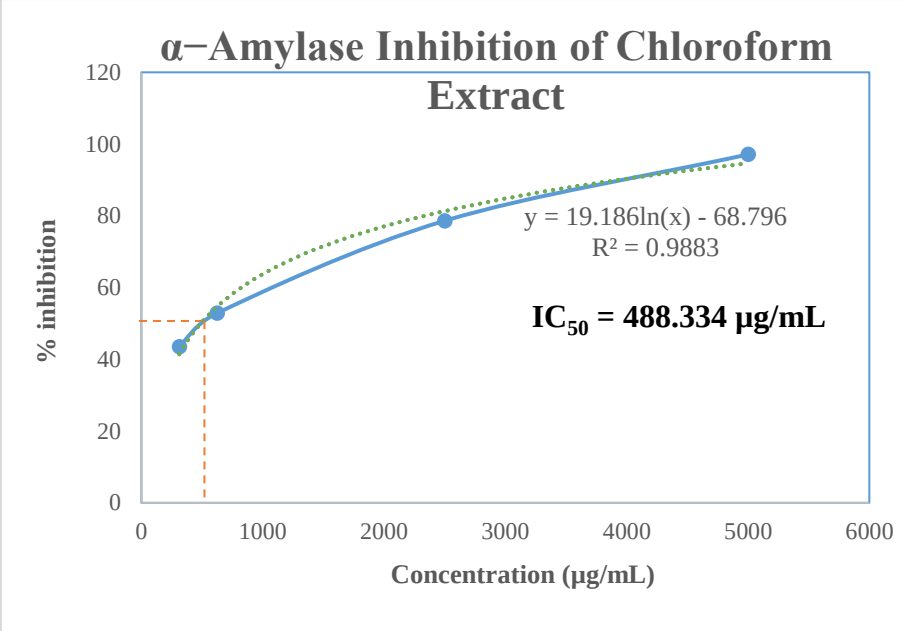


Figure 19: α−Amylase inhibition of chloroform extract of *A. aspera*

The level of α−amylase inhibition brought on by the ethyl acetate extract is depicted in the following table.

Table 17: α−Amylase inhibition of ethyl acetate extract of *A. aspera*

Sample	Concentration (µg/mL)	% Inhibition
Ethyl Acetate extract of *A. aspera*	5000	89.27
	2500	71.26
	1250	45.21
	625	42.91
	312.5	34.48
	IC_{50}	**895.157 µg/mL**

Figure 20 shows a graphical representation of the inhibition of α−amylase by *A. aspera* ethyl acetate extract, with an IC_{50} of 895.157 µg/mL.

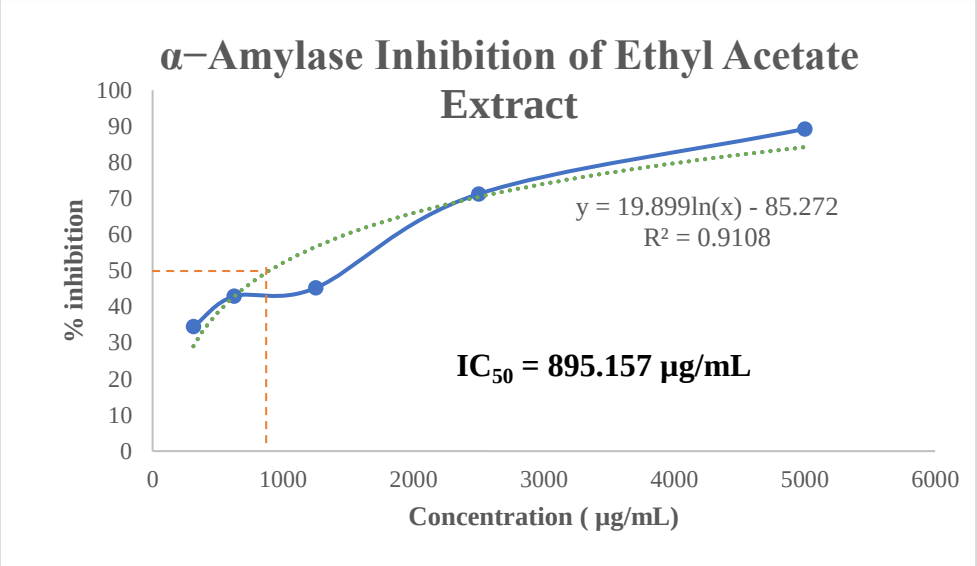


Figure 20: α−Amylase inhibition of ethyl acetate extract of *A. aspera*

The following table shows how much the methanol extract of *A. aspera* inhibited α−amylase.

Table 18: α−Amylase inhibition of methanol extract of *A. aspera*

Sample	Concentration (µg/mL)	% Inhibition
Methanol extract of *A. aspera*	5000	87.36
	2500	62.74
	1250	54.36
	625	43.09
	IC_{50}	**986.338 µg/mL**

The IC_{50} for the methanol extract of *A. aspera* was 986.338 µg/mL. This is depicted in the following figure:

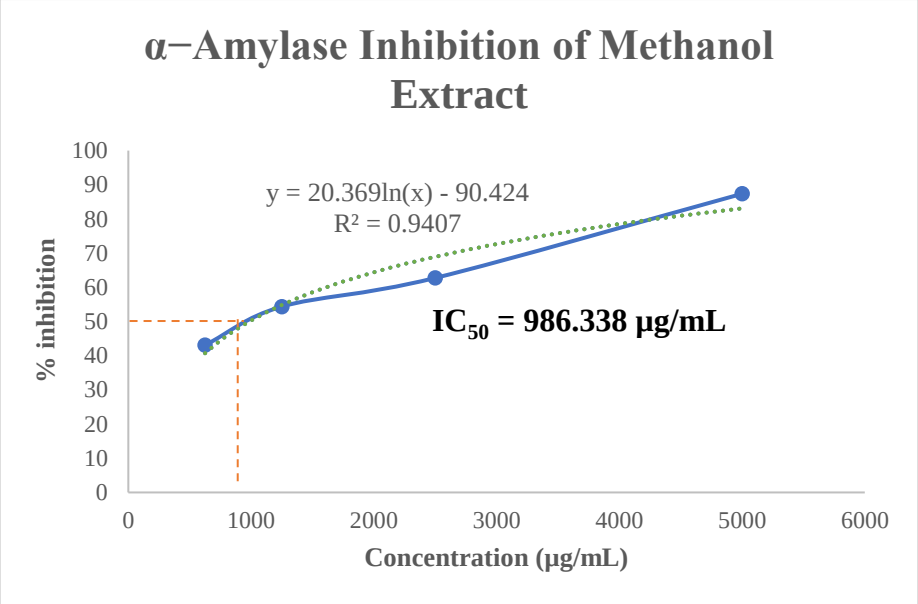


Figure 21: α−Amylase inhibition of methanol extract of *A. aspera*

The following table shows how much the distilled water extract of *A. aspera* inhibited α−amylase.

Table 19: α−Amylase inhibition of distilled water extract of *A. aspera*

Sample	Concentration (µg/mL)	% Inhibition
Distilled Water extract of *A. aspera*	5000	64.6
	2500	54.57
	1250	49.85
	625	42.47
	312.5	40.11
	IC_{50}	**1204.717 µg/mL**

Figure 22 shows a graphical representation of the inhibition of α−amylase by *A. aspera* distilled water extract, with an IC_{50} of 1204.717 µg/mL.

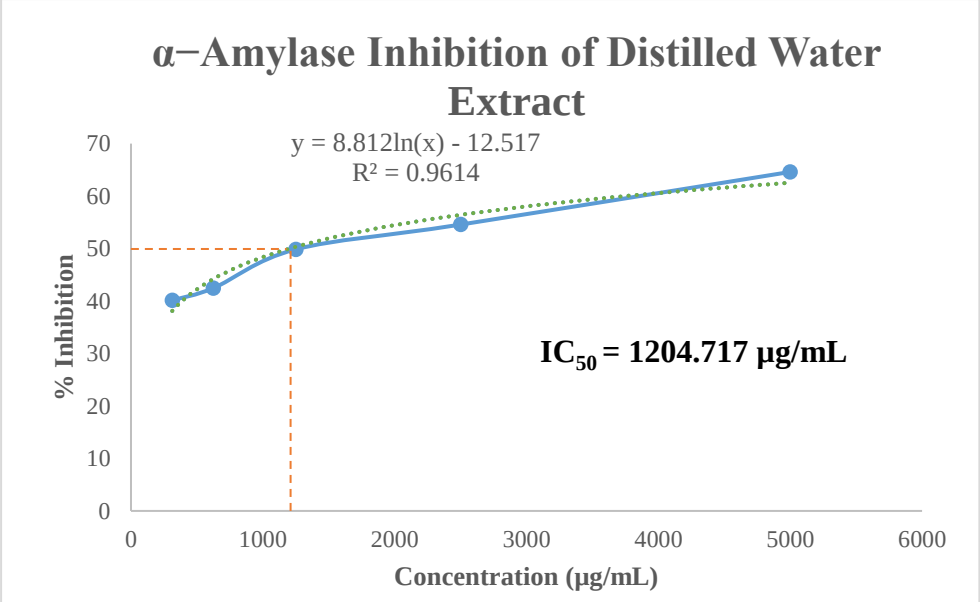


Figure 22: α−Amylase inhibition of distilled water extract of *A. aspera*

Following is a bar graph that compares the relative α−Amylase inhibition activity of various extracts.

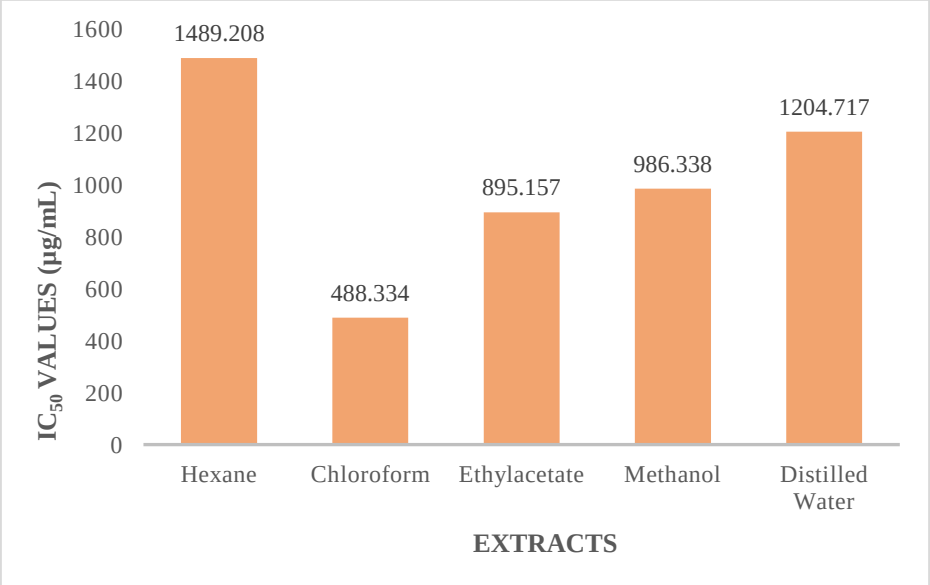


Figure 23: Comparative α−Amylase inhibition activity of different extracts

Chloroform (IC_{50}, 488.334 µg/mL) extract therefore appeared to be a potentially effective α−amylase inhibitors. The α−amylase inhibitory activity of ethyl acetate, methanol, distilled water and hexane extracts was found to be quite low.

The α-amylase inhibition demonstrated by the extracts was notably high compared to the standard acarbose, suggesting a potential effectiveness in their activity (Yashoda *et al*., 2021).

4.9 Brine Shrimp Lethality Assay (BSLA)

Freshly hatched live nauplii were exposed to concentrations of 1000−62.5 µg/mL to evaluate the LC_{50} values for hexane, chloroform, ethyl acetate, methanol, and distilled water extracts, respectively. Between the extract concentration and the degree of lethality, a clear relationship was seen. When given a dose of 1000 µg/mL, most brine shrimp larvae died, whereas 62.5 µg/mL caused the fewest to perish.

The tables and graphs below show the relationship between the degree of lethality and the various extract concentrations.

The table below shows the results of the determination of the LC_{50} value for the hexane extract:

Table 20: Calculation of mortality % of hexane extract

Sample	**Concentration (µg/mL)**	**No. of alive nauplii**			**% Mortality**
Hexane extract of *A. aspera*	1000	0	0	0	100
	500	0	1	1	93.33
	250	2	2	3	76.67
	125	6	6	5	43.33
	62.5	8	7	7	26.67
	LC_{50}	**132.55 µg/mL**			

The LC_{50} value of the hexane extract of *A. aspera* was 132.55 µg/mL. This is shown in the figure below:

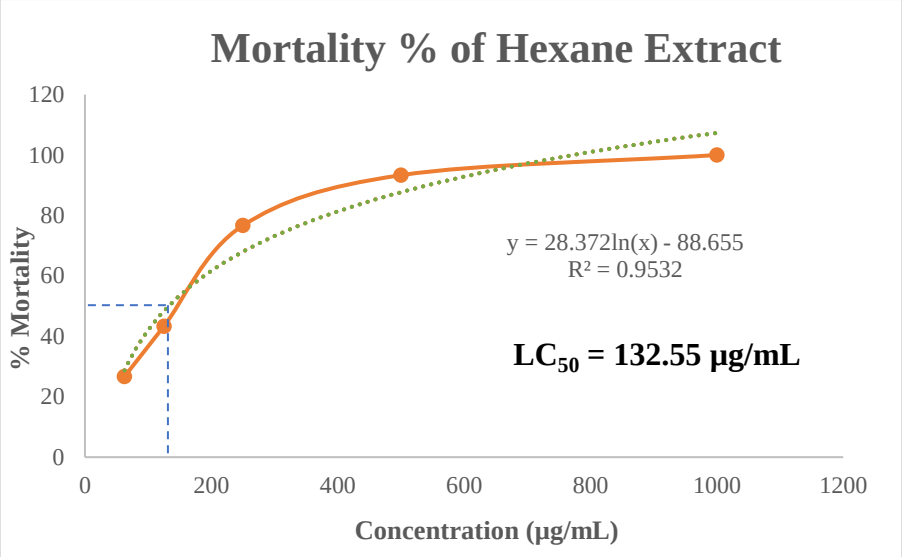


Figure 24: Calculation of mortality % of hexane extract

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

Table 21: Calculation of mortality % of chloroform extract

Sample	Concentration (µg/mL)	No. of alive nauplii			% Mortality
Chloroform extract of *A. aspera*	1000	0	0	0	100
	500	3	2	2	76.67
	250	5	6	6	43.33
	125	7	7	8	26.67
	62.5	9	10	8	10
	LC_{50}	**240.086 µg/mL**			

The LC_{50} value of the chloroform extract of *A. aspera* was 240.086 µg/mL. This is shown in the figure below:

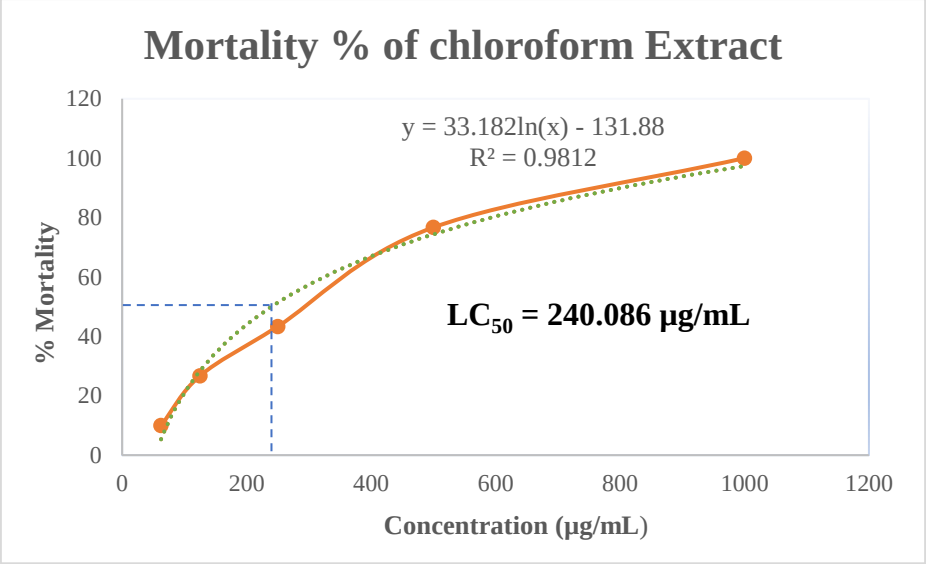


Figure 25: Calculation of mortality % of chloroform extract

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

Table 22: Calculation of mortality % of ethyl acetate extract

Sample	**Concentration (µg/mL)**	**No. of alive nauplii**			**% Mortality**
Ethyl Acetate extract of *A. aspera*	1000	0	0	0	100
	500	0	0	0	100
	250	0	0	0	100
	125	1	0	2	90
	62.5	2	3	2	76.67
	LC_{50}	**12.18 µg/mL**			

The LC_{50} value of the ethyl acetate extract of *A. aspera* was 12.18 µg/mL. This is shown in the figure below:

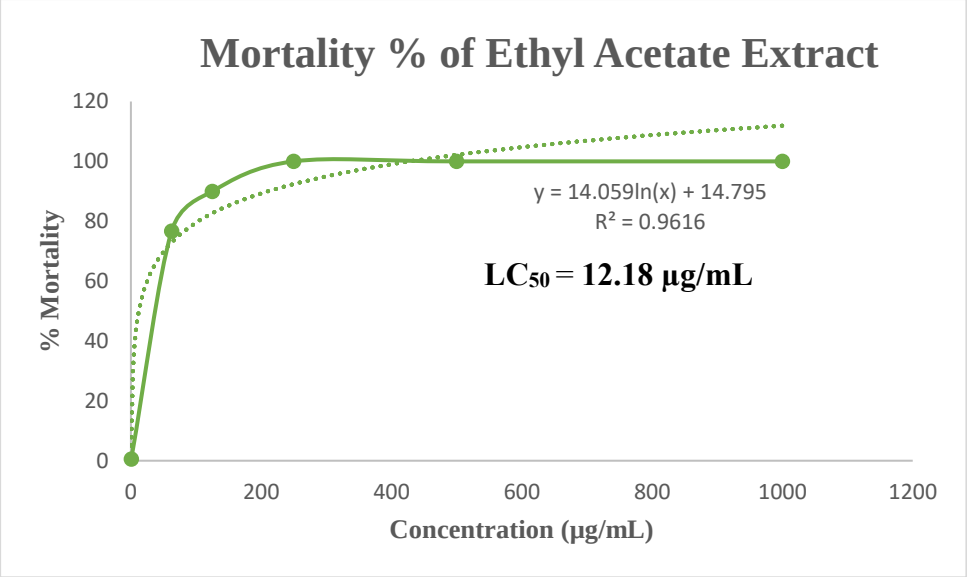


Figure 26: Calculation of mortality % of ethyl acetate extract

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

Table 23: Calculation of mortality % of methanol extract

Sample	**Concentration (µg/mL)**	**No. of alive nauplii**			**% Mortality**
Methanol extract of *A. aspera*	1000	0	0	0	100
	500	1	2	2	83.33
	250	3	4	3	66.67
	125	7	6	6	36.67
	62.5	10	8	8	13.33
	LC_{50}	**182.36 µg/mL**			

The LC_{50} value of the Methanol extract of *A. aspera* was 182.36 µg/mL. This is shown in the figure below:

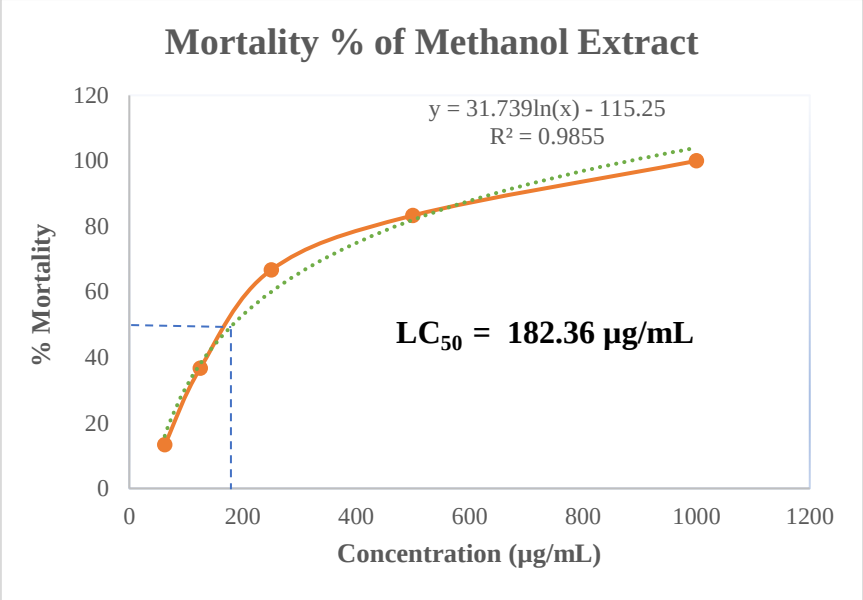


Figure 27: Calculation of mortality % of methanol extract

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

Table 24: Calculation of mortality % of distilled water extract

Sample	Concentration (µg/mL)	No. of alive nauplii			% Mortality
Distilled water extract of *A. aspera*	1000	0	1	0	96.67
	500	5	7	6	40
	250	7	8	8	23.33
	125	8	9	9	13.33
	62.5	10	9	9	6.67
	LC_{50}	**399.81 µg/mL**			

The LC_{50} value of distilled water extract of *A. aspera* was 399.81 µg/mL. This is shown in the figure below:

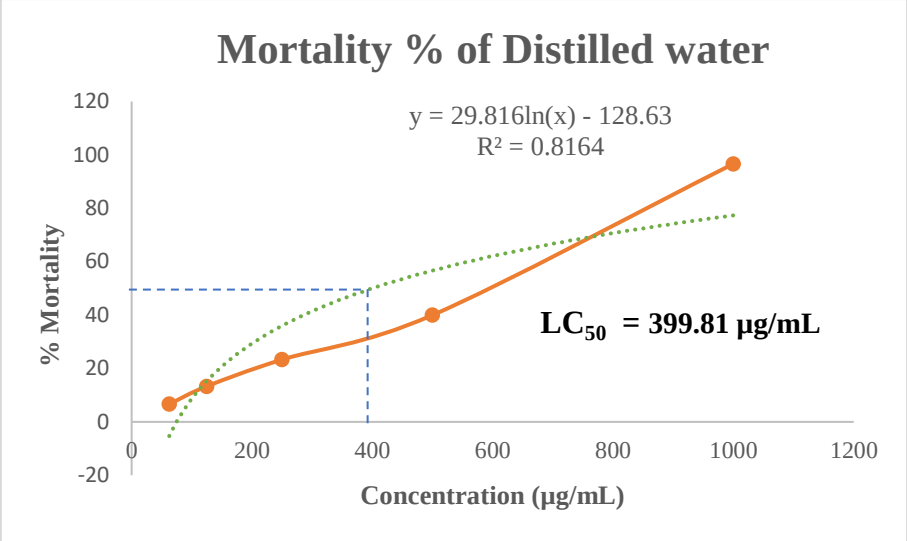


Figure 28: Calculation of mortality % of distilled water extract

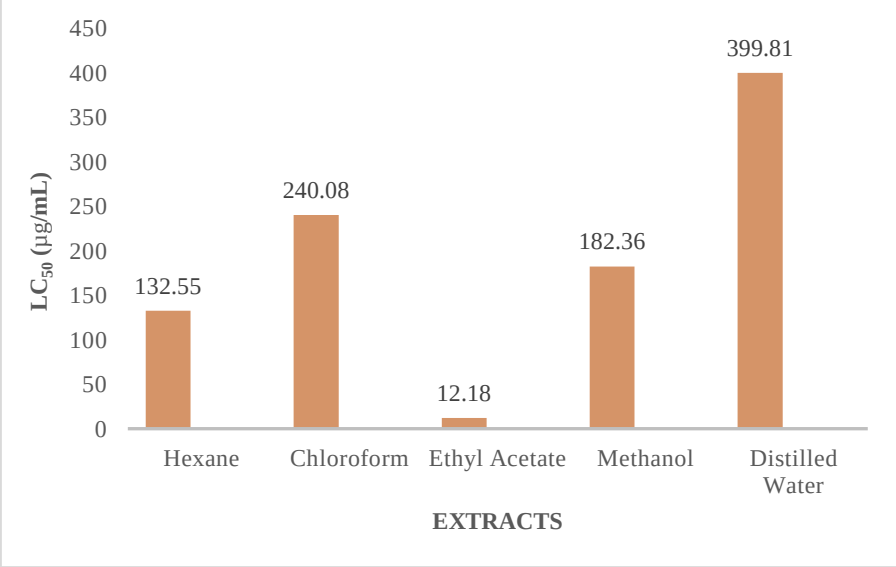


Figure 29: Comparative LC_{50} values of different extracts

From above, hexane, chloroform, ethyl acetate, and methanol extracts were discovered to be pharmaceutically potent and harmful to brine shrimp larvae and had LC_{50} values of 132.55 µg/mL, 240.08 µg/mL, 12.18 µg/mL, 182.36 µg/mL, 399.81 µg/mL respectively. Hexane showed the strongest % mortality, and distilled water extract showed the least.

The value of LC_{50} less than 250 µg/mL is significantly active (Jakir, 2013).

4.10 UV Spectroscopic Measurements

UV−Vis measurement, in particular. Finding unsaturation in organic compounds is frequently accomplished by spectroscopy. According to (Nazir *et al*., 2016), it can offer

important details regarding a compound's electronic structure, such as the conjugation of double bonds and the existence of π-bonds.

As shown below, a chromatogram in several extracts was used to demonstrate the observed data.

4.10.1 UV Spectroscopic Measurement of Hexane Extract

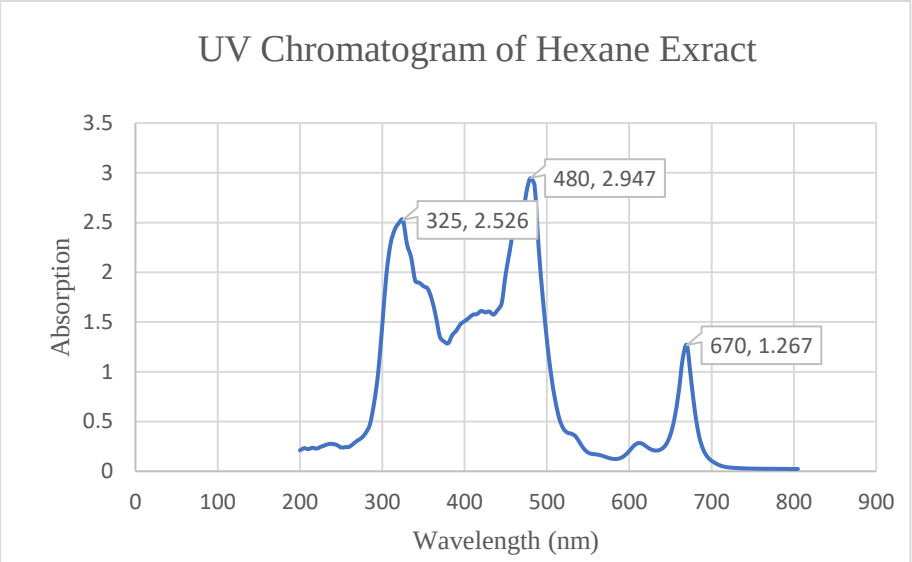


Figure 30: UV chromatogram of hexane extract

4.10.2 UV Spectroscopic Measurement of Chloroform Extract

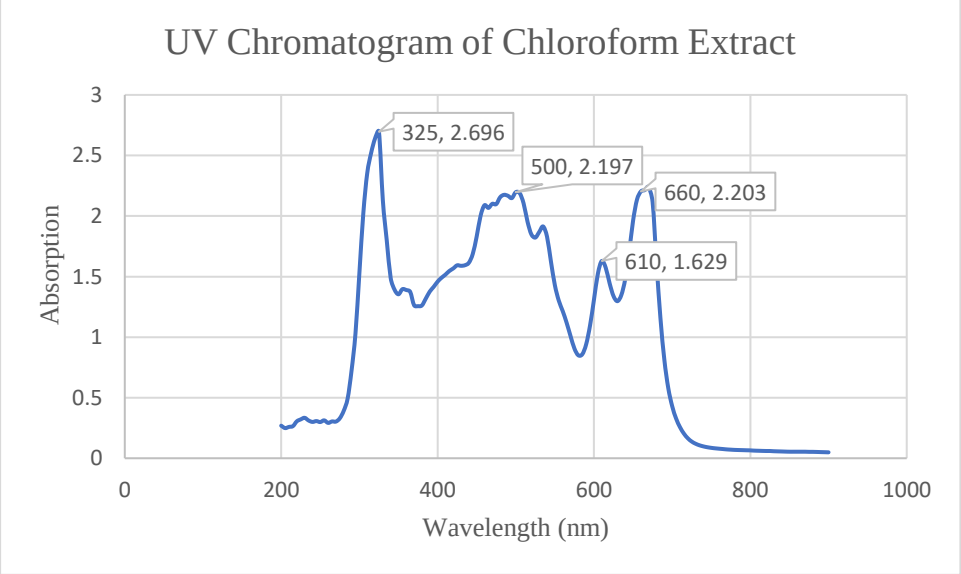


Figure 31: UV chromatogram of chloroform extract

4.10.3 UV Spectroscopic Measurement of Ethyl Acetate Extract

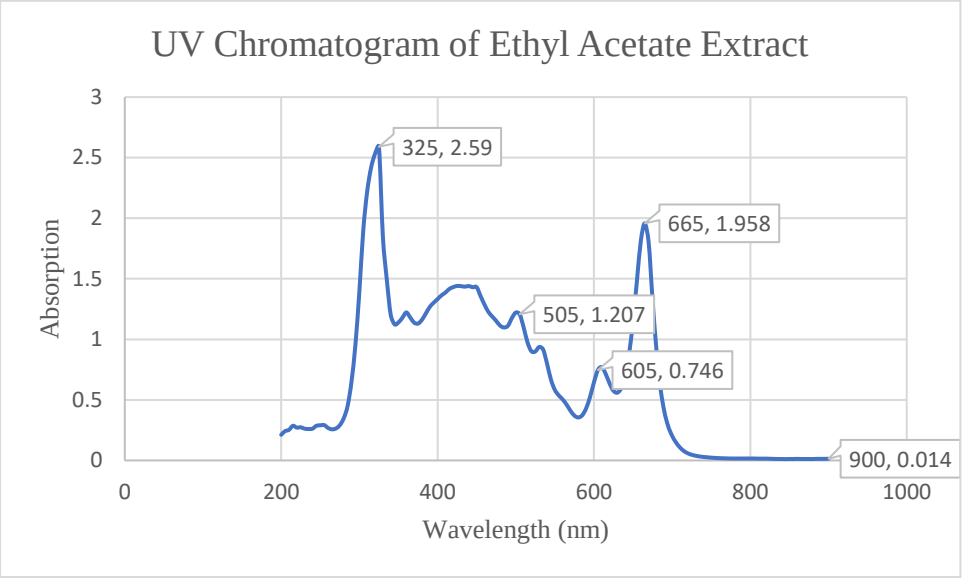


Figure 32: UV chromatogram of ethyl acetate extract

4.10.4 UV Spectroscopic Measurement of Methanol Extract

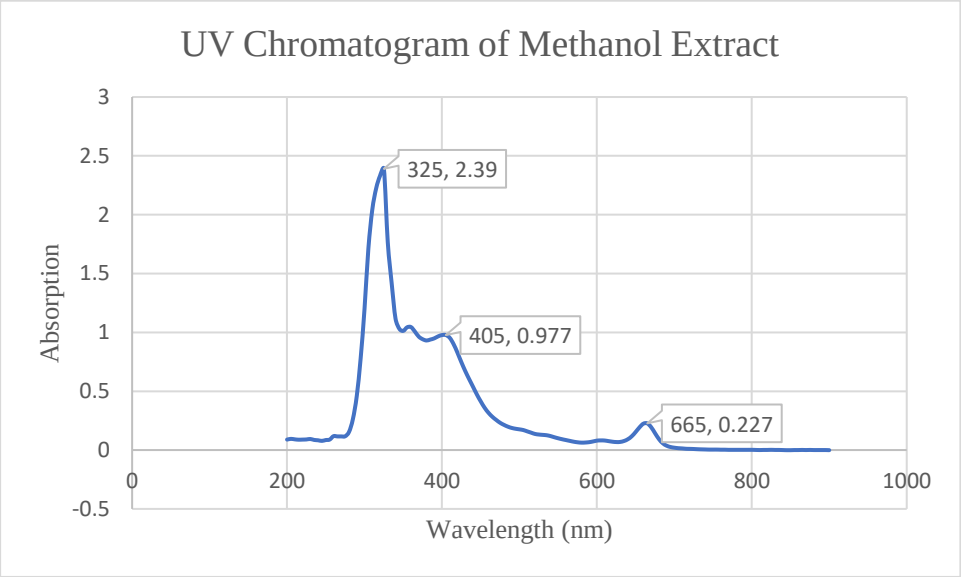


Figure 33: UV chromatogram of methanol extract

4.10.5 UV Spectroscopic Measurement of Distilled Water Extract

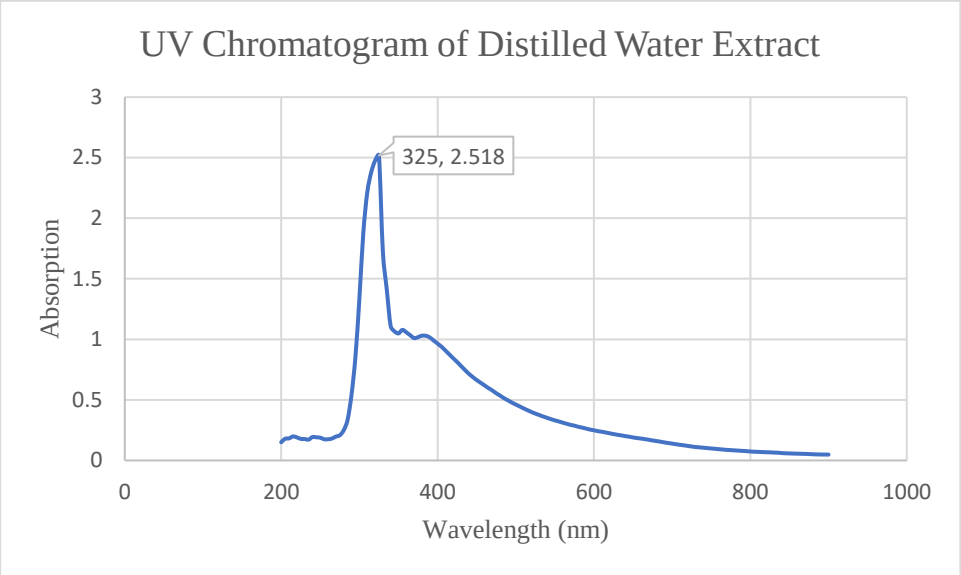


Figure 34: UV chromatogram of distilled water extract

4.11 FT-IR Analysis

The extract's active components were identified and their characteristic peaks and functional groups were detected using the FT-IR spectrum. This was done by examining peak values in the infrared radiation range. When analyzing organic compounds using FTIR spectroscopy, important details like the sample's bonding, aromatic and aliphatic structures, and functional groups can be found. The chemical structure of the sample is revealed by the FTIR spectrum, which also verifies the presence of different functional groups and organic molecules. It is possible to determine the binding between heteroatoms and carbon by examining the stretching frequency in the FTIR spectrum. FTIR spectroscopy is an effective method for identifying organic compounds and learning more about their structure and composition overall (Zulhussnain *et al*., 2020).

4.11.1 FT-IR Spectrum of Hexane Extract of *A. aspera* plant

The following table presents the observed wavenumbers of hexane extract together with their corresponding absorbance, kind of stretching, appearance, presence of a functional group, and frequency range. Peak values are shown in the figure for different functional groups.

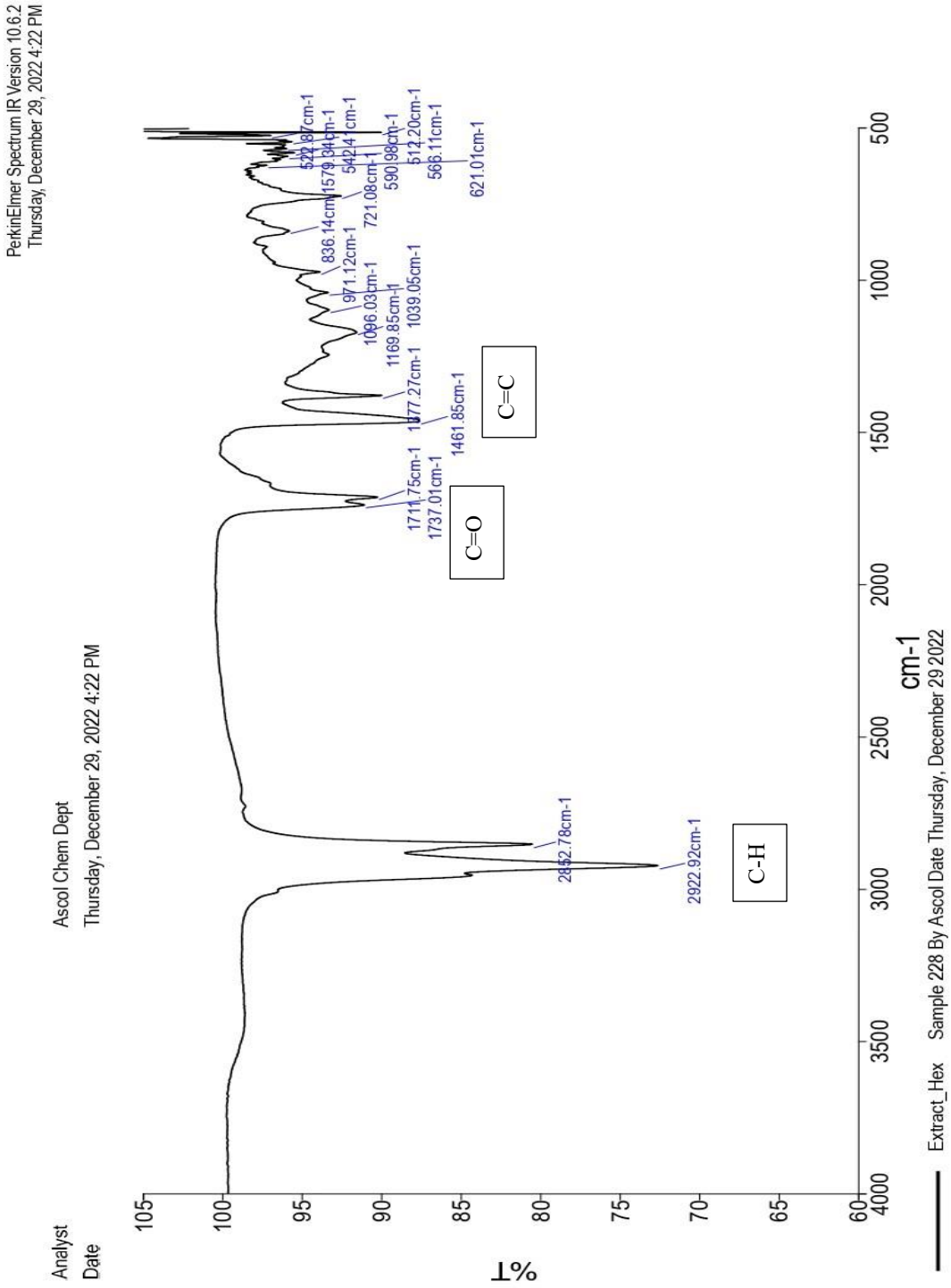


Figure 35: FT-IR peak values of hexane extract of *A. aspera* plant

Table 25: FT-IR peak values and functional groups of hexane extract *A. aspera*

Sample	Absorption (cm^{-1})	Types of Stretching	Appearance	Functional Group	Frequency Range (cm^{-1})
Hexane extract of *A. aspera*	1096.03	C-O stretch	medium	Alkyl-substituted ether	1150–1050
	1169.85	CN stretch	strong	Secondary amine	1190–1130
	1377.27	C-C stretching	strong	Aromatic compounds	1300-1200
	1461.85	C=C stretching	strong	Substituted aromatic rings	1500-1450
	1711.75	(C=O) stretching	strong	Carbonyl group	1725–1700
	1737.01	(C=O) stretching	medium	Ester	1750–1725
	2852.78	C-H stretching vibration	strong	Alkanes or alkyl group	3000-2800
	2922.92	C-H stretching vibration	strong	Alkanes or alkyl group	3000-2800

The FT-IR standard chart was compared, and in hexane extract, strong bands were found in 2922.92 cm^{-1} and 2852.78 cm^{-1} for C-H stretching, 1711.75 cm^{-1} for C=O stretching, 1461.85 for C=C stretching, 1377.27 cm^{-1} for C-C and 1169.85 cm^{-1} for CN stretching. The medium bands were 1737.01 cm^{-1} for C=O and 1096.03 cm^{-1} for C-O stretching.

4.11.2 FT-IR Spectrum of Chloroform Extract of *A. aspera* plant

The chloroform extract's FT-IR properties are displayed in the table and figure below. The table's appearance, stretching type, associated functional group, and absorption wavenumber are mentioned.

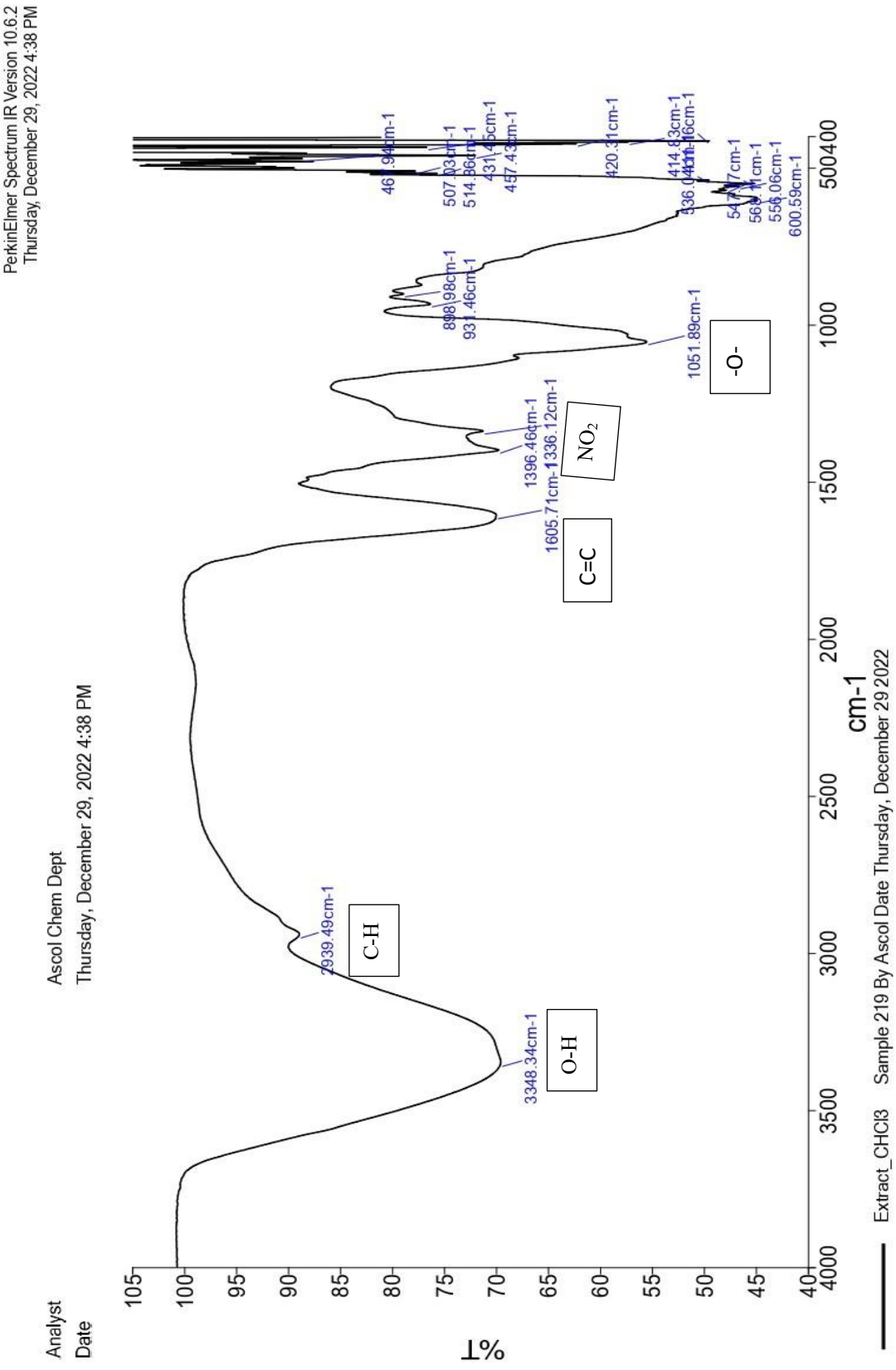


Figure 36: FT-IR peak values of chloroform extract of *A. aspera* plant

Table 26: FT-IR peak values and functional groups of chloroform of *A. aspera*

Sample	Absorption (cm^{-1})	Types of Stretching	Appearance	Functional group	Frequency Range (cm^{-1})
Chloroform extract of *A. aspera*	1051.89	Cyclohexane ring vibrations	medium	Cyclo alkane	1055–1000
	1336.12	OH in-plane bend	strong	Primary or secondary alcohol	1350–1260
	1396.46	N-O stretching	medium	Aliphatic nitro compounds	1380–1350
	1605.71	C=C stretch	strong	Alkenyl	1680–1620
	2939.49	C-H stretching vibration	strong	Alkanes or alkyl group	3000-2800
	3348.34	-O-H stretch	weak	Aliphatic primary amine	3400–3380

4.11.3 FT-IR Spectrum of Ethyl Acetate Extract of *A. aspera* plant

A table and an IR graph were used to summarize the FT-IR spectroscopy records of the ethyl acetate extract below.

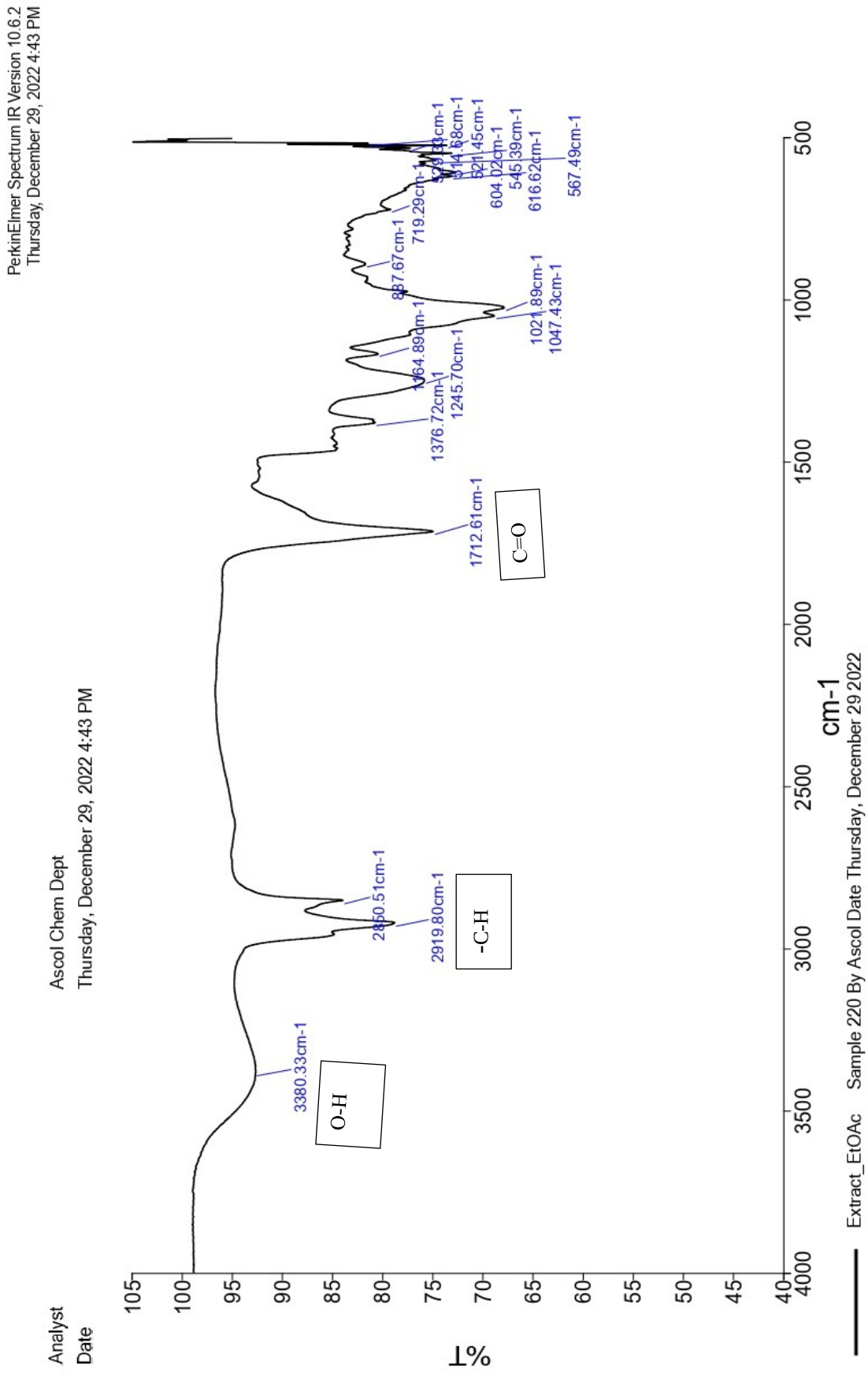


Figure 37: FT-IR peak values of ethyl acetate extract of *A. aspera* plant

Table 27: FT-IR peak values and functional groups of ethyl acetate of *A. aspera*

Sample	Absorption (cm^{-1})	Types of Stretching	Appearance	Functional group	Frequency Range (cm^{-1})
Ethyl Acetate extract of *A. aspera*	1021.89	Cyclohexane ring vibrations	weak	Cyclo alkane	1055–1000
	1047.43	Cyclohexane ring vibrations	medium	Cyclo alkane	1055–1000
	1164.89	CN stretch	medium	Secondary amine	1190–1130
	1245.70	OH in-plane bend	strong	Primary or secondary alcohol	1350–1260
	1376.72	N-O stretching	strong	Aliphatic nitro compounds	1380–1350
	1712.61	C=O stretch	strong	Carboxylic acid	1725–1700
	2850.51	-C-H stretching vibration	strong	Alkanes or alkyl group	3000-2800
	2919.80	-C-H stretching vibration	medium	Alkanes or alkyl group	3000-2800
	3380.33	-O-H stretch	weak	Polymeric hydroxy group	3400–3200

4.11.4 FT-IR Spectrum of Methanol Extract of *A. aspera* plant

The table and picture below illustrate the FTIR spectroscopy report, showing the different methanol extract characteristics peaks:

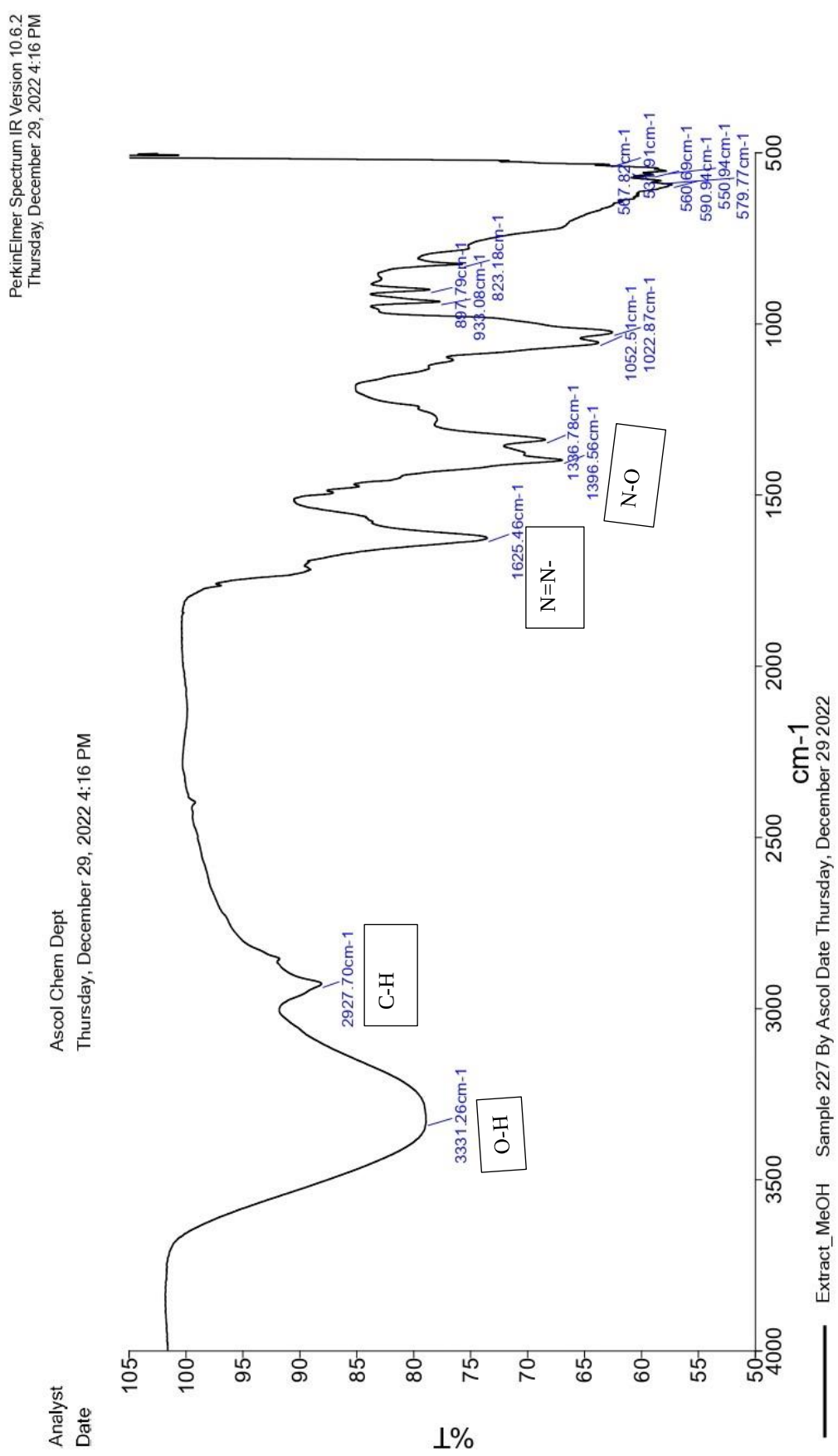


Figure 38: FT-IR peak values of methanol extract of *A. aspera* plant

Table 28: FT-IR peak values and functional groups of methanol of *A. aspera*

Sample	Absorption (cm^{-1})	Types of stretching	Appearance	Functional group	Frequency Range (cm^{-1})
Methanol extract *A. aspera*	897.79	C-O-O- stretch	weak	Peroxides	890–820
	1022.87	Cyclohexane ring vibrations	strong	Cyclo alkane	1055–1000
	1052.51	CN stretch	weak	Primary amine	1090–1020
	1336.78	OH in-plane bend	strong	Primary or secondary alcohol	1350–1260
	1396.56	N-O stretching	strong	Aliphatic nitro compounds	1380–1350
	1625.46	(-N=N-)	medium	Open-chain azo	1630–1575
	2927.70	-C-H stretching vibration	medium	Alkanes or alkyl group	3000-2800
	3331.26	H-bonded OH stretch	weak	Hydroxy compound	3570–3200

CHAPTER V: CONCLUSION AND RECOMMENDATION

5.1 Conclusions

A. aspera extracts are abundant sources of secondary metabolites, according to phytochemical studies. Phytochemical screening of all the ultrasonic-mediated extracts revealed the presence of flavonoids, terpenoids, alkaloids, phenolic compounds and volatile oils.

The presence of various compounds in extracts was confirmed by TLC, which revealed numerous spots in hexane, chloroform, ethyl acetate, methanol and distilled water extract, respectively.

The analysis of phenolic and flavonoid compounds in various solvent extracts revealed striking differences in their concentrations. Phenolic compounds were found in significantly higher amounts in the hexane, chloroform, ethyl acetate, and methanol extracts compared to the distilled water extract. Specifically, the hexane, chloroform, ethyl acetate, and methanol extracts contained 15.863 mg GAE/g, 10.991 mg GAE/g, 11.076 mg GAE/g, and 2.358 mg GAE/g of phenolic compounds, respectively. In contrast, the distilled water extract exhibited a substantially lower phenolic content, measuring only 0.307 mg GAE/g.

Similarly, the flavonoid content varied across different solvent extracts. The chloroform, hexane, ethyl acetate, and distilled water extracts displayed higher concentrations of flavonoids compared to the methanol extract. Specifically, the chloroform, hexane, ethyl acetate, and distilled water extracts contained 173.715 mg QE/g, 205.039 mg QE/g, 160.676 mg QE/g, and 8.617 mg QE/g of flavonoids, respectively. In contrast, the methanol extract showed a relatively lower concentration of flavonoids, measuring at 6.411 mg QE/g.

The assay for DPPH scavenging revealed that the methanol extract had the highest antioxidant potency (IC_{50} of 300.66 µg/mL) when compared to the other extracts such as chloroform, hexane, methanol, and distilled water, which had IC_{50} of 18.33 µg/mL for standard ascorbic acid. With an IC_{50} of 1273.83 µg/mL, distilled water extract was the least effective antioxidant.

The results of the evaluation of the chloroform extract's antibacterial activity revealed that it significantly inhibited the growth of Gram-positive bacteria *Staphylococcus*

aureus (ZOI, 1.3, 1.2, and 1.1 cm), while no antibacterial activity was shown by the other extracts.

Based on the results of the α-amylase inhibition test, chloroform extract (IC_{50}, 488.334 µg/mL) seemed to have the highest potential for effectiveness among all extracts. It was discovered that the extracts of ethyl acetate, methanol, distilled water and hexane had very little α-amylase inhibitory activity

Hexane, Chloroform, Methanol, and Distilled water extracts exhibited notable lethality towards brine shrimp larvae.

5.2 Recommendation

The plants' phytochemical analysis revealed the presence of numerous biologically active substances. These different secondary metabolites are what give medicinal plants their therapeutic qualities. To extract a greater variety of phytochemicals in higher amounts, it is preferable to prepare plant extracts in other major solvents using different extraction techniques than those outlined here. Column chromatography, LC-MS, HPLC & Spectral analysis can be used to extract pure compounds. Due to the plant's high extraction capacity, a wide range of active ingredients for various biological activities can be produced; these constituents could be utilized to create a potent medication. To extract novel active compounds from this plant, more investigation is required. This could result in a novel approach to treating several illnesses.

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APPENDIX

1: Reagents Used for Phytochemical Screening

Mayer's Reagent: 1.358 g of $HgCl_2$ was dissolved in 60 mL of water and mixed with a solution of 5 g of Potassium iodide (KI) in 10 mL of water.

Dragendorff's Reagent:

Solution I: 1.07 g basic bismuth nitrate and 20 g tartaric acid were dissolved in 80 mL distilled water.

Solution II: 16 g potassium iodide was dissolved in 40 mL distilled water.

Mix equal volumes of solution I and solution II, which is an actual Dragendorff"s reagent.

Wagner's Reagent: 16.6 g of KI was dissolved in 100 mL of distilled water followed by the addition of a few crystals of iodine to the solution and stirred properly.

Molisch's Reagent: 3.75 g of α-Naphthol is dissolved in 25 mL of ethanol 99 %.

Legal's Reagent: 3 drops of sodium hydroxide (NaOH) were mixed with 10 mL methanol which was then added to the solution containing 10 mL of pyridine and 10 mL of 10% sodium nitroprusside.

Benedict's Reagent:

Solution I: 50 g of crystalline sodium carbonate, 50 g of crystalline sodium citrate, and 31.25 g of potassium thiocyanate were dissolved in 200 mL of hot distilled water.

Solution II: 4.5 g of CuSO4 was dissolved and made 25 mL solution.

Solution III: 5% solution of potassium ferrocyanate was prepared by dissolving 5 g of potassium ferrocyanate in 100 mL water.

Finally, the Benedict reagent was prepared by mixing solution I, solution II, and solution III.

Fehling's Reagent:

Fehling A: 31.66 g of CuSO4 was dissolved in water to produce a 500 mL solution.

Fehling B: 176 g of sodium-potassium tartrate and 77 g of sodium hydroxide were dissolved in water to produce a 500 mL solution.

Finally, an equal volume of solution I and II were mixed to prepare Fehling's solution.

Concentrated Sulfuric Acid Solution: 36 N concentrated sulfuric acid solution was used.

Dilute Sulfuric Acid Solution: Concentrated sulfuric acid was diluted 10 times with water to produce the dilute sulfuric acid solution.

Concentrated Hydrochloric Acid: 12 N concentrated hydrochloric acid solutions were used.

1% Dilute Hydrochloric Acid: 1 mL of conc. HCl acid was dissolved in 100mL of water

Ferric Chloride Solution: 15 g of ferric chloride hexahydrate ($FeCl_3.6H_2O$) was dissolved in 100 mL of distilled water.

Ammonia Solution: 25% of the ammonia solution was used.

Copper Acetate Solution: 19.97 g of copper acetate was dissolved in 100 mL of distilled water.

Sodium Hydroxide Solution: 20 g of NaOH was dissolved in 100 mL of distilled water.

Lead Acetate Solution: 10 g of lead acetate was dissolved in 100 mL of CO_2-free water.

1 M Na_2CO_3 Solution: 10.6 g of Na_2CO_3 was dissolved in little distilled water in a 100 mL volumetric flask and diluted to the mark .by adding distilled water.

Preparation of 2% $AlCl_3$ Solution: 2 g of $AlCl_3$ crystals were dissolved in little distilled water in a 100 mL volumetric flask and diluted to the mark by adding distilled water.

2: PHOTOGRAPHS DURING THESIS WORK

Plant *A. aspera*

Shaded Drying plant

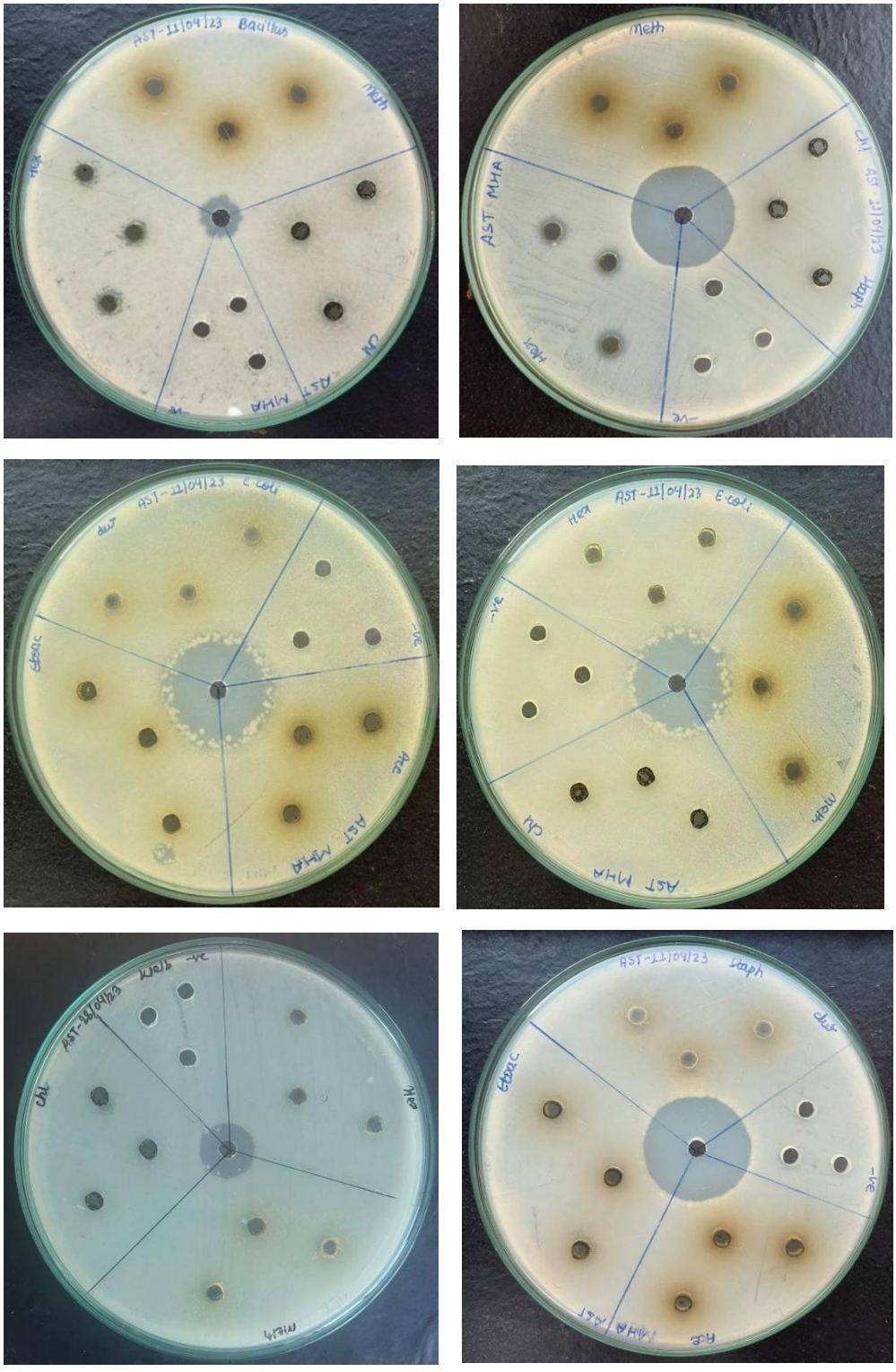
Plant Powder

Sonication

Filtration

Extract Concentration over the Water Bath

Various Extracts



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