GC-MS, ANTIBACTERIAL, ANTIOXIDANT, TPC, TFC, CYTOTOXICITY AND FTIR ANALYSIS OF EXTRACTS AND ESSENTIAL OIL OF *AGERATINA ADENOPHORA* (SPRENG.) R.M. KING & H. ROB. FROM PALPA DISTRICT OF NEPAL

A DISSERTATION

SUBMITTED FOR THE

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

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BOARD OF EXAMINER AND CERTIFICATE OF APPROVAL

This dissertation entitled "**GC-MS, Antibacterial, Antioxidant, TPC, TFC, Cytotoxicity and FTIR Analysis of Extracts and Essential Oil of *Ageratina adenophora* (Spreng.) R.M. King & H. Rob. from Palpa District of Nepal.**" prepared and submitted by Arjun Thapa, under the supervision of Asst. Prof. Dr. R. L. (Swagat) Shrestha, Department of Chemistry, Amrit Campus, Tribhuvan University, Kathmandu, Nepal is hereby submitted for the partial fulfillment of the requirements for the Master of Science (M.Sc.) Degree in Chemistry and has been approved for the award of a degree.

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RECOMMENDATION LETTER

This is to recommend that **Mr. Arjun Thapa** has carried out a dissertation work entitled "**GC-MS, Antibacterial, Antioxidant, TPC, TFC, Cytotoxicity and FTIR Analysis of Extracts and Essential Oil of *Ageratina adenophora* (Spreng.) R.M. King & H. Rob. from Palpa District of Nepal.**" for the partial fulfillment of the requirements of a Master of Science Degree in Chemistry under my supervision. To the best of my knowledge, this work has not been submitted to any other degree.

He has fulfilled all the requirements laid down by the Amrit Campus, Institute of Science and Technology (IOST), Tribhuvan University, Kathmandu, Nepal for the submission of the dissertation for the partial fulfillment of the requirements for the Master of Science Degree in Chemistry.

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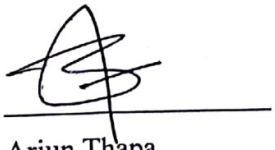
DECLARATION

I, **Arjun Thapa**, hereby declare that the dissertation entitled "**GC-MS, Antibacterial, Antioxidant, TPC, TFC, Cytotoxicity and FTIR Analysis of Extracts and Essential Oil of *Ageratina adenophora* (Spreng.) R.M. King & H. Rob. from Palpa District of Nepal.**" is being submitted to the Department of Chemistry, Amrit Campus, Institute of Science and Technology (IOST), Tribhuvan University, Kathmandu, Nepal for the partial fulfillment of the requirements for the Master of Science Degree in Chemistry, presented herein is my genuine work carried out under the supervision of Asst. Prof. Dr. R. L. (Swagat) Shrestha, Department of Chemistry, Amrit Campus, Kathmandu. This dissertation is done originally by me and has not been published or submitted elsewhere for the requirement of a degree program. Any literature, data, or works done by others and cited in this dissertation has been given due acknowledgment and listed in the reference section.



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LIST OF ABBREVIATIONS

A. adenophora	*Ageratina adenophora*
AgNPs	Silver nanoparticles
C	Concentration
CE	Chloroform Extract
Conc.	Concentrated
DMSO	Dimethyl sulfoxide
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EAE	Ethyl acetate Extract
EDX	Energy-dispersive X-ray
ME	Methanolic Extract
EO	Essential Oil
FTIR	Fourier Transform Infrared
GAC	Gallic acid concentration
GAE	Gallic acid equivalent
GC-MS	Gas Chromatography- Mass Spectrometry
HE	Hexane Extract
IC_{50}	Inhibitory Concentration of drug for killing 50%
IR	Infrared
kPa	Kilopascal
MRSA	Methicillin-resistant *Staphylococcus aureus*
nm	Nanometer
NMR	Nuclear Magnetic Resonance
QC	Quercetin concentration
QE	Quercetin equivalent
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
rpm	Revolution per minute
RSA	Radical Scavenging Activity
S	Second
SEM	Scanning Electron Microscope
TEM	Transmission Electron Microscopy
TFC	Total Flavonoid Content

TPC	Total Phenolic Content
TLC	Thin Layer Chromatography
UV	Ultra-violet
XRD	X-ray diffraction
ZOI	Zone of Inhibition

LIST OF SYMBOLS

°C	Degree Celsius
α	Alpha
β	Beta
γ	Gamma
μ	Mu
%	Percentage

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ABSTRACT

Ageratina adenophora (Spreng.) R.M. King & H. Rob. is an important perennial medicinal herb that belongs to the family Asteraceae. It has scented leaves that have historically been used as a medicine. The plants of *A. adenophora* were collected from the Palpa district and were air-dried. The goal of this study was to look into GC-MS analysis, phytochemical screening, total phenolic content, total flavonoid content, antibacterial and antifungal activity, toxicity, Thin layer chromatography, and column chromatography of *A. adenophora* extracts and essential oil. Clevenger-type hydro distillation apparatus was used to extract the essential oil. The air-dried powdered plant materials of *A. adenophora* were extracted with methanol, hexane, chloroform, and ethyl acetate solvents for the extract. Qualitative phytochemical analysis of methanol, hexane, chloroform, and ethyl acetate extracts of *A. adenophora* plants showed the presence of alkaloids, flavonoids, phenols, steroids, quinones, saponins, tannins, cardiac glycosides, carbohydrate, terpenoids, proteins, and amino acids. The GC-MS study of chloroform extract revealed ten different possible compounds, whereas essential oil revealed fourteen. 1-naphthalenol (24.33%) and α–bisabolol (18.01%) were the most numerous components in the chloroform extract, and 1-napthalenol (24.56%) and α-bisabolol (14.74%) were the most abundant components in the essential oil of *A. adenophora*. Free radical scavenging activity was evaluated using (DPPH) free radical. The antioxidant activity of essential oil was determined to be 50.24 % at 15 mg/mL of sample solution, while the antioxidant activity of chloroform extract was 62.84 % at 2 mg/mL of sample solution. The IC_{50} value of essential oil was found to be 17.21 mg/mL whereas that of chloroform extract was found to be 1.46 mg/mL. The TPC content of the essential oil and extract was found to be 53.42 mg gallic acid/g of equivalent and 89.75 mg gallic acid/g of equivalent. The TFC content in essential oil and chloroform extract was found to be 3.372 mg quercetin equivalent/g of dry extract and 49.252 mg quercetin equivalent/g of dry extract respectively. The essential oil, as well as chloroform extract, also showed LC_{50} values of 64.56 µg/mL and 174.78 µg/mL respectively. Antibacterial activity was shown to *E. coli, Proteus vulgaris,* and *Staphylococcus subsp. aureus* in both chloroform extract and essential oil of *A. adenophora*. There was no evidence of antifungal action. In FTIR analysis of chloroform leaf extracts of *A. adenophora* studies revealed the presence of these functional groups: 1° amine (N-H), the carboxylic acid (-COOH), alkane (C-H), aldehyde (-CHO). A single spot was seen in the solvent system of ethyl acetate and hexane (5:95), (10:90), (20:80) by the use of column chromatography.

Keywords: *Ageratina adenophora*, Essential oil, GC-MS, Antibacterial, Antioxidant, TPC, TFC, Cytotoxicity

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CHAPTER 1: INTRODUCTION

1.1. About Nepal

Nepal is a landlocked country in South Asia with China within the north and Republic of India within the south, east, and west, while Bangladesh is located within only 27 km of its southeastern tip, and the kingdom of Bhutan is separated from its by the state of geographical area. The country occupies 147,516 km^2 of land and lies between coordinates more or less 28^oN and 84^oE. Officially the Federal Democratic Republic of Nepal is a sovereign country in the world. It is chiefly within the Himalayas however conjointly included components of the indo-Gangetic Plain. It is the 49^{th} largest country by population and 93^{rd} largest country by space. By some measures, Hinduism is practiced by a larger majority of individuals in this country than in other nations. Buddhism, although a minority religion within the country is coupled traditionally with Asia countries because the birth place is mystic Siddhartha Gautama, who because the Gautam Buddha gave birth to the Buddhist tradition. It is a mountainous country within the central Himalayas set within the lap of the great Himalayas in South East Asia, which concerns one-third of (800 km) of the complete length of the range of the mountain chain.

Nepal alone claims eight out of the top ten tallest mountains within the world, as well as Mount Everest (8,848.86 m). Apart from the mountains, deep gorges, watercourse valleys, and flat lands, it provides a singular assemblage of very different habitats and great biodiversity within a small geographical area. In addition to the time from tropical heat to cold equivalent to Polar Regions, average annual precipitation varies from as little as 160 mm in the rain shadow north of the Himalaya to as much as 5,500 mm on windward slopes. Biodiversity is a reflection of its unique geographical position and altitudinal and climatic variances. The country's biological richness comprises both the Indo–Malayan and Paleoartic realms. It also includes endemic Himalayan Flora and Fauna[1,2].

1.2. Diversity of Flora in Nepal

Nepal is a country that is incredibly wealthy in diversity. It is ranked 25^{th} and 11^{th} positions in diversity richness within the world and Asia, severally. It

occupies 0.1 % of the world space, however, harbor3.2% and 1.1% of the world's identified flora and fauna as a result of its distinctive physical properties[3]. A total of 118 different ecosystems have been identified in Nepal, including 112 forest ecosystems, four cultivation ecosystems, one water body ecosystem. A total of 807 algae, 2025 fungi, 771 lichens, 1150 bryophytes, 534 pteridophytes, 28 gymnosperms, and 6,653 species of angiosperms have been reported in Nepal. Out of those about 370 species of flowering plants are considered endemic to Nepal.

According to the Nepal biodiversity strategy published in 2002 by the Ministry of Forests and Soil conservation, the British Museum has over 40,000 specimens, the University of Tokyo about 100,000 specimens, and the National Herbarium and Plant Laboratories of Kathmandu, 150,000 specimens. In addition, approximately 10,000 specimens are housed in different institutions of Tribhuvan University. Biodiversity is closely linked with the livelihoods and economic well-being of most Nepalese people. The economy of Nepal is very much dependent on the use of natural resources[4,5].

1.3. *Ageratina adenophora* (Spreng.) R.M. King & H. Rob.

Ageratina adenophora (Spreng.) R.M. King & H. Rob. is usually called the sticky snakeroot; other common names are eupatory, crofton weed, catweed, sticky agrimony, mexican devil, and sticky eupatorium, hemp agrimony, white thoroughwort, and in numerous parts of the globe in Nepal as banmara or kalimunte (killer of the forests)[6–9]. It is a perennial herb natively found in Mexico now distributed worldwide[10–13], and Nepal is cosmopolitan within the Himalayan region[7,14–16]. It is invasive to over 30 countries that have tropical and subtropical zones of the globe including Nepal, India, Indonesia, Vietnam, Africa China, and Myanmar[7,17–20]. It belongs to the Asteraceae family, one of the largest families of vascular plants that were found in open and deforested areas of central and eastern Nepal at an altitude of 500-2000 m[11,21–23]. It is a controversial weed in forest plantations because it infests disturbed areas and prevents the self-seeding of cultivated trees[24–26]. It grows to 1 or 2 meters (3.3 or 6.6 ft) high with trailing purplish to chocolate-

brown branches that strike roots upon contact with soil, leading to dense thickets. Each flower head is up to 0.5 cm within diameter and creamy white. They are followed by a little brown seed with a white feathery 'parachute'[27–30]. It is a profusely branching under shrub growing up to 90-120 cm in height with a few ascending branches; leaves simple, dark green, opposite, subsessile, lanceolate, subentire and glabrous types[16,20,31–34] and everyone grows to about 10 cm in length. Flowers are borne terminally in compound clusters during summer and spring[26,35,36]. The toxic weed that causes damage to farm lands, pasture fields, and forests then it has been observed that other plants were rarely found in the proximity because it suppresses the expansion of different plants within the vicinity thanks to its allelopathy, additionally to its strong adaptability under diverse environmental conditions[24,27,37,38]. It is seldom attacked by bacteria, fungi, and insects, suggesting that rich bioactive secondary metabolites that may be defense-related that exist within this plant[39–41]. Previously, structurally diverse chemicals including terpenoids, phenylpropanoids, flavonoids, coumarins, sterols, and alkaloids were reported from this species, a number of which were shown to possess allelopathic, phytotoxic and antifeedant activities[42,43]. It is accounted for to own different therapeutic properties and see the standard medications[44]. *A. adenophora* leaf accustomed treat the various diseases, as an example, wounds, a sleeping disorder, jaundice and ulcers[7,35,45]. Until this time, there is no effective medication produced for anticancer drugs.

1.4. Morphology of *A. adenophora*

- **Scientific name**: *Ageratina adenophora* (Spreng.) R.M. King & H. Rob.

Other Scientific Names[6]

- *Ageratina trapezoideum (*Kunth) R.M. King & H. Rob.
- *Eupatorium adenophora* Spreng

International Common Names

- English: Crofton weed, Sticky snakeroot

Local Common Names

- Nepali: Banmara, Kalimunte

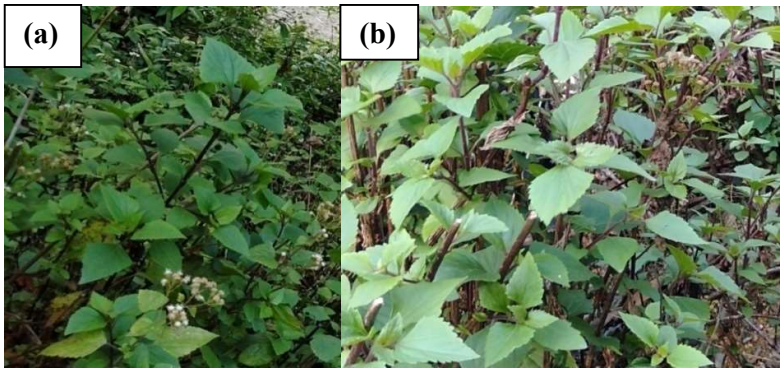


Figure 1: (a) Digital photo of *A. adenophora* plant, and (b) Leaves of *A. adenophora*

Leaves: opposite, triangular to rhomboid, with 4-10 and 2-9 cm, acuminate, with a long petiole, serrated on the margin, densely pubescent, dark green on the upper surface, and violet on the lower body.

Flowers: white, sometimes pink or purple, arranged in dense flowerheads (10-60 flowers) of 5-10 mm diameter, with a campanulate involucre, tubular, with 5 lobes.

Fruits: black cypselas, glabrous and 2mm long, with a 3-4 mm white pappus.

Flowering: March to July.

1.5. Classification of *A. adenophora*

Domain	: Eukaryota
Kingdom	: Plantae
Phylum	: Spermatophyta
Subphylum	: Angiospermae
Class	: Dicotyledonae
Order	: Asterales
Family	: Asteraceae/Compositae
Genus	: *Ageratina*
Species	: *A. adenophora*
Binomial name	: *A. adenophora* (Spreng.) R.M. King & H. Rob.

1.6. Traditional use of *A. adenophora*

A. adenophora has been used in the traditional system of medicine indifferent parts of the world. Plant essential oils generally have a broad spectrum of bioactivity because of the presence of several active ingredients or secondary metabolites, which work through various modes of action. It is licensed for numerous healthful properties and finds therapeutic applications in ancient medicines as an anti-inflammatory, antimicrobial, antiseptic, analgesic, antipyretic, and coagulant and phenol barbitone induced sleep enhancer. *A. adenophora* leaf juice is employed to prevent harm of cut and wounds, forming clots. Root juice is prescribed to treat fever. Pure juice of the leaf is poured into the eye to treat insomnia. A decoction of the plant has been recommended to treat jaundice and ulcers. Conventionally, stewing of leaves has been applied on cut wounds to abate trauma and used against infection of gum and toothache. A decoction of leaves is given to cure stomachache among the tribal people of Meghalaya and Nagaland states of India. The Local populace of Kurseong and Darjeeling hill region in the Eastern Himalayas use leaves of the plant for remedial purposes against oral and skin sores. Traditional practitioners of Darjeeling Himalaya prescribe the young leaves and shoots of the plant against dysentery. Nainital of Kumaun region of Uttarakhand state in India, leaf juice is used in blood clotting. In Garhwal region of the state, leaf paste is applied on cuts and wounds, and paste mixed with mustard oil is useful for ulcer. In Nepal, leaf juice is employed antiseptic to treat cuts and wounds. In Nigerian traditional medicine, it is used to treat fever, diabetes, and inflammation, and it also possesses pneumotoxic and hepatotoxic effects[43,46–48]. The *A. adenophora* mainly contains flavanoids, alkaloids, phenolic, glycosides, tannins, saponins, and glycosides[43].

1.7. Objectives of the Study

The objectives of this study are as follows:

General Objective

- To find out the phytochemical constituents and medicinal value of plant extracts and essential oil of *A. adenophora*.

Specific Objectives

- To extract constituents in the leaf with the different solvent systems (methanol, hexane, chloroform, and ethyl acetate).
- To obtain essential oil from fresh leaf powder by hydrodistillation method.
- To identify chemical constituents present in the essential oil as well as in extract of leaf powder by GC-MS technique.
- To perform phytochemical screening.
- To study the antibacterial; antifungal activity of the plant.
- To study the antioxidant activity of the plant by using DPPH free radical scavenging assay.
- To determine the total phenolic content and total flavonoid content.
- To conduct the cytotoxic activity against Brine shrimp.
- To study the FTIR spectrum.

CHAPTER 2: LITERATURE REVIEWS

Tripathi *et al*., (2018) reported *A. adenophora* has been used traditionally as folklore medicine across the world. In the traditional system of medicine, it is regarded as antiinflammatory, antimicrobial, antiseptic, analgesic, antipyretic, blood, and coagulant. The phytochemical and pharmacological studies demonstrated that *A. adenophora* possesses a wide spectrum of pharmacological activities, such as anti-inflammatory, analgesic, antipyretic, antioxidant, antibacterial, antifungal, antitumor, antioxidant, antiseptic, and cytotoxic activities which could be attributed to the presence of an array of phytochemicals of various groups including terpenoids, phytosterols, alkaloids, flavonoids, phenolic acids, coumarins, phenylpropanoids, sesquiterpene lactones, polysaccharides, and essential oil[49].

Rajalakshmi *et al*. (2016) studied the secondary metabolites of *A. adenophora* reported flavonoids, alkaloids, tannins, saponins, steroids, and terpenoids. The antioxidant activity of the plant extracts was determined by using phospho-molybdenum assay, Nitric oxide free radical scavenging assay, and FRAP assay. The aqueous extract showed a better free radical antioxidant power assay (FRAP) in *A. adenophora* at a concentration of 1000 mg/mL of the extract[50].

King *et al*., (2013) and Tripathi and Saini., (2019) reported that *A. adenophora* is used for the treatment of diabetes, and extracts of the whole plant are used for epilepsy and in the combination of psychoneurosis, depression, irritability, insomnia, anxiety, and stress. This plant also showed antispasmodic, antiseptic, antibacterial, antimalarial, antitumor, antirheumatic, and hepatoprotective properties. *A. adenophora* has not only been used as an edible plant (spices) but also as a folk medicine resource. Hence it was thought worthy to investigate the phytochemical constituent of the Petroleum ether, chloroform, and methanol extracts of *A. adenophora* leave. The preliminary phytochemical screening exploitation of the quality phytochemical tests detected the presence of alkaloids, steroids, flavonoids, reducing sugars, tannins, saponins, terpenoids, anthraquinones, cardiac glycosides, and phenols in numerous solvent extracts. The methanol extract leaves revealed a total

phenolic content of 30.0 mg gallic acid equivalent/g and 510.0 mg quercetin equivalent/g. The total antioxidant capacity ranges from 16.98 to 94.87% of standard ascorbic acid at concentrations starting from 0.05 to 1 mg/mL of the plant extract in methanol[21,46].

Pala-Paul *et al*., (2002) reported the analysis of the essential oils of the aerial components of *A. adenophora* by GC-MS and found the major constituents as p-cymene (11.6%), α-phellandrene (5.7%), γ-curcumene (5.0%), δ-2-carene (5.0%), camphene (4.8%), and endo-bornyl acetate (4.4%)[23].

Chauhan *et al*., (2015) studied the analysis of the GC-MS of *A. adenophora* oils and found that it was conjointly dominated by monoterpenoids (59.77%) having p-cymene (14.56%), phellandrene (12.25%), camphene (10.42%), and bornyl acetate (9.76%) because of the major constituents[51].

Subba *et al*., (2013) studied the essential oils of *A. adenophora* by GC–MS within which the yield of oil 4.5% on the resent weight basis by hydro distillation, 45 volatile compounds were known and also the major compound was found to be torreyol (16.8%) and also the sesquiterpene fraction was higher than the monoterpene one. The essential oil showed medicament or antibacterial activity against each gram-positive (*Staphylococcus aureus* and *Klebsiella pneumoniae*) and gram-negative (*Escherichia coli* and *Proteus Vulgaris*) microorganism[7].

Nadaf *et al*., (2018) reported the *A. adenophora* of leaf, stem, flower, and root extracts were characterized qualitatively for the presence of alkaloids, flavonoids, tannins, saponins, and terpenoids[52].

Adebisi *et al*., (2019) reported an analysis of the GC-MS of *A. adenophora* oils with the constituent byα-epi-cadinenol (16.63%), O-cymene (13.54%), bornyl acetate (7.70%), β-phellandrene (7.46%), and σ-2-carene (5.77%) were the main terpenoids. The essential oils showed promising toxicity (median deadly concentration, LC_{50} = 3176.54 mg L^{-1}) and repellent activity (median repellent concentration, RC_{50} = 2070.99 mg L^{-1}) to larvae of *Plutella xylostella* within 24 h. Among fractions, hexane fraction was simpler effective to

Plutellaxylostella (LC_{50} = 5056.74 mg L^{-1}), whereas methanol fraction to *Aphis craccivora* (LC_{50} = 1175.83 mg L^{-1})[53].

Balami *et al*., (2019) *A. adenophora* studied mycelial growth of selected ecologically important soil fungi (*Alternaria alternata, Trichoderma harzianum, Aspergillus niger, Chaetomium funicola, and Fusarium oxysporum*) by using extracts obtained from *A. adenophora* leaves, litter, and root. All types of extracts showed an inhibitory effect on fungal growth. The degree of inhibition varied with fungal species, extract type, and concentration. It is proposed that the inhibitory activities of *A. adenophora* to the soil fungi could bring changes in soil fungal diversity and their composition. It could be a reason that affects soil characteristics, native plant species, and ecosystem functioning by *A. adenophora* [54].

Chauhan *et al*., (2019) reported the composition of essential oils was investigated by GC/MS analyses. The GC/MS analysis of the oils of the aerial parts of *A. adenophora* revealed that oxygenated sesquiterpenes (25.9-41.87 %) and sesquiterpenes hydrocarbons (22.54-33.15%) were the major class of compounds in the oils. The essential oils of *A. adenophora* is rich in amorph-4-en-7-ol (12.03-19.48%) followed by p-cymene (3.18-15.44%), bisabolol (5.25-9.39%), bornyl acetate (4.17-7.93 %), phellandrene (1.79-7.25%), camphene (2.50-6.83%), β-bisabolene (3.36-6.64%)[55].

Liu *et al*., (2017) studied the *A. adenophora* with essential oil which was analyzed by GC-MS in which the components identified as the major components were 10Hβ-9-oxo-agerophorone (37.03%), 10Hα-9-oxo-agerophorone (37.73%) and 9-oxo-10, 11-dehydro-agerophorone (23.41%)[56].

Katoch *et al*., (2013) reported the composition of essential oils was investigated by GC/MS analyses from Chromolaena aerial parts which were rich in 1-phellandrene (11.73%) and torreyol (11.41%), whereas, in the stem, the percentage of these two compounds was 9.65 and 9.84, respectively. Phellandrene (8.46%) and anthemol (5.33%) were the main constituents of

essential oil from roots. Bornyl was present in significant amounts both in the aerial parts (7.34%) as well as stem (5.33%)[6].

Padalia *et al*., (2009) studied the *A. adenophora* with essential oil that was analysis by GC-MS in which the components identified such as amorph-4-en-7-ol (17.7%), p-cymene (16.6%), 3-acetoxyamorpha-4,7(11)-dien-8-one (16.3%), bornyl acetate (15.9%), α-phellandrene (9.6%), camphene 8.9%), α-bisabolol (7.8%), α-cadinol (6.2%) and amorph-4,7(11)-dien-8-one (5.7%)[57].

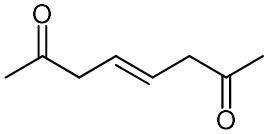
Pandey*et al*., (2014) reported the *A. adenophora* with essential oil that was analysed by GC-MS in which the components identified such as acoradiene (10.11%), bornyl acetate (10.60%), camphene (12.05%), p-cymene (11.6%) and α-phellandrene (8.57%) represent higher percent composition. *A. adenophora* oil showed potent antioxidant activity and IC_{50} values were 8.3 and 4.2 when tested by DPPH and β-carotene bleaching methods[48].

Vasanthi *et al*., (2013) reported the essential oil was dominated by sesquiterpenoids (81.90) represented by sesquiterpene hydrocarbons (36.50%) and oxygenated sesquiterpenoids (45.4%). The major compounds are copaen (19.72%), α-bisabolol (9.8%), 4,4dimethyl-3-(3-methylbut-3-enylidine)-2-methylene bicycle [4.1.0] heptane (8.9%) and azulenone (9.5%). The IC_{50} value of the oil towards the antioxidant activity was found to be 60.2±2.3, 71.25±1.5 µg/mL for the DPPH, ABTS radical scavenging assays respectively. Ascorbic acid was used as the standard substance and showed an IC_{50} value of 55.13±1.2 and 61.32±2.6 µg/mL for DPPH and ABTS radical scavenging activity respectively[20].

Ramu *et al*., (2018) studied the antioxidant activity and total phenolic content of the hydroalcoholic extract of *A. adenophora* roots using standard *in vitro* methods. The total phenol and total flavonoid were determined spectrophotometrically. Quercetin and Gallic acid were used as reference standards for these parameters. The total antioxidant activity of the extract was determined by phosphomolybdenum method. Results demonstrate that the extract has antioxidant activity. A correlation between the antioxidant activity

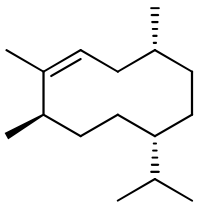
and the total phenolic content of the extract indicated that the phenolic compounds were the dominant contributors to the antioxidant activity of the extract[58].

Kundu *et al*., (2013) reported the Cadinene derivatives were extracted from leaves of *A. adenophora* using ethyl acetate. Five cadinene sesquiterpenes were isolated by column chromatography and Preparative Thin Layer Chromatography. Purified sesquiterpenes were spectroscopically elucidated as cadinan-3-ene-2,7-dione, 7-hydroxycadinan-3-ene-2-one, 5,6-dihydroxycadinan-3-ene-2,7-dione, cadinan-3,6-diene-2,7-dione and 2-acetyl-cadinan3,6-diene-7-one[59].

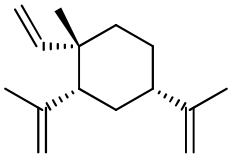


Cadinan-3-ene-2,7-dione

Kundu *et al*., (2016) studied the *A. adenophora* was hydro-distilled and the obtained essential oil was analyzed by GC-MS. Twenty-six essential oil constituents comprising sesquiterpenes and monoterpenes were identified. g-Cadinene was most abundant followed by germacrene-D and γ-elemene. The antioxidant activity of essential oil was comparable with the standards. The essential oil and the cadinene sesquiterpene-rich extract of *A. adenophora* have exhibited potential antioxidant activities[60].



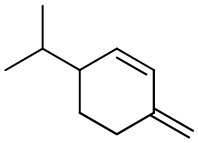
Germacrene-D



γ-elemene

Kurade *et al*., (2010) studied the *A. adenophora* oil, of the total identified volatile constituents (84.95%), six [1-napthalenol (17.50%), α-bisabolol (9.53%), bornyl acetate (8.98%), β-bisabolene (6.16%), germacrene-D

(5.74%) and α-phellandrene (3.85%)] represented the major ones. The essential oil of *A. adenophora* had antibacterial activity against *Arthrobacter protophormiae, Escherichia coli, Micrococcus luteus, Rhodococcus rhodochrous, and Staphylococcus aureus* with MBC values of 200, 100, 100, 12.5, and 200, respectively[61].



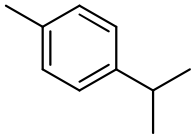
α-phellandrene

Shrestha and Bhattarai (2009) confirmed that ethanol, an organic solvent, the extract of *A. adenophora* has demonstrated antibacterial effect towards *Staphylococcus spp., Bacillus subtilis, Proteus mirabilis, Proteus spp., Staphylococcus aureus*, and water solvent extract showed antibacterial effect towards *Escherichia coli, Staphylococcus aureus, Staphylococcus spp., Proteus spp.* and *Bacillus subtilis*[62].

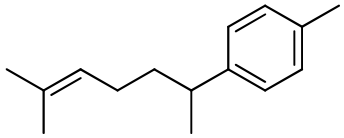
Maharjan (2011) reported the methanolic extract of *A. adenophora* was effective against *Klebsiella pneumonia, Staphylococcus aureus, Bacillus subtilis,* and *Escherichia coli* at different concentrations. But it was not able to inhibit *P. mirabilis* and extract of distilled water only inhibited *B. subtilis*[63].

Rajamani *et al*., (2014) studied the antibacterial activity on different leaf extracts (chloroform, methanol, petroleum ether, ethyl acetate, and water) of *A. adenophora* against three different human pathogens such as *Staphylococcus aureus, Klebsiella pneumonia,* and *Escherichia coli.* The different leaves extract of the plant *A. adenophora* were found to have maximum antibacterial activity. The study indicated chloroform extract showed a maximum zone of inhibition against *Klebsiella pneumonia* (10mm) and *Staphylococcus aureus* (9mm) respectively. Methanol extract showed a 7mm inhibition zone against *Klebsiella pneumoniae* and an 8mm inhibition zone against *Staphylococcus aureus* when compared with other extracts[64].

Weyerstahl *et al*., (1997) reported the composition of the essential oil of flowers of *A. adenophora*. The major constituents were α-phellandrene (15.3%), camphene (12.2%), bornyl acetate (10.6%), *p*-cymene (8.5%), γ-curcumene (4.5%) and 2-carene[65].



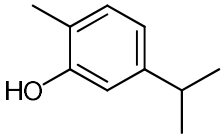
p-cymene



γ-curcumene

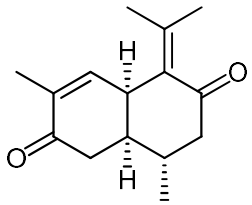
Mazumder *et al*., (2018) reported the qualitative and quantitative analysis of the petroleum ether; chloroform and methanolic extract of *A. adenophora* leaves were performed. The plant has a high reproductive capacity which is partly due to the well-developed root system. From the roots, important bioactive compounds such as benzofuran derivatives, chromene derivatives, and a monoterpene glucoside and sesquiterpenoid have been identified. Qualitative phytochemical analysis in the presence of carbohydrates, alkaloids, phenols, flavonoids, xanthoprotein, glycosides, tannins, steroids, and terpenoids. Quantification of the total tannins, total alkaloids, and total phenols was determined for the methanolic extracts of the leaves. A high number of tannins and phenols was detected[66].

Ahluwalia *et al*., (2014) studied the *A. adenophora* with essential oil that was analyzed by GC-MS in which the components identified such as sesquiterpenes (55.9%) with γ-cadinene (18.4%), γ-muurolene (11.7%), 3-acetoxyamorpha-4,7(11)-diene-8-one (7.4%) and bornyl acetate (6.3%) as the major constituents. The oil obtained from the roots contained both sesquiterpenes (34.3%) and monoterpenes (32.5%) in almost equal proportions with E,E-cosmene (19.9%), γ-muurolene (10.1%), isothymol (7.5%), β-cadinene (7.0%) and α-phellandren-8-ol (5.9%) as the major constituents[67].



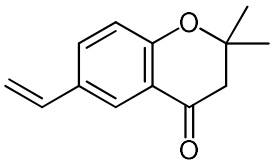
Isothymol

Liao *et al*., (2014) studied the *A. adenophora*, an invasive plant that has caused widespread poisoning of livestock and crop failures in China. 9-oxo-10, 11-dehydroageraphorone is the main toxin causing a series of respiratory and digestive system diseases in animals[68].

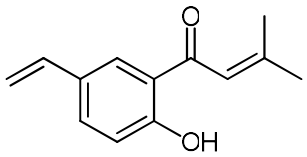


9-oxo-10, 11-dehydroageraphorone

Albuquerque *et al*., (2004) studied the composition of the essential oils of from leaves and roots of *A. adenophora*. The major constituents were identified β-Caryophyllene (12.4-41.7%), α-humulene (11.7-14.6%), γ-muurolene (10.4-19.0%), bicyclogermacrene (15.0- 17.5%), 2,2-dimethyl-6-vinylchroman-4-one (10.3-25.5%), and 2-senecioyl-4-vinylphenol (8.5-41.0%) were the most prominent constituents[69].

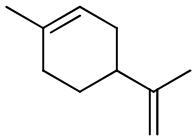


2,2-dimethyl-6-vinylchroman-4-one



2-senecioyl-4-vinylphenol

Antonio *et al*., (2017) studied antimicrobial activity as promising, especially antibacterial; antifungal activity. The species of *A.adenophora* are rich in terpenes, phytosterols, and sesquiterpene lactones, the latter being chemotaxonomic markers of the group, with broad anticancer, antiplasmodial and antimicrobial activity, making them promising for the development of new drugs[70].



1-methyl-4-prop-1-en-2-ylcyclohexene (terpenes)

CHAPTER 3: MATERIALS AND METHODS

3.1. Materials

3.1.1. Solvents

Methanol, Hexane, Chloroform, and Ethyl acetate were used as solvents for the preparations of plant extracts. All the solvents were of analytical grade and manufactured by Fisher chemical company, India.

3.1.2. Chemicals, Plant Materials, and Test Organisms

- TLC Aluminium sheets Silica gel 60 F_{254}
- Concentrated HCl
- Concentrated H_2SO_4
- Ammonia solution
- 2, 2-Diphenyl-1- picrylhydrazyl (DPPH)
- Mercuric chloride
- Potassium iodide
- NaOH
- Dimethyl Sulfoxide (DMSO)

Plant Material: Leaves of *A. adenophora*.

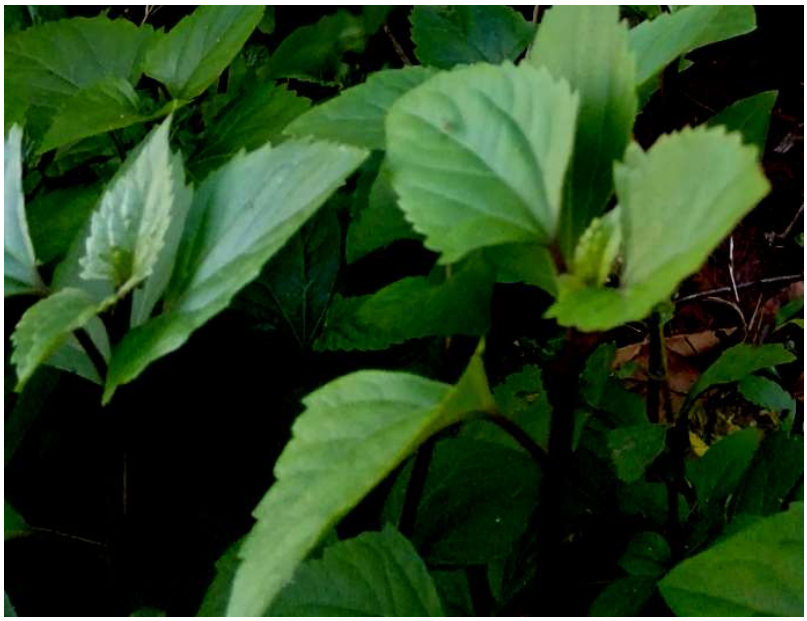


Figure 2: Leaves of *A. adenophora*

Test Organisms:

S. N.	Name of microorganisms;	KTCC No.
	Bacteria	
	Gram Positive	
1	*Bacillus subtilis*	ATCC 6051
2	*Enterococcus faecalis*	ATCC 8739
3	*Staphylococcus aureus*	ATCC 6538P
4	*Klebsiella pneumonia*	ATCC 700603
5	*Staphylococcus epidermidis*	ATCC 1228
	Gram Negative	
6	*Escherichia coli*	ATCC 29212
7	*Pseudomonas aeruginosa*	ATCC 9027
8	*Salmonella enterica Subsp.enterica pv Typhi*	ATCC 29630
9	*Shigella dysteneriae*	ATCC 13313
10	*Proteus vulgaris*	ATCC 6380
	Fungi	
1	*Candida albicans*	ATCC 2091
2	*Saccharomyces cerevisiae*	ATCC 18824

3.1.3. Instruments

The following instruments were used.

- Grinder
- Electronic balance
- Refrigerator
- Rotary evaporator
- UV-Chamber for TLC
- Digital Water Bath
- Hot Air Oven
- Digital Water Bath
- Soxhlet
- Cylindrical chromatographic column
- Separating funnel
- Iodine chamber
- TLC plate developing chamber
- Capillary tubes

- Solvent mixer

3.2. Methods

3.2.1. Collection of the Plant Part

About 100 kg of leaves of *A. adenophora* were collected from Ribdikot-7, Palpa, Nepal at about 2000 m altitude in October 2019.

Figure 3: Collection of *A. adenophora* leaves

3.2.2. Herbarium Preparation

The collected plant specimen was pressed on newspaper and left for a few days. The paper was changed every day until complete drying. The dried sample was mounted on the standard size of herbarium sheet, fixed, and appropriately labeled.

3.2.3. Identification of the Plant

The plant *A. adenophora* was identified by the Department of Botany, Amrit Science Campus, Lainchour, Kathmandu.

3.2.4. Drying and Grinding

The leaves of *A. adenophora* were separated from the branches. The fresh plant sample (leaves) were collected and washed under the running tap water to remove soil particles and other dust particles. The leaves were air-dried under laboratory conditions at room temperature for 16 days. The dried leaves samples were ground well into a fine powder with the help of a mixer grinder.

3.2.5. Preparation of Extracts

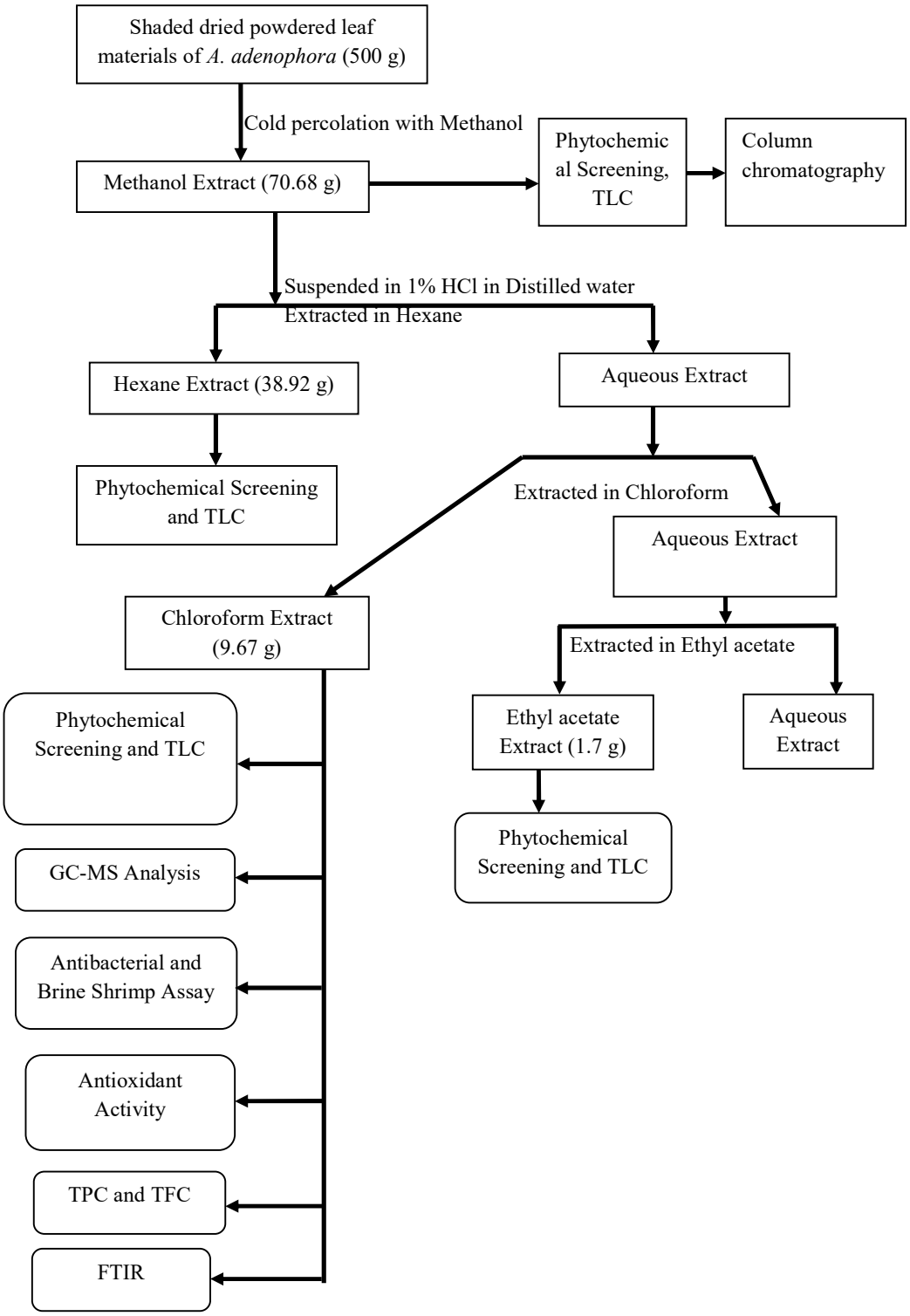
A total of four types of extracts using four different solvents of methanol hexane, chloroform, and ethyl acetate were collected from the plant leaf of *A. adenophora*.

Methanolic Extract For the collection of methanolic extracts, 70 g of *A. adenophora* powder was packed in a thimble and extracted in Soxhlet apparatus using 250 mL of Methanol. The temperature was kept between 60-80ºC. The samples in the thimble of the Soxhlet apparatus was kept boiling for approximately 4-5 hours till the solution becomes clear and the dark-coloured extract were collected at the bottom of the apparatus. This was then collected in a beaker and left to dry for 48 hours. The dried extract having a sticky appearance was stored in 15 mL vail at 4 ºC temperatures at in the refrigerator for further use. The whole process was repeated six times for the collection of a substantial amount of extracts for the study.	

3.2.6. Extraction Procedure

The powder of the leaves was first exhaustively extracted with 3L of Methanol by cold percolation process in a gas jar for 20 days for three times. The content was filtered using Whatmann filter paper no. 1. The filtrate, i.e., methanol extract, was concentrated using Rota evaporator. The small portion of methanol extract was subjected to various phytochemical tests. The remaining large amount was further proceeded, for successive extraction with n-Hexane in a separating funnel and vigorously with continuous release of air. The light Hexane fraction remained at the top, and heavy aqueous fractions at the bottom were separated. The crude Hexane extract was obtained from Hexane fraction using Rota evaporator and subjected to various phytochemical tests. The Aqueous fraction was mixed with chloroform for further extraction in a separating funnel. Similar to extraction by hexane, the mixture was shaken vigorously with continuous release of air with great care. The heavy Chloroform fraction and light aqueous fraction were separated. The separated chloroform fraction was concentrated using Rota evaporator as well as ethyl acetate and subjected to various phytochemical tests and the qualitative

analysis of constituents in the chloroform extract was carried out through thin layer chromatography. Methanol extracts were subjected to column chromatographic separation.



Scheme 1:Research process

3.2.7. Storage of Extracts

The extracts were collected in vials. In every vial, extracts were contained till the vials are filled. They were dried till the extract had a very sticky appearance. It shows the different amounts of extracts that were collected. When an assortment of extracts using a spatula, the vials were tightly stoppered and stored in the refrigerator at $4^{\circ}C$.

3.2.8. Preparation of Stock Solution for phytochemical assays

For working the extracts were dissolved, and a stock solution of 5 µg/L was made. This was done by mixing 0.5 g of the crude extract with 100 mL of solvent. All four stock solutions were made this way.

3.2.9. Phytochemical Screening Analysis

Phytoconstituents are the natural bioactive compound present in the plant. It is responsible for therapeutic and biological as well as the toxic activities of plants. The phytochemicals are located or concentrated mainly in the leaves, stem, flowers, roots, bark, fruits, and seeds depending on the family of the species. Hence, many phytochemicals or secondary metabolites are used as lead compounds for a new medicine or active pharmaceutical ingredients for commercial purposes. Therefore, studying phytochemical constituents helps to reveal the usage of plants[21,27]. These phytoconstituents work with nutrients and fibers to form an integrated part of the defense system against various diseases and stress conditions[71]. Preliminary screening of biochemical tests of all four extracts was done for testing various phytochemicals found in plants. The crude extracts were tested for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, tannins, and cardiac glycosides. The following biochemical tests have been performed to confirm the presence or absence of the secondary metabolites in the plant extract[50,72–75].

3.2.9.1. Tests for Alkaloids

For testing alkaloids, three different types of tests were carried out.

Mayer's Test: 1 mL of filtrates were treated with Mayer's reagent (Potassium Mercuric Iodide). The formation of a yellow-colored precipitate indicates the presence of alkaloids.

Hager's Test: 1 mL of extract was carefully mixed with 3 drops of freshly prepared Hanger's reagent in a test tube. The formation of yellow precipitates showed a positive result and the presence of alkaloids in the extract.

Wagner's Test: 1 mL of extract was mixed in a test tube with 3 drops of Wagner's reagent prepared beforehand. The formation of reddish-brown precipitate showed the presence of alkaloids.

Dragendorff's Test: 2 mL of extract was taken in a test tube with 0.2 mL dilute HCL and 1 mL of Dragendraff's reagent and left for a few mins. A positive result is indicated by the presence of an orange-brown precipitate.

3.2.9.2. Test for Terpenoids

Chloroform test: The plant extract was taken in a test tube with a few mL of chloroform and added concentrated sulfuric acid carefully to form a layer and observed for the presence of reddish-brown color.

Liebermann-Burchard test: 1 mL extracts were treated with chloroform, acetic anhydride, and added drops of H_2SO_4 and observed for the formation of dark green color.

3.2.9.3. Test for Flavonoids

Alkaline Reagent Test: Extracts were treated with a few drops of sodium hydroxide solution. The formation of intense yellow color, which becomes colorless on the addition of dilute acid, indicates the presence of flavonoids.

Lead acetate Test: Extracts were treated with a few drops of lead acetate solution. The formation of a yellow color precipitate indicates the presence of flavonoids.

Pew's Tests: To 2-3 mL extract, zinc powder was added, followed by dropwise addition of conc. HCl. The formation of purple-red or cherry color indicates the presence of flavonoids.

Shinoda Tests: Few pieces of magnesium were mixed with 2-3 mL extract followed by dropwise addition of concentrate HCl and boiled for 5 minutes. The formation of magenta color indicates the presence of flavonoids.

3.2.9.4. Test for steroids compounds

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

3.2.9.5. Test for Quinones

To 2 mL of extract, add a few drops of conc. H_2SO_4 or aqueous NaOH solution. Color formation indicates the presence of the quinoid compound.

3.2.9.6. Tests for Saponins

Froth Test: Extracts were diluted with distilled water to 20 mL, and this was shaken in a graduated cylinder for 15 minutes. The formation of a 1 cm layer of foam indicates the presence of saponins.

Foam Test: 0.5 g of the extract was shaken with 2 mL of water. If foam produced persists for ten minutes, it indicates the presence of saponins.

3.2.9.7. Test for Tannins

$FeCl_3$ Test: 5 mL of extract solution was allowed to react with 1 mL of 5% ferric chloride solution. Greenish black coloration indicated the presence of tannins.

Potassium Dichromate Test: 5 mL of the extract was treated with 1 mL of 10% aqueous potassium dichromate solution. The formation of yellowish-brown precipitate suggests the presence of tannins.

Lead Acetate Test: 5 mL of each type of extract and a few drops of freshly prepared 1% lead acetate were dissolved together. Yellow precipitate shows a positive result.

Gelatin Test: To the extract, 1% gelatin solution containing sodium chloride was added. The formation of a white precipitate indicates the presence of tannins.

3.2.9.8. Test for Phenols

Ferric Chloride Test: Equal amounts of 1% ferric chloride solution and 1% potassium ferrocyanide were mixed. To 2 mL extract, 3 drops of this freshly prepared mixture were added. The formation of a bluish-green or dark green color was taken as positive.

Liebermann's test: The extracts were heated with sodium nitrite, add H_2SO_4 solution diluted with water and add an excess of dilute NaOH and observed for the formation of deep red or green, or blue color.

3.2.9.9. Detection of carbohydrates

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

Benedict's Test: Filtrates were treated with Benedict's reagent and heated gently. Orange-red precipitate indicates the presence of reducing sugars.

Fehling's Test: Filtrates were hydrolysed with dilute HCl, neutralized with alkali, and heated with Fehling's A & B solutions. The formation of a red precipitate indicates the presence of reducing sugars.

3.2.9.10. Tests for Cardiac Glycoside

Extracts were hydrolysed with dil. HCl, and then subjected to test for glycosides.

Modified Borntrager's Test: Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and extracted with equal volumes of benzene. The benzene layer was separated and treated with ammonia solution. The formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

Killer-Killani Test: 1 mL of the extracts were dissolved in 1 mL of glacial acetic acid and cooled, after cooling, 2-3 drops of ferric chloride were added. To this solution 2 mL of conc. H_2SO_4 was added carefully along the walls of the test tube. The appearance of the reddish-brown colour ring at the junction of two layers indicates the presence of glycosides.

Legal's Test: Extracts were treated with sodium nitroprusside in pyridine and sodium hydroxide. The formation of pink to blood-red colour indicates the presence of cardiac glycosides.

3.2.9.11. Test for Proteins

The extract was diluted in 10 mL of distilled water and filtered with Whattmann no. 1 filter paper. 2 mL of filtrate was heated with a few drops of Millon's reagent (The reagent is made by dissolving metallic mercury in nitric acid and diluting with water). A reddish-brown coloration or precipitate indicates the presence of tyrosine residue which occurs in nearly all proteins.

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

3.2.9.12. Test for amino acids

Millon's test: To the test solution, 2 mL of Millon's reagent was added and observed for white precipitate.

Ninhydrin test: To the test solution, ninhydrin solution was added, boiled, and observed for the formation of violet color.

3.2.9.13. Detection of resins

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

3.2.9.14. Test for Triterpenoids

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

3.2.10. Extraction of Essential Oil

There are several methods for extracting essential oils. They can be broadly classified as conventional (traditional) methods and modern (non–traditional) methods. Traditional methods are mostly used on a commercial scale as these methods are cheap and easy. Here, the hydrodistillation method is used for the extraction of essential oil.

3.2.10.1. Hydro distillation

Hydrodistillation is the most simple and common technique for the extraction of essential oils (EO). In this method, the mixture of plant materials and water is heated, and the EO is evaporated. And the EO as vapour is condensed to get liquid oil. It is a particular type of distillation or a separation process for temperature-sensitive materials or natural aromatic compounds such as oils, resins, hydrocarbons, etc. which are insoluble in water and may decompose at their boiling point. The fundamental nature of hydrodistillation is that it enables a compound or mixture of compounds to be distilled at a temperature significantly below that of the boiling point of the individual constituent.

EO contains substances with boiling points up to 200°C or higher temperatures. In the presence of steam or boiling water, however, these substances are volatilized; at a temperature one at the point of 100°C, at atmospheric pressure. Clevenger apparatus designed by J.F. Clevenger in 1928 is generally used for hydrodistillation. Firstly, fresh and dried, botanical materials were placed in the plant chamber of the distillation and the EO to escape in vapour form[76].

The size of the round bottom flask to be used depends upon the nature of the plant and the percentage of volatile oil present. The temperature must be high enough to vaporize; the oil is present, yet not so high that it destroys the plants or burns the EO. Besides the steam, tiny droplets of EO evaporate and travel through a tube into the stills condensation chamber. Here, EO vapour gets condensed with the steam. The EO forms a film on the surface of the water. To separate the EO from the water, the film is then decanted or removed off the top. The remaining water, a byproduct of distillation, is named floral water, distillate, or hydrosol. It retains many of the therapeutic properties of

the plant, making it valuable in skincare for facial mists and toners (A solution containing chemicals that can change the color of a photographic print). In certain situations, floral water may be preferable to be a pure EO, such as when treating a sensitive individual or a child, or when a more diluted treatment is required. Rose hydrosol, for example, is commonly used for its mild antiseptic and soothing properties, as well as its pleasing floral aroma[76].

Several things verify the ultimate quality of a steam distilled EO. Apart from the plant material, the most important are time, temperature and pressure, and the quality of the distillation equipment. EO are very complex products. Each is formed from several, generally a whole lot, of distinct molecules that come together to create the oils aroma and therapeutic properties. Some of these molecules are somewhat delicate structures that can be altered or destroyed by adverse environmental conditions. So, much like a fine meal is more flavorful when made with patience, most oils benefit from a long, slow 'cooking' process. Longer distillation times may give more complete oil. It is also possible; however, that longer distillation time may lead to the accumulation of more artifacts than usual[76].

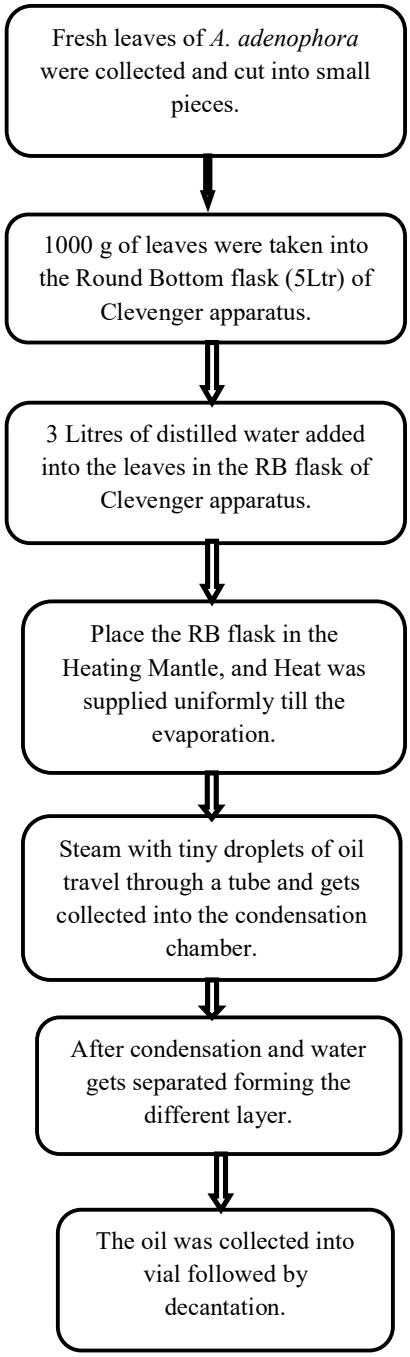
Hydrodistillation approach has been used for the extraction of EO from *A. adenophora.* The EO was obtained from dried leaves by hydrodistillation in a 1000 mL Clevenger apparatus for 6 hours. This process was repeated several times. The EO obtained was separated from water and dried over anhydrous (Na_2SO_4) and was stored in sealed vials under refrigeration before analysis. The size of the round bottom flask to be used depends upon the nature of the plant and the percentage of volatile oil present.

3.2.10.2. Determination of Percentage Yield

The percentage yield of the extract was calculated using the formula below:

$$\text{Percentage (\%) yield} = \frac{\text{Weight of the extracted oil (g)}}{\text{Dried weight of the sample (g)}} \text{ x } 100$$

3.2.10.3. Flow Chart Diagram Showing Research Process for Extraction of Essential Oil from *A. adenophora*



Scheme 2**:** Flow chart diagram showing essential oil extraction

3.2.11. Chromatographic Separation of Compound

Chromatography is the collective term for a collection of laboratory techniques for the separation of mixtures into their components. All forms of

chromatography work on a similar principle and have a stationary section that may be a solid or a liquid supported on a solid and a mobile unit that may be a liquid or a gas. The mixture to be separated is dissolved during a fluid called the mobile phase, which carries it through a structure holding another material referred to as the stationary section. The mobile phase flows having the components of the mixture with it through the stationary phase. The various traveling speeds cause the separation of the various constituents of the mixture. The separation relies on differential partitioning between the mobile and stationary phases[77]. Chromatography may be preparative or analytical. The purpose of preparative chromatography is to separate the components of a mixture for more advanced use (and is thus a form of purification). Analytical chromatography is done, typically with smaller amounts of material, and is for measuring the relative proportions of analyses in a mixture. The two are not mutually exclusive[78]. A TLC plate is a sheet of glass, metal, or plastic which is coated with a thin layer of a solid adsorbent (stationary phase). A small amount of the mixture to be analyzed is spotted near the bottom of this plate, and the plate is placed in a shallow pool of a solvent in a developing chamber[77].

TLC can be classified by the mechanism of separation: adsorption (physical sorption of the solutes onto the sorbent particles), partition (dissolution of the solutes into a stationary liquid on the sorbent), ion exchange (attraction of ions to groups bearing opposite charge on the sorbent), and size exclusion or gel 22 permission (rejection or retention based on size or shape). Most often, adsorption and partition are involved in TLC as they both involve the same type of forces, induced dipole-dipole, and hydrogen bonding. TLC is also a more versatile, cheap, and simple method for analytical and preparative applications than HPLC[79–81].

The chloroform extract of *A. adenophora* was carried out through thin layer chromatography to observe the qualitative analysis of constituents present in that extract. Here TLC was performed on TLC aluminium sheets Silica gel 60 F_{254} pre-coated TLC plates of E. Merck Company. Pre-coated TLC aluminium plates with a thickness of 0.2 mm. The plates were developed in different

solvents ratios by increasing the polarity of hexane to ethyl acetate gradually. The plates were visualized in a UV fluorescence lamp.

3.2.12. Gas Chromatography-Mass Spectrometry

This technique is known as a hyphenated technique in which gas chromatography is coupled with mass spectrometry. This method identifies the composition of a mixture of organic compounds based on their molecular mass and volatility. The GC-MS consists of two instrumental parts that are gas chromatography and mass spectrometer. The first instrumental part is the gas chromatograph which separates the constituents of a mixture with the help of a temperature-controlled capillary column. Gas chromatography separates the constituents based on the boiling points (volatility) and the molecular weights. The component which has a high volatility rate passes from the column earlier, and those constituents which have high boiling points and high molecular weights give later. The second instrumental part is the mass spectrometer. Each pulse breaks down in this part and provides the mass fragmentation pattern (mass spectra). Then the mass spectra are matched with the available database for the confirmation of structure[9].

3.2.12.1. Analytical Condition for GC/MS

GC/MS analysis was performed on a gas chromatography-mass spectrometer GCMS-QP 2010 under the following condition: injection volume 1µL with split ratio 1: 90; Helium as a carrier gas with a Rtx-5MS column of dimension 30m× 0.25mm×0.25 µm, temperature-programmed at 80℃ and 300℃ with a hold time of 2.0 and 5.0 min while the ion source temperature and interface temperature maintained to 200℃ and 250℃ respectively. Identification was accompanied by a comparison of MS.

Analytical Line for GC-MS Analysis

Column Oven Temperature	:	80.0ºC
Injection Temperature	:	220.00ºC
Injection Mode	:	Split
Flow Control Mode	:	Linear velocity
Pressure	:	67.7 kPa
Total Flow	:	18.5 mL/min

Column Flow	:	1.03 mL/min
Linear Velocity	:	37.4 cm/sec
Purge Flow	:	2.0 mL/min
Split Ratio	:	15.0
High Pressure Injection	:	OFF
Carrier Gas Saver	:	OFF
Splitter Hold	:	OFF
Ion Source Temperature	:	200.00ºC
Interface Temperature	:	250.00ºC
Solvent Cut Time	:	4.00 min
Detector Gain Mode	:	Relative
Detector Gain	:	1.10 kV + 0.00kV
Threshold	:	0
Analytical Line for MS		
Start Time	:	4.00 min
End Time	:	40.33 min
ACQ mode	:	Scan
Event Time	:	0.50 sec
Scan Speed	:	1000
Start m/z	:	40.00
End m/z	:	500.00
Sample Inlet Unit	:	GC
Use MS Program	:	OFF

3.2.12.2. Analysis of Essential oil and Chloroform Extract

The GC-MS analysis of essential oil and the little amount of concentrated extract of chloroform obtained from the Rota evaporator was dissolved in chloroform and subjected to GC-MS analysis. The GC-MS analysis has been carried out at the Department of Food and Technology and Quality Control, Babarmahal, Kathmandu, Nepal.

3.2.13. Antibacterial Activity

In physical screening, the effect of the crude plant extract or fraction at a fixed dose level in species of the organism was studied. In this work, the

antibacterial assay was performed. Agar well diffusion method was used in the study of screening and the evaluation of the antibacterial activity of essential oil and crude plant extracts. Inhibition of the bacterial growth was tested by the agar well diffusion method and measured in the form of the zone of inhibition(ZOI) as mentioned in the standard protocol[9].

The ability of the plant extracts/fractions/compounds to kill or inhibit the growth of pathogenic microorganisms; was determined by antibacterial activity. It provides the rationale for the selection of potentially bioactive compounds. The antibacterial screening of plant extracts was carried out by the agar well diffusion method based on the procedure given by the chemist[82–84]. In this method, the average diameter of zone of inhibition (ZOI) produced by plant extracts on particular pathogenic bacteria was measured for the estimation of the antibacterial activity of the extract. Antibacterial susceptibility tests measure the ability of an antibacterial agent to inhibit bacterial growth *in vitro*. There are mainly two methods for antibacterial susceptibility tests. They are the diffusion method and dilution method. Of these, the diffusion-based approach is commonly known as Kirby-Bauer's method. It is quite a suitable test of antibacterial activity[11,54]. To test the antibacterial property of the sample, a good diffusion method was used. Positive and negative control was used to compare the results. This method follows the following procedures:

3.2.13.1. Preparation of Stock/ Working Solution

For this process, we took crude oil as well as 10% concentrated oil solution. For 10% solution, 0.1 mL (i.e. 100 µL) of essential oil of the plant was dissolved in 1000 µL DMSO to make 10% concentration solution in an Eppendorf tube. The tubes were sealed and stored in the refrigerator at 4^0c until use.

25 mg of plant extract (Essential oil and chloroform extract) was dissolved in 500 µL methanol to make the concentration of 50 mg/mL stock solution in an Eppendorf tube. From the stock solution, the extract was diluted in autoclaved distilled water and made a 25 mg/mL concentration working solution. After making the stock/working solution, the tubes were sealed and stored in the

refrigerator at 37°C until use. The tube was capped, sealed, and stored in cool condition until use.

3.2.13.2. Collection of Standard Culture

Active cultures of six standard strains of bacteria were provided by Research Institute for Bioscience and Biotechnology (RIBB), Nakhkhu, Lalitpur, Nepal, and 12 standard strains of bacteria were supplied by the Ministry of Forests and Environment, Department of Plant Resources (Biological Section), Banaspati Marga, Thapathali. The following organisms were included in the study; gram-positive *bacteria* and gram-negative bacteria. All micro-organism was cultured in Nutrient Broth and kept viable by sub-culturing in Nutrient Agar. The purity of organisms was maintained by sub-culturing using the streak plate technique.

3.2.13.3. Preparation of Standard Culture Inoculum

It was prepared from primary culture plates as described below: The isolated colony was sub-cultured on nutrient agar plates with the inoculating loop aseptically. It was then transferred to a tube containing 9 mL of sterile nutrient broth and incubated for 24 hours at 37°C.

3.2.13.4. Preparation of Media

A) Nutrient Agar

It was added in distilled water in the ratio of 28 g/liter in appropriate sizes of a conical flask and boiled with continuous shaking and autoclaved at 121°C for 15 minutes. Sterilized media was allowed to cool about 50°C. They were distributed in the sterile Petri-plates of the size of 90 mm diameter in the ratio 25 mL per plate aseptically and labelled properly. Plates were left as such for solidification.

B) Nutrient Broth

The nutrient broth is used for growing these pathogenic bacteria. 1.3 g of nutrient broth was dissolved in some distilled water and diluted to 100 mL. It was sterilized by autoclaving at 121°C for 15 minutes. It was cooled and 9 mL of it was poured inside a screwed capped bottle and again sterilized.

C) Muller Hinton Agar

3.42 g of media was dissolved in 100 mL of distilled water and sterilized by autoclaving at 121°C for 15 minutes. It was then allowed to cool about 50°C and poured into Petri-plates in 15 mL per plate and the plates were left as such for solidification.

D) Screening and Evaluation of Antibacterial Activity

Already prepared sterile Muller-Hinton Agar (MHA) plates were dried to remove excess moisture from the surface of the media. The sterile cotton swab was dipped into the prepared inoculums and the excess of inoculums were removed by pressing and rotating against the upper inner inside wall of the tube above the liquid level and then swabbed carefully all over the plates. The plate was rotated through an angle of 60º after each swabbing. Finally, the swab was passed round the edges of the Agar surface. The inoculated plates were left to dry for a day in laminar airflow.

The wells were made in the incubated media plates with the help of a sterile cork borer (4 mm) and labeled properly. Then 50 µL of the working solution of the plant extracts and essential oil were loaded into the respective wells with the help of a micropipette. Streptomycin was used as a control in the separate well. The plates were then left for an hour with the lid closed so that extracts diffuse into the media. The plates were incubated overnight (18-24 hours) at 37°C.

The plates were then observed for the zone of inhibition around the well which is suggested by a clean zone without growth was noted. The ZOI was measured with the help of the ruler and the mean was recorded for the estimation of the potency of the antibacterial substance.

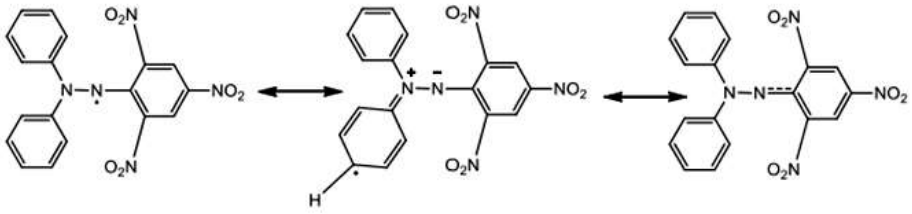
3.2.14. Antioxidant Activity

An antioxidant is an important substance that can protect the body from cellular damages by free radical-induced oxidative stress and those compounds capable of either delaying or inhibiting the oxidation processes which occur under the influence of atmospheric oxygen or reactive oxygen species. They are used for the stabilization of polymeric products,

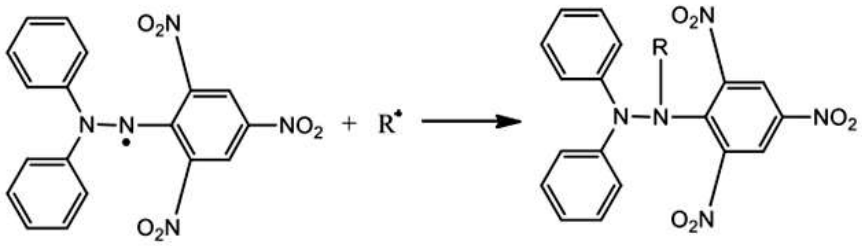
petrochemicals, foodstuffs, cosmetics, and pharmaceuticals[41,48,85]. Free radicals, namely reactive oxygen species (ROS) and reactive nitrogen species (RNS), are known to cause damage to lipids, proteins, enzymes, and nucleic acids leading to cell or tissue injury implicated in the process of ageing. These free radicals and oxidative stress cause degenerative diseases such as inflammation, cancer, atherosclerosis, diabetes, liver injury, Alzheimer, Parkinson, and coronary heart pathologies. Several pieces of evidence indicate that oxidative stress can lead to cell and tissue injury. There occurs depletion of antioxidants during oxidative stress. The ROS and RNS include diverse reactive entities namely superoxide (O_2-.), hydroxyl (OH.), peroxyl (ROO.), peroxynitrite (. ONOO-), and nitric oxide (NO.) radicals as well as non-free radical species such as hydrogen peroxide (H_2O_2), nitrous acid (HNO_2) and hydrochlorous acid (HOCl)[27,85–87].

3.2.14.1. Principle of DPPH Assay

DPPH (2,2-Diphenyl-1-picrylhydrazyl-hydrate) free radical method is an antioxidant assay based on electron transfer that produces a violet solution in alcohol and change to a colorless solution due to the presence of an antioxidant molecule[88]. The molecule 2, 2-Diphenyl-1- picrylhydrazyl (DPPH) is characterized as a stable free radical by the delocalization of the lone pair of the electron over the molecule as a whole, to resist dimerization, as would be the case with most other free radicals. The delocalization gives rise to the deep violet color, characterized by an absorption band in methanol solution centered at about 517 nm. When a solution of DPPH is mixed with a substance that can donate a hydrogen atom, then this gives reduced form with the change of the violet color to the pale color at the end due to the still presence of pecryl residue[89].



Stable DPPH free radical



DPPH (dark purple) Derivatives of DPPH (pale yellow)

Reaction of DPPH free radical

3.2.14.2. Preparation DPPH Solution

2, 2- Diphenyl-1-picrylhydrazyl (DPPH) has a molecular weight of 394.32 g/mol. Thus, 100 mL of 0.2 mM solution of DPPH was prepared to weigh 4 mg of the DPPH carefully weight and dissolving it with methanol and finally maintain the volume to 100 mL.

3.2.14.3. Measurement of DPPH Free Radical Scavenging Activity

The percentage of radical scavenging activity was calculated using the following formula:

Percentage scavenging= $\frac{(A_0 - A_T)}{A_0} \times 100\%$

Where, A_O= Absorbance of the DPPH

A_T= Absorbance of the DPPH free radical solution containing the sample extract.

The 50% inhibitory concentration IC_{50} value was indicated as the effective concentration of the sample that was required to scavenge 50% of the DPPH free radicals. IC_{50} values were calculated using the dose inhibition curve in the

logarithm range by plotting the extract concentration versus the corresponding scavenging effect.

3.2.14.4. General Protocol for Antioxidant Assay

1 mg sample, when dissolved in 1 mL solvent, gives the solution of a concentration of 1 mg/mL and 2 mg in 1 mL solvent gives the solution of concentration 2 mg/mL So 10 mg of the sample (essential oil and Chloroform extract of *A. adenophora* to be tested was dissolved in 5 mL methanol to get stock solution of concentration 2 mg/mL (2000 µg/mL). Different concentrations (1500, 1000, 500, 250, and 125 µg/mL) of the 1000 µL (1 mL) extracts were prepared by a two-fold dilution method using a stock solution.

Table 1: Preparation of different concentration test samples for antioxidant assay

Concentration (µg/mL)	Extract Solution	Distilled water	Final volume
1500	750 µL (2000 µg/mL)	250 µL	1000 µL
1000	500 µL (2000 µg/mL)	500 µL	1000 µL
500	500 µL (1000 µg/mL)	500 µL	1000 µL
250	500 µL (500 µg/mL)	500 µL	1000 µL
125	500 µL (250 µg/mL)	500 µL	1000 µL

3.2.15. Total Phenol Content Assay

Phenolic compounds are secondary metabolites that are derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants[90]. About 8000 phenolic compounds have been identified as biologically active ingredients [91,92]. Plants produce polyphenols for several purposes such as plant pigmentation; reproduction and protection against bacterial pathogens or UV light[93].

The 'phenolic' or 'polyphenol' is defined chemically as a substance having an aromatic ring with one (phenol) or more (polyphenol) hydroxylsubstituent[93]. Most naturally occurring phenolic compounds are present as conjugates with mono- and polysaccharides, linked to one or more of the phenolic groups, and may also occur as functional derivatives such as glycosides, esters, and methyl esters. Phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, anti-artherogenic,

antiinflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective, vasodilatory effects, antimutagenic, anticarcigenic, and ability to modify the gene expression. Phenolic compounds have been associated with the health benefits derived from consuming high levels of fruits and vegetables[58,90,91,94–96].

3.2.15.1. Principle of Total Phenolic Content Assay

The content of total phenolic compounds of the different plants was determined by Folin-Ciocalteau Reagent. The Folin-Ciocalteau Reagent measures a sample's reducing capacity. The exact chemical nature of the Folin-Ciocalteau Reagent is not known, but it is believed to contain Folin-Ciocalteau phenol reagent consists of a mixture of the heteropoly acids, phosphomolybdic and phosphotungstic acids in which the molybdenum and the tungsten are in the 6+ oxidation state. The sequence of reversible one or two-electron reductions leads to the formation of the molybdenum blue and the tungsten blue, possibly (Phenol-$MoW_{11}O_{40})_4$, and the mean oxidation state of the metals is between 5 and 6[21,97]. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron transfer reaction that occurs between reductants and Mo (VI).

3.2.15.2. Preparation of Folin-Ciocalteau Reagent

1 mL of Folin-Ciocalteau reagent was taken in a beaker and 10 times dilution was done with distilled water.

3.2.15.3. Preparation of Standard Gallic Acid Solution

1 mg gallic acid was dissolved in 1 mL distilled water, so the concentration of the solution is 1 mg/mL or 1000 µg/mL. This is called the stock solution. Then the different concentrations (100, 80, 60, 40, 20, and 10 µg/mL) of the gallic acid solution were prepared by two-fold dilution method.

3.2.15.4. Measurement of Total Phenolic Content (TPC)

The concentration of total phenolic content in the sample was determined as milligram of gallic acid equivalent by using the following equation:

$$\text{TPC} = \frac{(C \times V)}{m}$$

Where, C= concentration of gallic acid from curve (mg/mL)

V= volume of extract (mL)

m= weight of plant extract (g)

3.2.15.5. General Protocol for Total Phenolic Content

0.1 mL of sample (1 mg/mL in methanol) was mixed with 1 mL of Folin-Ciocalteu phenol reagent (1:10 dilution with water) and 0.8 mL of aqueous 1 M Na_2CO_3 solution. The reaction mixture was allowed to stand for about 15 minutes in dark and then the absorbance of the reactants was measured at 765 nm against a blank (methanol). Gallic acid was used as standard. Total phenolic content is expressed as mg of gallic acid equivalents per gram of dried extract.

3.2.16. Total Flavonoid Content Assay

Flavonoids are a broad class of low molecular weight polyphenols with diphenylpropanes (C_6-C_3-C_6) skeletons, secondary plant phenolics characterized by the flavan nucleus. Flavonoids are large families which are widely distributed in the leaves, seeds, bark, and flowers of plants. Over 4,000 flavonoids have been identified to date. In plants, these compounds afford protection against ultraviolet radiation, pathogens, and herbivores. Most of the beneficial health effects of flavonoids are attributed to their antioxidant, chelating abilities, and a reduced incidence of heart disease[95,98–101]. Flavonoids include six major subgroups that are found in higher plants: the chalcones, flavones, flavonols, flavandiols, anthocyanins, and condensed tannins (or proanthocyanidins) and aurones[93].

3.2.16.1. Principle Total Flavonoid Content Assay

The content of total flavonoids of plant extract was determined by the well-known aluminium chloride colorimetric method. In this method, aluminium chloride forms a complex with hydroxyl groups of flavonoids present in the sample. This complex has a maximum absorbance of 420 nm.

3.2.16.1. Preparation of Standard Quercetin Solution

1 mg quercetin was dissolved in 1 mL distilled water, so the concentration of the solution is 1 mg/mL or 1000 μg/mL. This is called the stock solution. Then

the different concentrations (80, 60, 40, 20, 10, and 5 µg/mL) of quercetin solution were prepared by two-fold dilution method.

3.2.16.2. Measurement of Total Flavonoid Content (TFC)

The concentration of total flavonoid content in the sample was determined as milligram of quercetin equivalent by using the following equation:

$$\text{TFC} = \frac{C \times V}{m}$$

Where, C= concentration of quercetin from curve (µg/mL)

V= volume of extract (mL)

m = weight of plant extract (g)

3.2.16.3. General Protocol for Total Flavonoid Content

1 mL of sample (0.1 mg/mL in methanol) was mixed with 1 mL of $AlCl_3$ (dissolved in methanol) and kept for 1 hr and absorbance was measured at 415 nm against the blank (methanol). Quercetin was used as standard. Total flavonoid content is expressed as mg of quercetin equivalents per gram of dried sample.

3.2.17. Brine Shrimp Lethality Bioassay

Brine shrimp (*Artemia salina*, fairy shrimp, or sea monkeys) lethality assay is commonly used to check the cytotoxic effect of bioactive chemicals. It is a rapid and comprehensive bioassay for the bioactive compounds of natural and synthetic origin. It is a preliminary toxicity screening of plant extracts, fungal toxins, heavy metals, cyanobacteria toxins, pesticides, cytotoxicity testing of dental material, and nanostructures. The method utilizes *in vivo* lethality in a simple zoological organism (brine nauplii) as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products. This assay was first proposed by Michael *et al*. in 1956 and subsequently modified by others. This lethality assay has been successfully employed as a bioassay guide for active cytotoxic and antitumor agents. It is a rapid (24 hours), inexpensive and simple test as no aseptic techniques are required. It easily utilizes a large number of organisms for statistical validation and requires no special equipment and a relatively small amount of sample (2-20 mg or less). The brine shrimp lethality bioassay also indicates antifungal

effects, pesticidal effects, teratogenic effects, toxicity to the environment, and many more[102–108].

3.2.17.1. Preparation of Artificial Seawater

Artificial seawater needed for the entire bioassay was freshly prepared by dissolving the following chemicals in distilled water as given in Table 3.

Table 2: Composition of artificial seawater

S.N.	Composition	Amounts (g/L)
1	NaCl	23.50
2	Na_2SO_4	4.00
3	KCl	0.68
4	H_3BO_3	0.027
5	$MgCl_2.2H_2O$	10.68
6	$CaCl_2.2H_2O$	1.78
7	$NaHCO_3$	0.197
8	Na_2EDTA	0.0003

3.8 g sea salt (without iodine) was weighed, dissolved in 100 mL of distilled water and filtered off to get clear solution.

3.2.17.2. Hatching of Brine Shrimp

The test organism *Artemia salina* leaches (brine shrimp eggs) were hatched in seawater inside a conical flask. Two days were allowed to hatch the shrimp and to be matured as nauplii. The constant oxygen supply was carried out through the hatching time. The hatched shrimps are attracted to light (phototaxis). The nauplii were taken from the fish tank by a pipette and diluted in fresh clear seawater to increase visibility and 10 nauplii were taken carefully by micropipette.

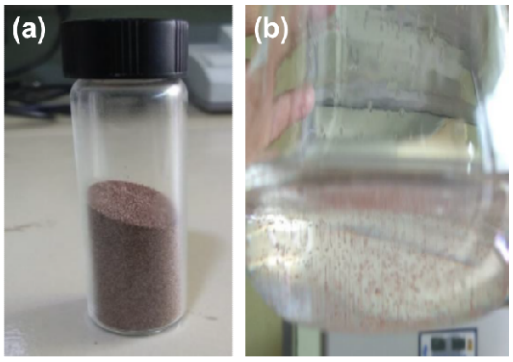


Figure 4: (a) Brine shrimp eggs, and (b) Hatched shrimps

3.2.17.3. Preparation of Test Solutions with Samples of Experimental Plants

10 mg of the test sample (essential oil and chloroform extract of *A. adenophora* were taken and dissolved in 1 mL of pure dimethyl sulfoxide (DMSO) and finally the volume was made to 10 mL with seawater. Thus, the concentration of the stock solution was 1000 µg/mL. Then the solution was serial diluted to 500, 250, 125, 62.5, 31.25, 15.625 µg/mL with seawater. Then 2.5 mL of plant extract solution was added to 2.5 mL of seawater containing 10 nauplii.

Table 3: Preparation of different concentration test samples for brine shrimp lethality assay

Concentration (µg/mL)	Extract Solution	Seawater containing 10 Nauplii	Final volume
500	2.5 mL (1000µg/mL)	2.5 mL	5 mL
250	2.5 mL (500 µg/mL)	2.5 mL	5 mL
125	2.5 mL (250 µg/mL)	2.5 mL	5 mL
62.5	2.5 mL (125 µg/mL)	2.5 mL	5 mL
31.25	2.5 mL (62.5 µg/mL)	2.5 mL	5 mL
15.625	2.5 mL (31.25 µg/mL)	2.5 mL	5 mL

3.2.17.4. Preparation of Control Group

Control groups were used in the cytotoxicity study to validate the test method and ensure that the results obtained were only due to the activity of the test agent and the effects of the other possible factors were nullified. 50 µL of DMSO was added to each of three premarked test tubes containing 4.95 mL of simulated seawater and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

3.2.17.5. Counting of Nauplii

After 24 hours, the test tubes were inspected using a magnifying glass against a black background and the number of survived nauplii in each tube was counted. From this data, the percent (%) of the lethality of the brine shrimp nauplii was calculated for each concentration. The effectiveness of the

concentration-mortality relationship of plant products is usually expressed as a median lethal concentration (LC_{50}). This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure time and is determined by the logarithm regression method from plotting % mortality against corresponding concentration[103].

$$\% \text{ mortality} = \frac{N_o - N_1}{N_1} \times 100$$

Where,

N_O= Number of Nauplii taken

N_1= Number of Nauplii dead

3.2.18. Fourier Transform Infrared Spectrophotometer (FTIR) Analysis

The functional groups present in these chemical constituents of plants are usually identified by FTIR. This helps in structure elucidation with other methods and gained importance to identify medicines in pharmacopoeia of many countries. Fourier Transform Infrared Spectrophotometer (FTIR) is perhaps the most powerful tool for identifying the types of chemical bonds (functional groups) present in compounds. The wavelength of light absorbed is characteristic of the chemical bond as can be seen in the annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. Dried powder of different solvent extracts of each plant material was used for FTIR analysis. 10 mg of the dried extract powder was encapsulated in 100 mg of KBr pellet, to prepare translucent sample discs. The powdered sample of each plant specimen was loaded in FTIR spectroscope (Shimadzu, IR Affinity 1, Japan), with a Scan range from400 to 4000cm^{-1} with a resolution of 4 cm^{-1}[109].

For liquid samples, the easiest is to place one drop of the sample between two plates of sodium chloride. The drop forms a thin film between the plates. Solid samples can be milled with potassium bromide (KBr) and then compressed into a thin pellet which can be analyzed. Otherwise, solid samples can be dissolved in a solvent such as methylene chloride, and the solution is then placed onto a single salt plate. The solvent is then evaporated off, leaving a thin film of the original material on the plate[110].

3.2.19. Column Chromatography

3.2.19.1. Column Chromatography of methanol extracts of *A. adenophora* leaves

Chromatography comprises a group of techniques for separating molecular mixtures based on the differential affinities of the solutes between two immiscible phases. One of the phases is a fixed bed of large surface area termed as stationary phase, the other mobile phase moves over the surface of the fixed phase. Chromatographic techniques were classified according to the nature of the stationary phase and mobile phases[78,111–114].

Before the isolation process, the crude extract was examined on a TLC plate (Silica Gel 60 F_{254} Merck) to find the best solvent systems for column chromatography. The solvent used were hexane, chloroform, ethyl acetate, and methanol[112,115–119]. The fraction with a single component (one spot) that appeared in the TLC plate was treated as a possible pure secondary metabolite. The combined fractions which contain the same single component were then allowed to air-dried or evaporated to dryness to obtain a pure secondary metabolite[115]. The Chemical Structure Elucidation Identification of the isolated secondary metabolite was obtained using various spectroscopes[111,120].

Column chromatography was used to get the fraction of plant extracts. (2 g) of the extract was subjected to column chromatography to separate the extracts into their component fractions. Silica gel was used as the stationary phase, and the solvent system ethyl acetate: hexane as mobile phase. In the setting up of the column chromatography, the lower part of the glass column was stocked with cotton with the aid of a glass rod. The sample was prepared by adsorbing 2.0 g of the extract to 10 g of silica gel in methanol then allowed to dry, the dry powder was gently layered on top of the column then glass wool was put on top to avoid splashing of the solvent system when pouring it into the column. The elution of the extract was done with solvent system ethyl acetate: hexane 9:1, 8:2, 7:3, 6:4, and 5:5. The eluted fractions were collected in conical flasks. The conical flasks were collected as eluted fractions[120].

3.2.19.2. Thin Layer Chromatography (TLC)

TLC study was done for bioactive isolation and to find out the probable number of compounds present in a fraction. On the pre-coated TLC plate, test samples (after dissolving in respective solvents) were applied in the form of spots with the help of a fine capillary. Spots were marked on the top of the plate for their identification. Rectangular glass chambers were used for chromatography. To avoid insufficient chamber saturation and undesirable edge effect, a smooth sheet of filter paper was placed (in U shape) in the TLC chamber and was allowed to be in the developing solvent. Several developing solvent systems were tried during the study. The most informative and satisfactory resolution was taken as the final solvent system. The plates were observed under UV light and after the development of plates, they were air-dried and numbers of spots were noted. As soon as the fractions were eluted, it was analyzed by using a ready-made TLC plate with suitable mobile solvent according to the polarity of eluting. The developed chromatogram was observed under UV and also derivatized with detecting agent[120].

The Thin Layer Chromatography was developed in Twin through the chamber with silica gel 60 F_{254} pre-coated aluminum plate of 0.2 mm thickness using ethyl acetate: methanol (1:1) as the developing solvent system and retention factor R_f value was calculated. Distance moved by the molecule the spot).

$$\text{Rf value} = \frac{\text{Distance move by the molecule(located by the spot)}}{\text{Distance moved by the mobile phase(solvent front)}}$$

CHAPTER 4: RESULTS AND DISCUSSION

4.1. Amount of Extracts Collected

The essential oil and extraction of *A. adenophora* leaves were done using four different solvents of varying polarity such as hexane; ethyl acetate, methanol, and chloroform were used for extraction and their effectiveness in extracting the active compounds responsible for the biological activity of *A. adenophora* leaves were evaluated through the determination of extract yields. It was crucified using Rota-evaporator and dried using the waterbath. As shown in Table 4 the number of extracts collected in grams after drying them in vails.

Table 4: Number of extracts collected from each experiment

Plant Names	**Parts Used**	**Method of Extraction**	**Solvents**	**Colour & Consistency**	**Average Extractive Value (% w/w)**
A. adenophora	Leaves	Cold Percolation	Methanol	Dark green	70.68 g
			Hexane	Dark green	38.92 g
			Chloroform	Light green	9.67 g
			Ethyl acetate	Brownish green	1.7 g
		Continuous hot Percolation by Soxhlet Apparatus	Methanol	Dark green	60 g
		Hydro-distillation using Clevenger-type apparatus		Yellowish green (oil)	10 mL

4.2. Phytochemical Screening Analysis

The phytochemical screening analysis was done to check the secondary metabolites present in the plant extract of *A. adenophora* that was collected. After performing several tests, the secondary metabolites and their phytoconstituents present in them were found. A total of phytochemical tests were performed to observe 4.78 pH value of methanol extracts, alkaloids, flavonoids, phenols, steroids, quinones, saponins, tannins, cardiac glycosides, carbohydrates, terpenoids, proteins, and amino acids in different solvent extracts.

The results of the phytochemical screening have been summarized in Table5.

Table 5: Results of the Phytochemical Screening of Methanol (ME); Hexane (HE); Chloroform (CE); and Ethyl acetate (EAE) extract of *A. adenophora* leaves

S. N	Detection	Test name	Reference	Observation	Results			
					ME	HE	CE	EAE
1	pH test	Blue litmus paper Red litmus paper pH value test	Turns to red if acidic Turn to blue if basic	Red No change	Acidic 4.78	-	-	-
2	Alkaloids	Mayer's	White/Pale yellow ppt.	Yellow ppt.	+	+	+	+
		Dragendorff's	Yellow /brown ppt	Orange-brown ppt.	+	+	+	+
		Wagner's test	Yellow /brown ppt	Reddish-brown ppt.	+	+	+	+
3	Flavonoids	Sodium hydroxide	Yellow ppt	Yellow ppt	+	+	+	+
		Lead acetate test	Yellow ppt	Yellow ppt	+	+	+	+
		Shinoda test	Green colour	Green colour	+	+	+	+
4	Phenols	Ferric chloride test	Bluish Black /Greenish Yellow colour	Greenish-yellow colour	+	+	+	+
5	Steroids	Salkowski test	Golden-yellow /wine red colour (triterpenes)	Golden colour	+	+	+	+
6	Quinones	Sodium hydroxide and H_2SO_4	Green colour	Green colour	+	+	+	+
7	Saponins	Froth test	Frothing	Frothing present	+	+	+	+
8	Tannins	Gelatin test	White ppt.	White ppt.	+	+	+	+
		5 % $FeCl_3$	Brownish green colour	No appear	-	-	-	-

9	Cardiac Glycosides	Killer-Kilani test	The Reddish-brown ring at the junction of two solvents	Ring formation	+	+	+	+
		Molisch's test	A red-violet ring appears which disappears with the addition of alkali.	Colour change	+	+	+	+
10	Carbohydrates	Fehling's test	Red/Brick red ppt.	Red ppt	+	+	+	+
		Molisch's test	Violet ring at the junction	Ring formation	+	+	+	+
		Benedicts test	Brick red ppt	Red colour	+	+	+	+
11	Terpenoids	Chloroform and conc. H_2SO_4	The reddish-brown colour at the interface	Reddish-brown colour formation	+	+	+	+
12	Proteins and Amino acid	Xanthoproteic test	Yellow color (Proteins)	Yellow colour	+	+	+	+
		Ninhydrin test	Blue color (Amino acids)	Blue colour (Amino acids)	+	+	+	+
13	Resin	Acetone-water test	Turbidity	No turbidity	-	-	-	-

'+'indicates the presence and '- 'the absence

4.3. GC-MS Spectra Analysis

4.3.1. GC-MS Spectra Analysis of Chloroform Extract and Essential oil

GC-MS chromatogram of the chloroform extract and essential oil of *A. adenophora* shows the presence of 10 and 14 Significant compounds, respectively. The GC-MS chromatogram of chloroform extract and essential oils of *A. adenophora* is presented below:

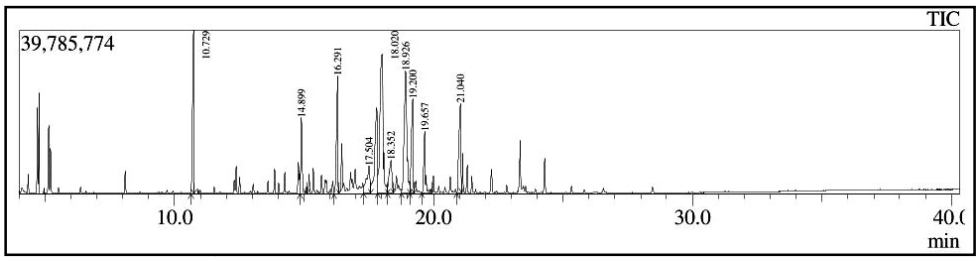


Figure 5: Chromatogram of chloroform extract of *A. adenophora*

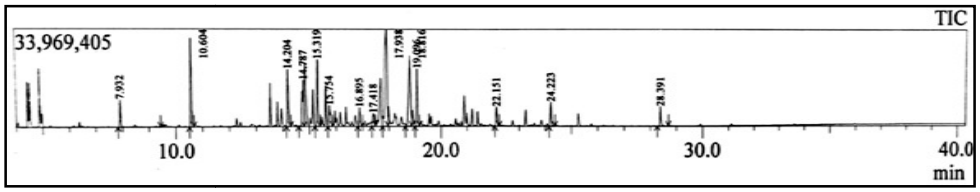


Figure 6: Chromatogram of essential oil of *A. adenophora*

The chemical constituents of chloroform extract of *A. adenophora* are analyzed by GC-MS coupled with a mass library search revealed the presence of 10 major compounds. Major compounds analyzed by GC-MS analysis are shown below:

Table 6: List of Compounds detected in the chloroform extract

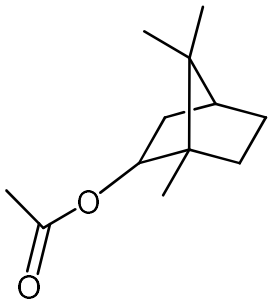
S. N	Name of the compound	Retention time	Molecular formula	Molecular weight	Area (%)
1	Bornyl acetate	10.729	$C_{12}H_{20}O_2$	196	10.21
2	Naphthalene	14.899	$C_{15}H_{24}$	204	5.22
3	Cyclohexanemethanol	16.291	$C_{15}H_{26}O$	154	8.53
4	Nerolidol	12.567	$C_{15}H_{26}O$	222	5.24
5	1-Naphthalenol	14.483	$C_{15}H_{26}O$	222	24.33
6	1H-Cycloprop[e]azulen-7-ol	14.917	$C_{15}H_{24}O$	220	4.93
7	α–Bisabolol	15.150	$C_{15}H_{26}O$	222	18.01
8	2H-Cyclopropa[g]benzofuran	15.275	$C_{15}H_{22}O$	218	8.90
9	3a,7-Methano-3aH-cyclopentacyclooctene	15.450	$C_{15}H_{24}$	204	5.08
10	Cyclohexane	15.700	$C_{14}H_{24}$	192	9.55

The chemical constituents of essential oils of *A. adenophora* analyzed by GC-MS coupled with a mass library search revealed the presence of 14 major compounds. Major compounds analyzed by GC-MS analysis are shown below:

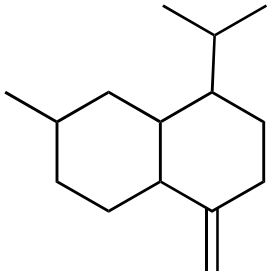
Table 7: List of Compounds detected in the essential oil

S.N.	Name of the compound	Retention time	Molecular formula	Molecular weight	Area (%)
1.	Borneol	7.932	$C_{10}H_{18}O$	136	2.84
2.	Bornyl acetate	10.604	$C_{12}H_{20}O_2$	152	10.89
3.	β- Farnesene	14.204	$C_{15}H_{24}$	152	6.59
4.	Epizonarene	14.787	$C_{15}H_{24}$	152	8.61
5.	Cyclohexene	15.319	$C_{15}H_{24}$	154	7.81
6.	Thujen-2α-yl acetate	15.754	$C_{12}H_{18}O_2$	196	3.45
7.	Caryophyllene oxide	16.895	$C_{15}H_{24}O$	204	2.49
8.	Nerolidol	17.418	$C_{15}H_{26}O$	204	2.29
9.	1-Naphthalenol	17.938	$C_{15}H_{26}O$	294	24.56
10.	α-Bisabolol	18.816	$C_{15}H_{26}O$	204	14.74
11.	9H-Cycloisolongifolene	19.096	$C_{15}H_{22}O$	204	8.56
12.	Bicyclo [5.1.0] octan-2-one	22.151	$C_{16}H_{24}O$	204	2.29
13.	6-(1-Hydroxymethylvinyl)-4	24.223	$C_{12}H_{22}O_2$	234	2.59
14.	9,19-Cycloergost-24(28)-en-3-ol	28.391	$C_{32}H_{52}0_2$	468	2.29

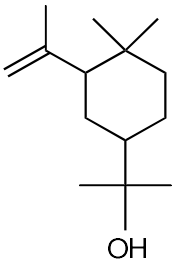
4.3.2. Structure of Compounds Detected from GC-MS Analysis of Chloroform extract of *A. adenophora*



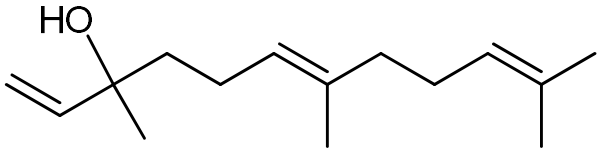
Bornyl acetate



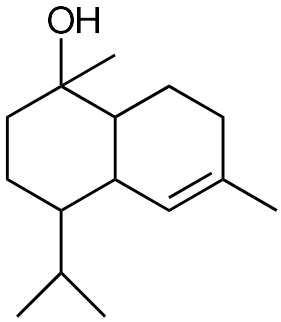
Naphthalene



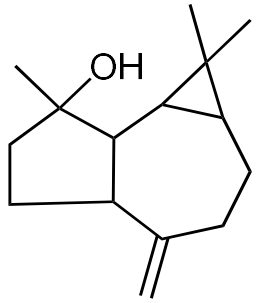
Cyclohexanemethanol



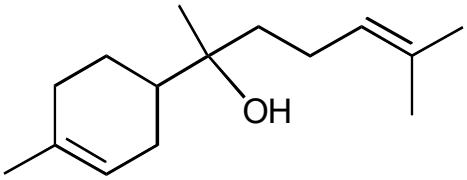
Nerolidols



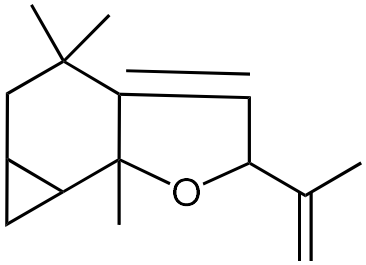
1Naphthalenol



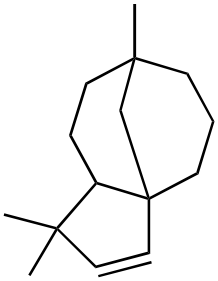
1H-Cycloprop[e]azulen-7-ol



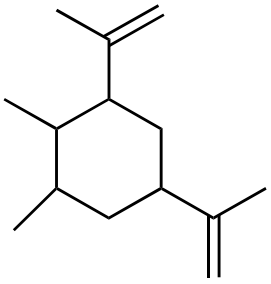
α–Bisabolol



3a,7-Methano-3aH-cyclopentacyclooctene

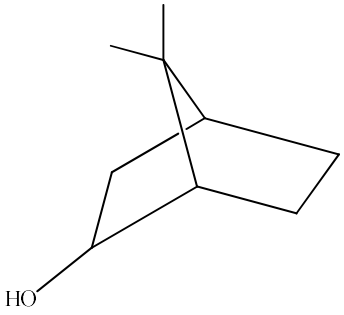


2H-Cyclopropa[g]benzofuran

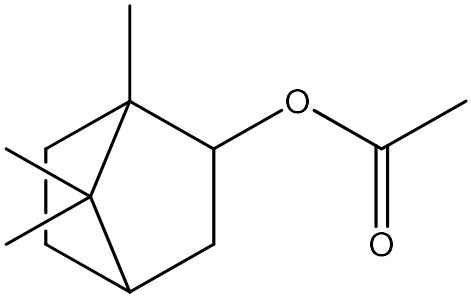


Cyclohexane

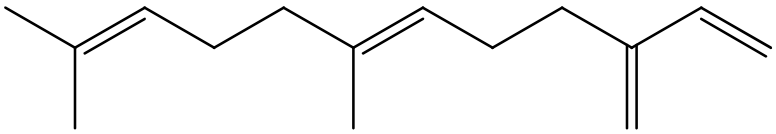
4.3.3. Structure of Compounds Detected from GC-MS Analysis of Essential oil of *A. adenophora*



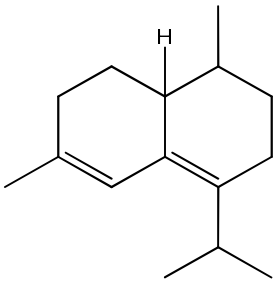
Borneol



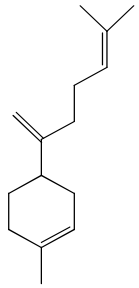
Bicyclo [2.2.1] heptan-2-ol



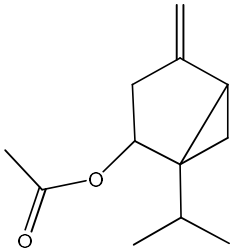
β- Farnesene



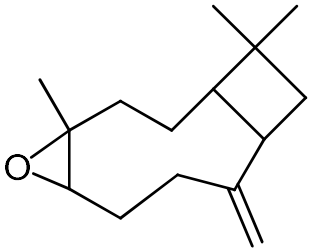
Espizonarene



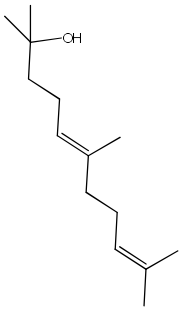
Cyclohexene



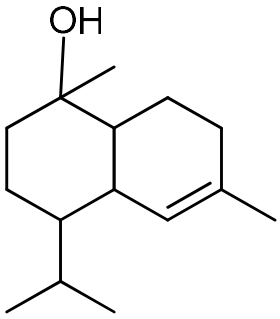
Thujen-2*α*-yl acetate



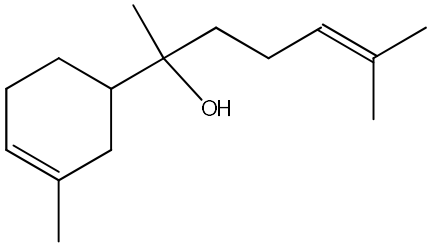
Caryophyllene oxide



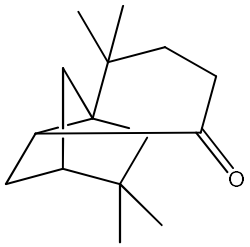
Nerolidol



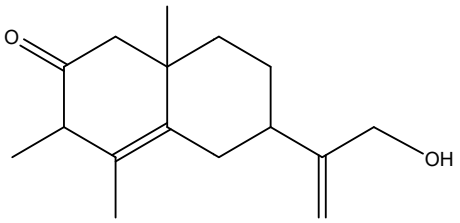
1–Naphthalenol



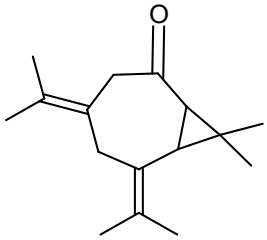
Bisabolol



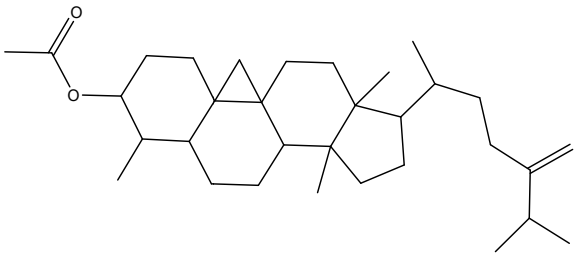
9H-Cycloisolongifolene



6-(1-Hydsroxymethylvinyl)-4



Bicyclo [5.1.0] octan-2-one



9,19-Cycloergost-24(28)-en-3-ol

4.4. Mass Spectral Data of Constituents Identified by GC-MS

4.4.1. Mass Spectral Data of Constituents Present in Chloroform Extract; identified by GC-MS

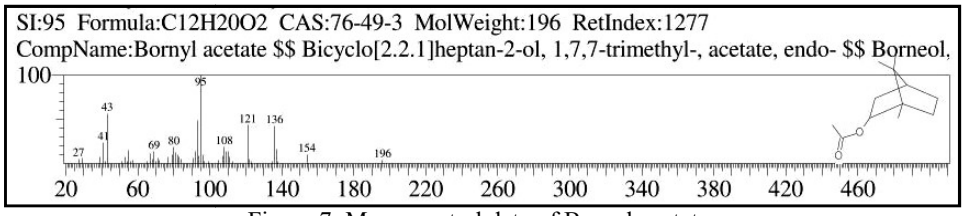


Figure 7: Mass spectral data of Bornyl acetate

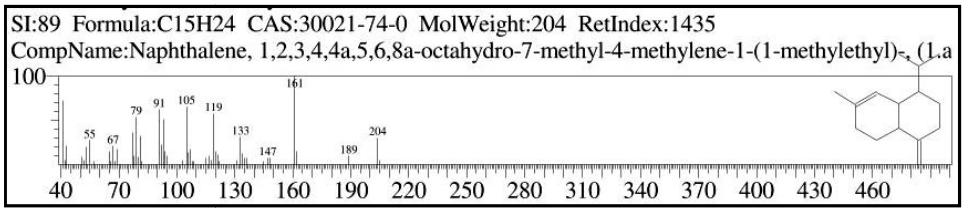


Figure 8: Mass spectral data of Naphthalene

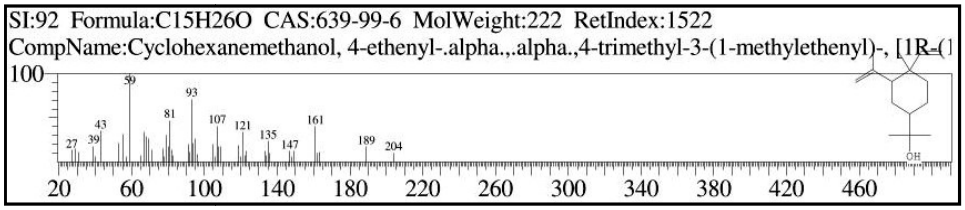


Figure 9: Mass spectral data of Cyclohexanemethanol

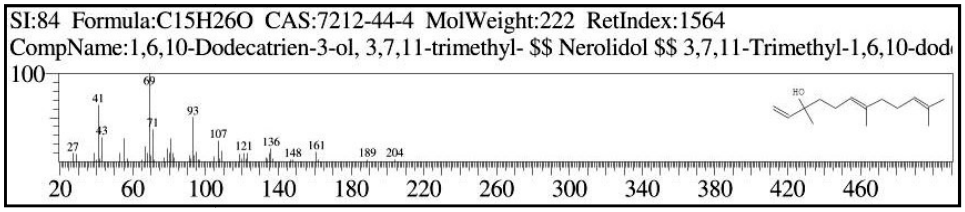


Figure 10: Mass spectral data of Nerolidol

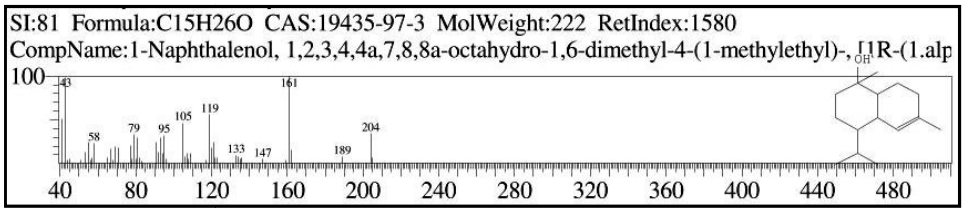


Figure 11: Mass spectral data of 1-Naphthalenol

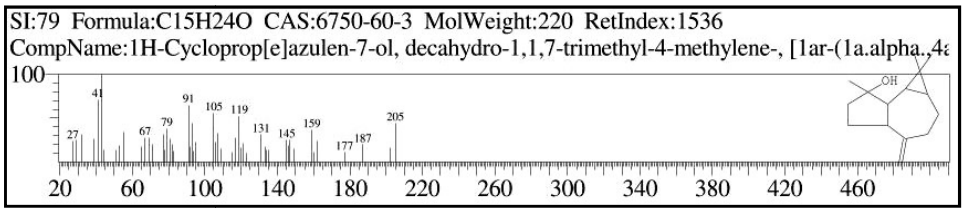


Figure 12: Mass spectral data of 1H-Cycloprop[e]azulen-7-ol

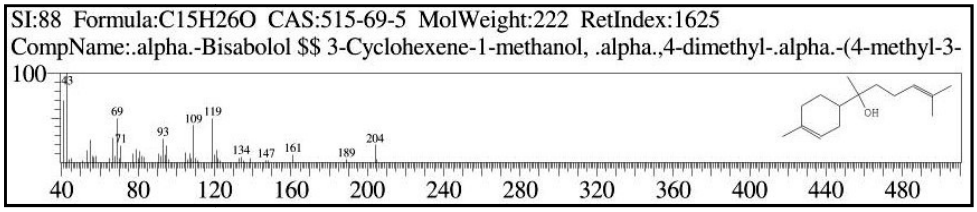


Figure 13: Mass spectral data of α–Bisabolol

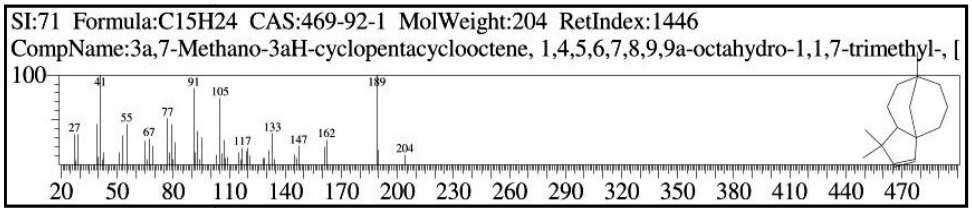


Figure 14: Mass spectral data of 3a,7-Methano-3aH-cyclopentacyclooctene

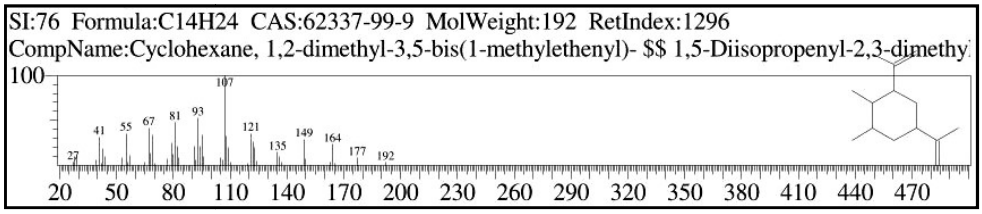


Figure 15: Mass spectral data of Cyclohexane

4.4.2. Mass Spectral Data of Constituent Present in Essential Oil identified by GC-MS

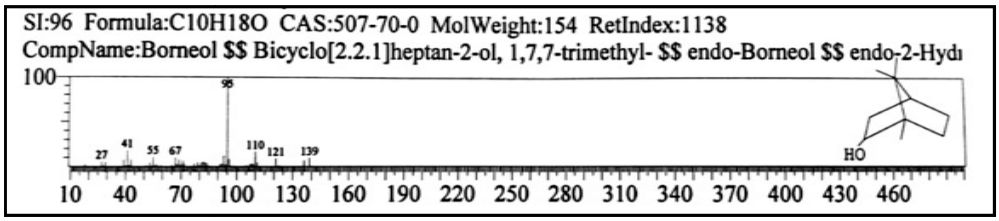


Figure 16: Mass spectral data of Borneol

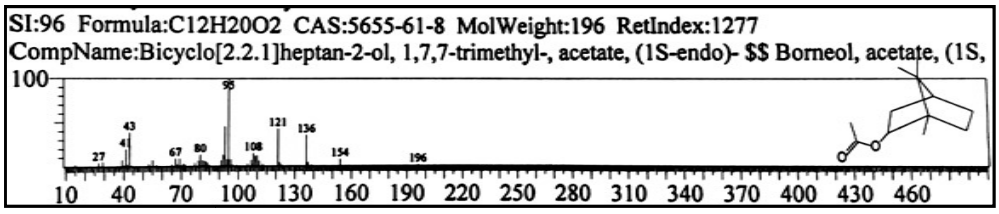


Figure 17: Mass spectral data of Borneol acetate

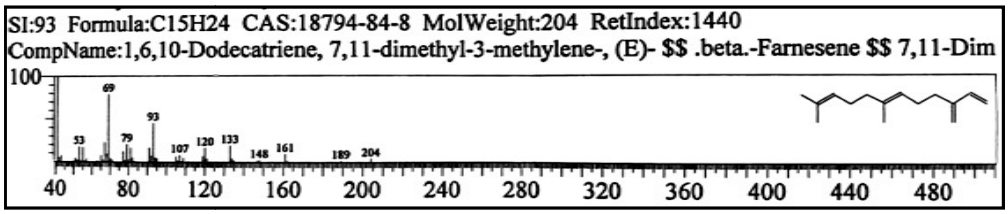


Figure 18: Mass spectral data of β-Farnesene

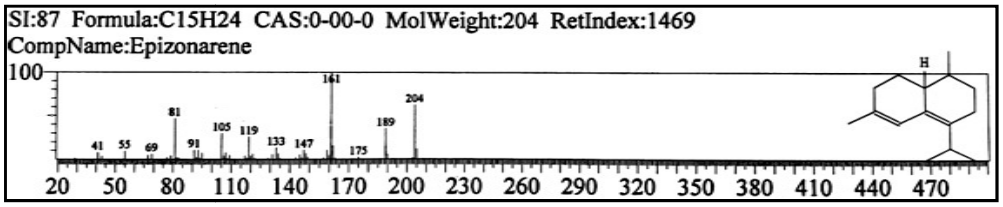


Figure 19: Mass spectral data of Epizonarene

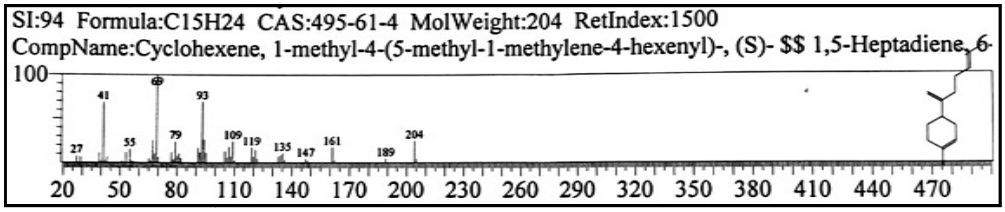


Figure 20: Mass spectral data of Cyclohexene

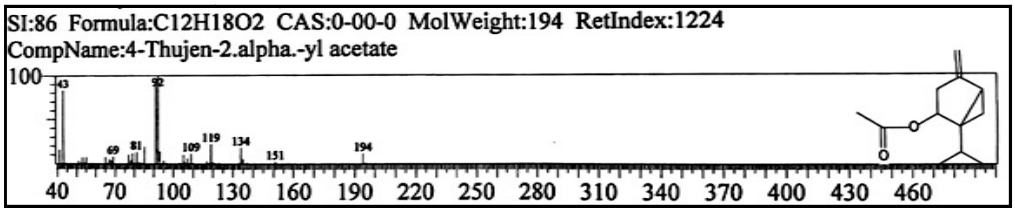


Figure 21: Mass spectral data of Thujen-2α-yl acetate

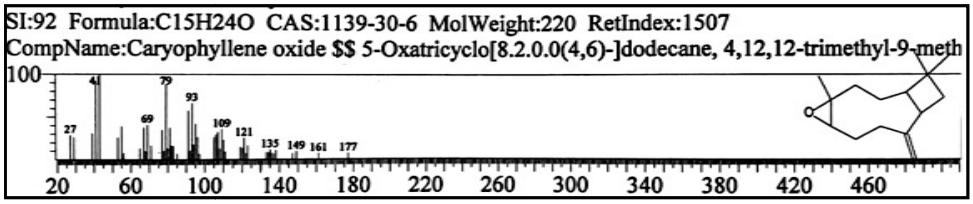


Figure 22: Mass spectral data of Caryophyllene oxide

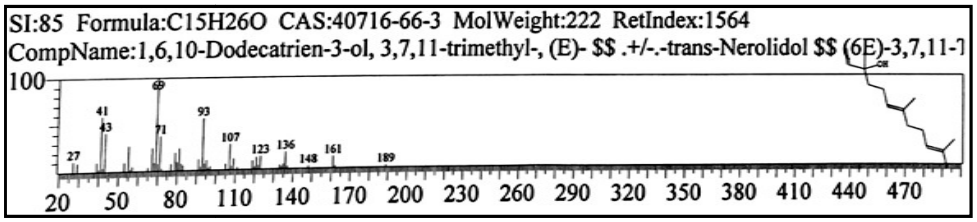


Figure 23: Mass spectral data of Nerolidol

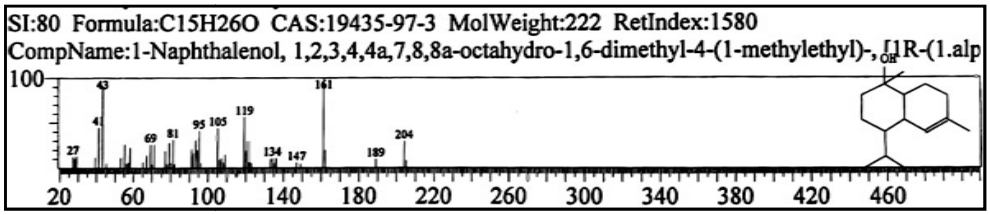


Figure 24: Mass spectral data of 1-Naphthalenol

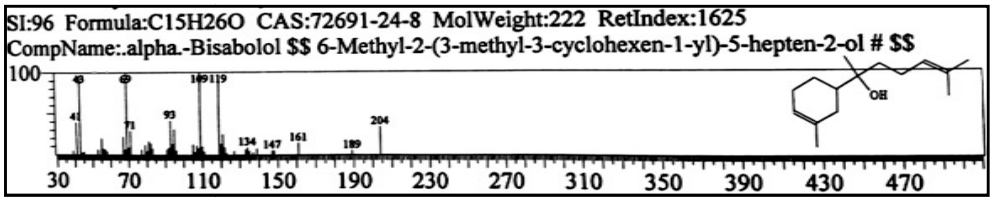


Figure 25: Mass spectral data of α-Bisabolol

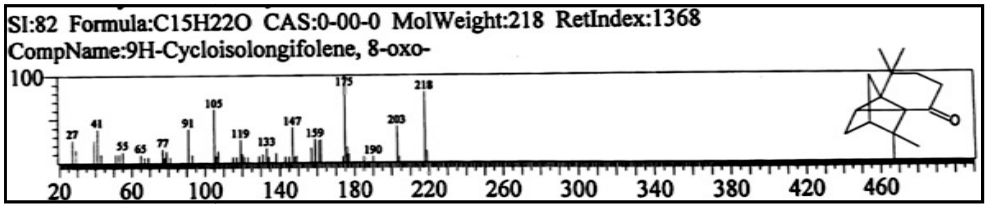


Figure 26: Mass spectral data of 9H-Cycloisolongifolene

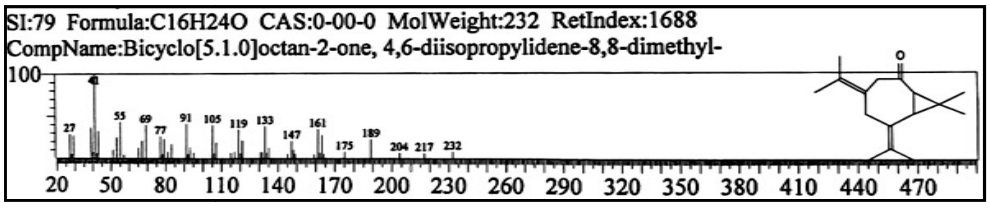


Figure 27: Mass spectral data of Bicyclo [5.1.0] octan-2-one

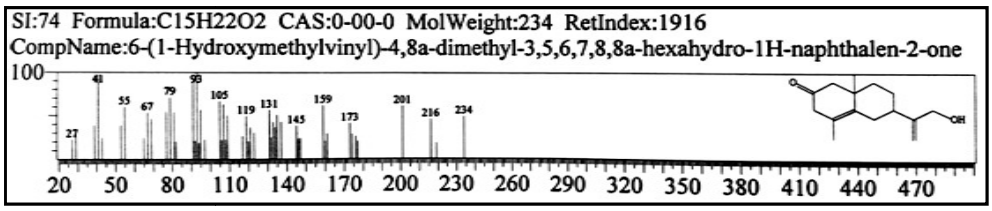


Figure 28: Mass spectral data of 6-(1-Hydroxymethylvinyl)-4

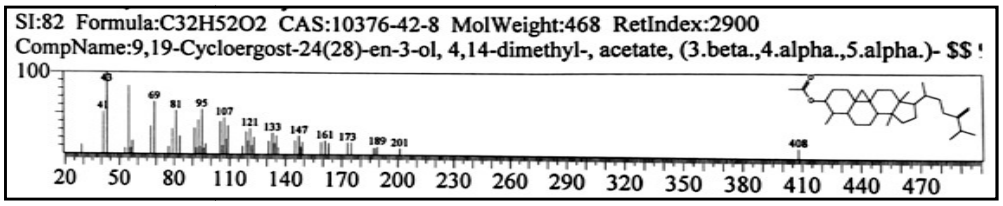


Figure 29: Mass spectral data of 9.19-Cycloergost-24(28)-en-3-ol

4.5. Antibacterial Screening Analysis

Plants are rich sources of essential phytoconstituents which has been proved from various previous studies. Each constituent has its effect against microorganisms. However, the antimicrobial activity of both chloroform extract and essential oil varies according to the species of microorganisms[22,121–123].

Table 8: Antimicrobial activity shown by chloroform extract of *A. adenophora* in diameter (mm) of inhibition zone

Test Organism	**Zone of inhibition**		
	Positive control (Chloramphenicol)	**Negative Control (DMSO)**	**Extract of *A. adenophora* Activity**
Klebsiella pneumoniae ATCC 700603	26.58mm	No activity	8mm
Bacillus subtilis ATCC 6051	20.86mm	No activity	8mm
Pseudomonas aeruginosa ATCC 9027	20.86mm	No activity	6mm
Staphylococcus aureus ATCC 6538P	12.28mm	No activity	8mm
Escherichia coli ATCC 8739	14mm	No activity	12mm
Enterococcus faecalis ATCC 29212	26.22mm	No activity	No activity
Proteus vulgaris ATCC 6380	22.38mm	No activity	12mm
Shigella dysenteriae ATCC 13313	28.99mm	No activity	4mm
Salmonella enterica Subsp.enterica pv Typhi ATCC 29630	28.4mm	No activity	8mm
Staphylococcus epidermidis ATCC 1228	31.47mm	No activity	No activity

Fungi	Clotrimazole		
Candida albicans ATCC 2091	32.33mm	No activity	No activity
Saccharomyces cerevisiae ATCC 18824	24.35mm	No activity	No activity

Table 9: Antimicrobial activity shown by the essential oil of *A. adenophora* in diameter (mm) of the inhibition zone

Test Organism*(80µl)*	**Zone of inhibition**		
	Positive control	**Negative Control (DMSO)**	**Oil Activity**
Klebsiella pneumoniae KCTC 2242	10mm	No activity	8mm
Bacillus subtilis KACC 17047	7mm	No activity	7mm
Pseudomonas aeruginosa KACC 10232	No activity	No activity	No activity
Staphylococcussubsp.Aureus KCTC 1916	12mm	No activity	12mm
Micrococcus leutus KACC 13377	7mm	No activity	7mm
Enterobacter cloacae subsp. disolvens KACC 13002	No activity	No activity	No activity
Fungi			
Aspergillus flavus	No activity	No activity	No activity
Fusariumspp.	No activity	No activity	No activity

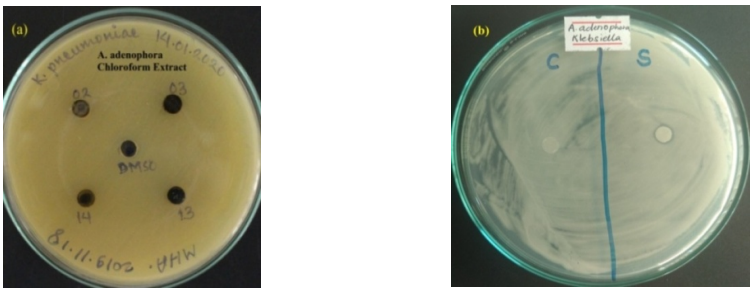


Figure 30: Antibacterial activity shown by chloroform extract (a) and essential oil (b) against *Klebsiella pneumoniae*

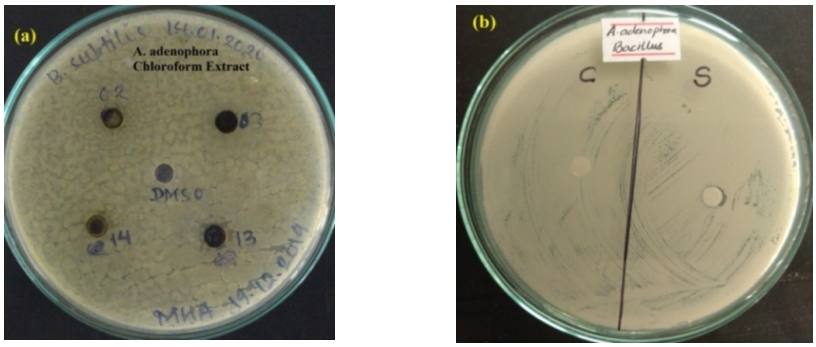


Figure 31: Antibacterial activity shown by chloroform extract (a) and essential oil (b) against *Bacillus subtilis*

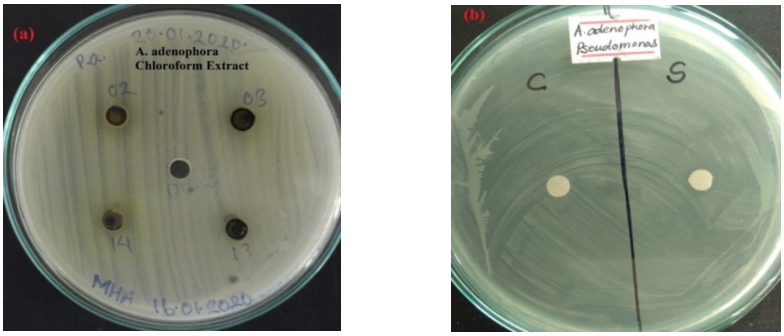


Figure 32: Antibacterial activity shown by chloroform extract (a) and essential oil (b) against *Pseudomonas aeruginosa*

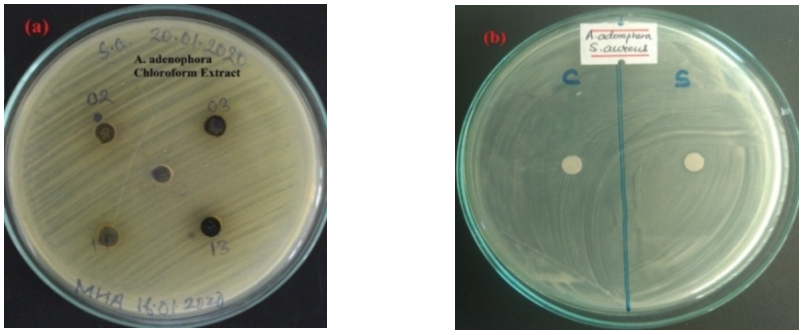


Figure 33: Antibacterial activity shown by chloroform extract (a) and essential oil (b) against *Staphylococcus aureus*

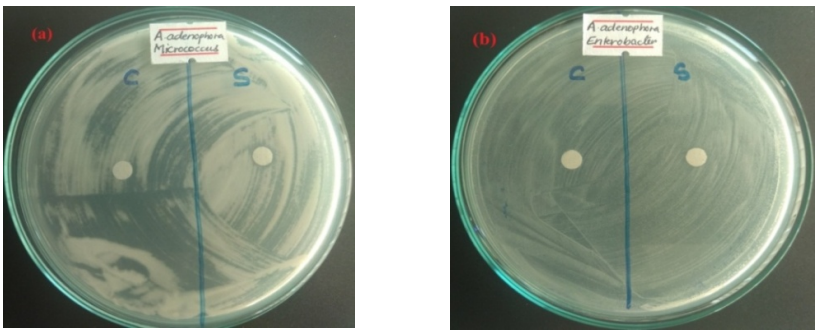


Figure 34: Antibacterial activity shown by essential oil (a) and (b) against *Micrococcus leutus and Enterobacter cloacae subsp. Dissolvens*respectively.

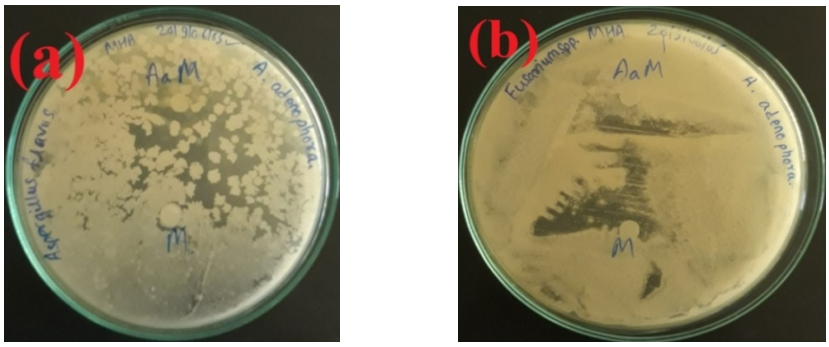


Figure 35: Antifungal activity shown by essential oil (a) and (b) against *Aspergillus flavus and Fusarium spp.* respectively.

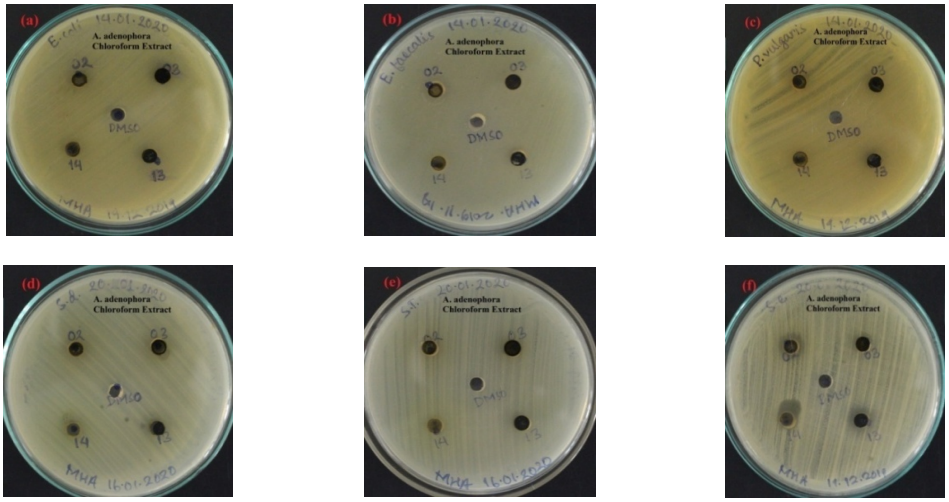


Figure 36: Antibacterial activity shown by chloroform extract (a), (b), (c), (d), (e), (f) against *Escherichia coli, Enterococcus faecalis, Proteus vulgaris, Shigella dysenteriae, Salmonella enterica subsp.enterica pv Typhi, Staphylococcus epidermidis respectively.*

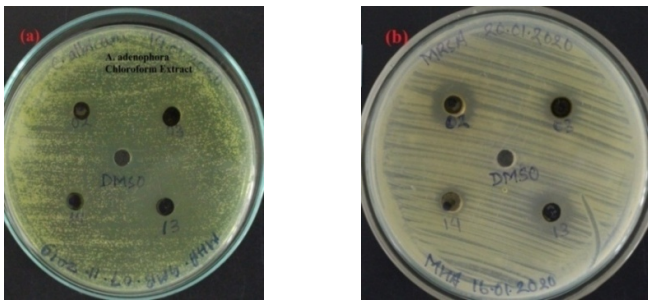


Figure 37: Antifungal activity shown by chloroform extract (a) and (b) against *Candida albicans and Saccharomyces cerevisiae* respectively.

4.6. Antioxidant Screening Analysis

The antioxidant capacity of plants is associated with the activity of "free radical scavenging enzymes" (superoxide dismutase, catalase, peroxidase, etc.) and the contents of antioxidant substances mainly phenolic compounds,

carotenoids, tocopherol, and ascorbic acid[47]. The antioxidant potential is in an inverse relation with IC_{50} value, which can be calculated from the logarithmic regression of the % inhibition versus antioxidant activity. Lower the IC_{50} value indicates high antioxidant activity. All the calculations are based on the standard method[50,124,125]. Absorbance was measured at 517 nm.

The absorbance; of each solution was measured and recorded as follows

Table 10: Antioxidant activity of Chloroform Extract of *A. adenophora* sample by DPPH method

Sample	**Conc^n (µg/mL)**	**Absorbance (nm)**			**Average Absorbance (nm)**	**Percentage Scavenged**
Control		0.913	0.928	0.928	0.923	
A. adenophora (chloroform extract)	2000	0.341	0.344	0.345	0.343	62.838
	1500	0.489	0.492	0.491	0.49	46.912
	1000	0.537	0.542	0.541	0.54	41.495
	500	0.69	0.696	0.0697	0.693	24.918
	250	0.771	0.784	0.779	0.778	15.709

Table 11: Antioxidant activity of essential oil of *A. adenophora* sample by DPPH method

Sample	**Conc^n (mg/mL)**	**Absorbance (nm)**			**Average Absorbance (nm)**	**Percentage Scavenged**
Control		0.621	0.621	0.623	0.621	
A. adenophora (Essential Oil)	2.5	0.534	0.532	0.536	0.534	14.00
	5	0.496	0.496	0.496	0.496	20.13
	10	0.401	0.4	0.402	0.401	35.43
	12	0.348	0.347	0.345	0.346	44.28
	15	0.309	0.309	0.31	0.309	50.24

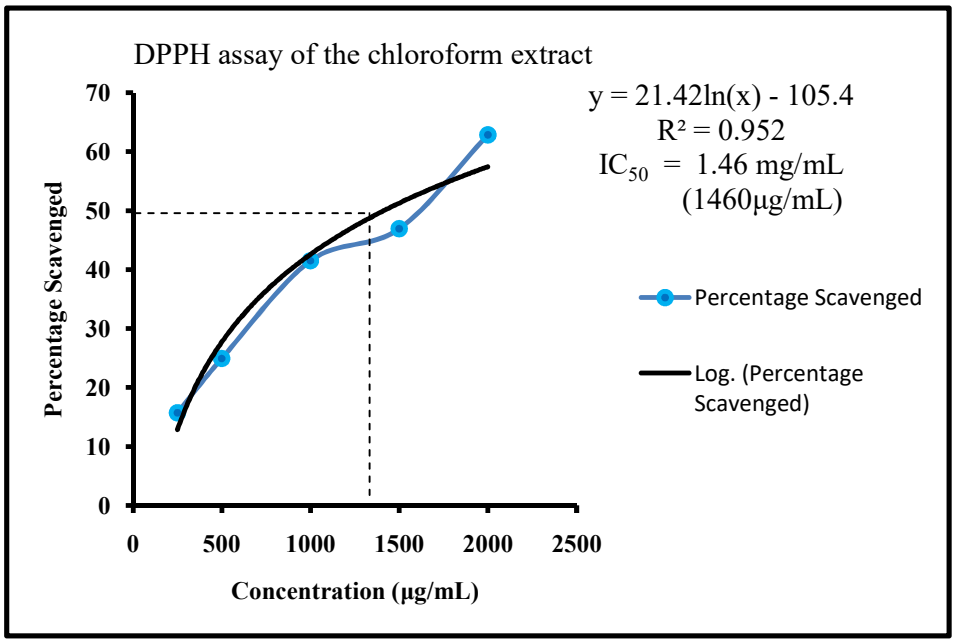


Figure 38: Graphical representation of the DPPH assay of the chloroform extract

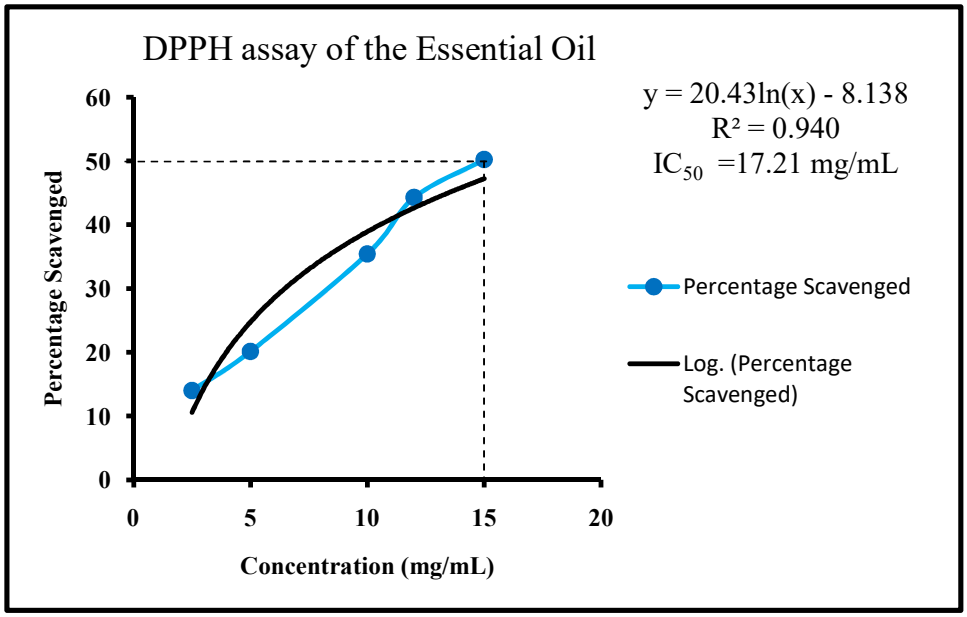


Figure 39: Graphical representation of the DPPH assay of the essential oil

The half-maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a substance inhibiting a specific biological or biochemical function. This quantitative measure indicates how much of a particular drug or other substance (inhibitor) is needed to inhibit a given biological process (or

component of a process, i.e., an enzyme, cell, cell receptor, or microorganism) by half. The value is typically expressed as molar concentration. It is commonly used as a measure of antagonist drug potency in pharmacological research. According to the FDA, IC_{50} represents the concentration of a drug that is required for 50 % inhibition *in vitro*. The IC_{50} value of chloroform extract and essential oil of *A. adenophora* was found to be 1.46 mg/mL and 17.21 mg/mL, respectively. This study shows that chloroform extract is more effective than that essential oil in the inhibition of a specific biological or biochemical function.

4.7. Total Phenolic Content Analysis

Phenolic compounds are ubiquitous secondary metabolites in plants. They are known to have antioxidant activity, and the activity of these extracts is likely due to these compounds[93]. The total phenolic content was determined as a milligram of gallic acid equivalent using the calibration curve of gallic acid.

The absorbance of each solution was measured and recorded as follows:

Table 12: Absorbance of gallic acid

Concentration (µg/mL)	**Absorbance (nm)**			**Average Absorbance (nm)**
10	0.034	0.025	0.03	0.03
20	0.117	0.125	0.11	0.117
40	0.267	0.27	0.261	0.266
60	0.412	0.462	0.415	0.429
80	0.606	0.6	0.59	0.6
100	0.8	0.787	0.78	0.789

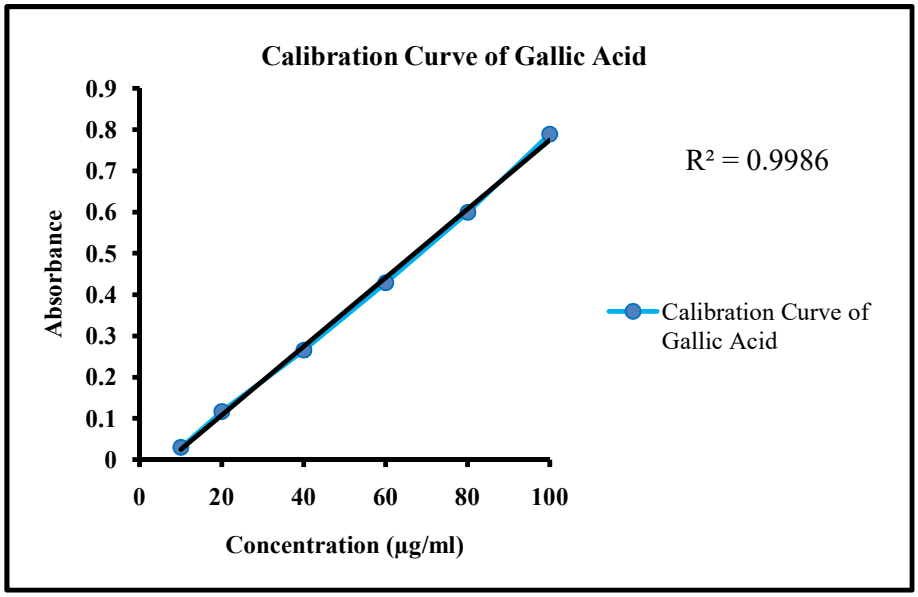


Figure 40: Calibration curve of gallic acid for total phenolic content determination

The concentration of gallic acid in chloroform extract of *A. adenophora* was determined by using an equation that was obtained from the standard gallic acid curve in figure 46. The equation is given below:

$y = 0.083x - 0.0584$

$R^2 = 0.9986$

Where, y = absorbance;

x = Gallic acid concentration (GAC) (µg/mL)

m = slope = 0.0083

c = y–intercept = -0.0584

$$x = \frac{y + 0.0584}{0.0083}$$

The total phenolic content with necessary data is shown in the following table.

Table 13: Total phenolic content in chloroform extract of *A. adenophora*

Sample Concentration (mg/mL)	**Weight of extract; per mL m (g)**	**Absorbance**	**GAC C (µg/mL)**	**GAC C (mg/mL)**	**TPC as GCE** $= \frac{c \times v}{m}$ **(mg/g)**
1	0.001	0.68	89.75	0.08975	89.75

Table 14: Total phenolic content in the essential oil of *A. adenophora*

Sample Concentration (mg/mL)	Weight of extract; per mL m (g)	Absorbance	GAC C (µg/mL)	GAC C (mg/mL)	TPC as GCE $= \frac{c \times v}{m}$ (mg/g)
1	0.001	0.385	53.421	0.05342	53.421

Based on table 13, table 14 and figure 40 the total phenolic content of chloroform extract and essential oil was tested as gallic acid equivalent by reference to a standard curve (y = 0.0083x – 0.0584, R^2 = 0.9986). The total phenolic content in the chloroform extract and essential oil of *A. adenophora was* found to be 89.75 mg gallic acid equivalent/g of dry extract; and 53.42 mg gallic acid equivalent/g of dry extract; respectively which is shown in table 15 and 16 respectively. This indicates that chloroform extract is known to possess more antioxidant activity than essential oil as the phenolic compounds are known to possess antioxidant activity.

4.8. Total Flavonoid Content Analysis

Flavonoids show a wide range of biochemical and pharmacological effects, including anti-inflammation, anti-oxidation, antithrombotic action, antiplatelet, and anti-allergic effects. They can inhibit enzymes like prostaglandin synthase, lipoxygenase, and cyclooxygenase closely related to tumorigenesis and induce detoxifying enzyme systems such as glutathione S-transferase. Quercetin inhibited oxidation and cytotoxicity of low-density lipoprotein *in vitro* and helps to reduce the risk for coronary heart disease or cancer[98]. Total flavonoid content was expressed as mg of quercetin equivalents per gram of dried sample.

Table 15: Absorbance of quercetin

Concentration (µg/mL)	Absorbance (nm)			Average Absorbance (nm)
5	0.77	0.059	0.045	0.06
10	0.135	0.118	0.114	0.122
20	0.439	0.433	0.404	0.425
40	0.99	0.976	0.914	0.96

80	1.933	1.934	1.836	1.901

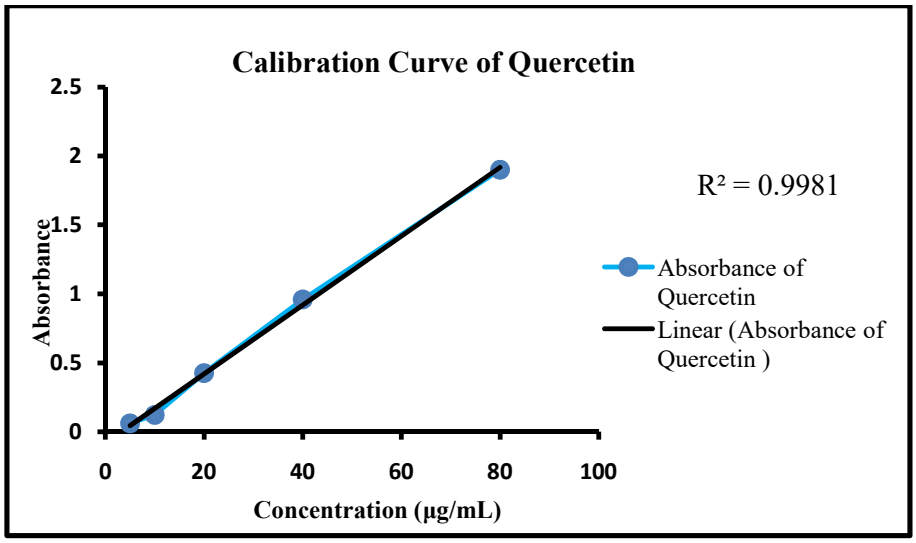


Figure 41: Calibration curve of Quercetin; for total flavonoid content determination

The concentration of quercetin; in the chloroform extract of *A. adenophora* was determined by using an equation that was obtained from the standard quercetin; curve in figure 41. The equation is given below:

$y = 0.025x - 0.0813$

$R^2 = 0.9981$

Where, y = absorbance

x = Quercetin concentration (µg/mL)

m = slope = 0.025

c = y-intercept = -0.0813

$$x = \frac{y + 0.0813}{0.025}$$

The total flavonoid content with necessary data is shown in the following table:

Table 16: Total flavonoid content in chloroform extract of *A. adenophora*

Sample Concentration (mg/mL)	**Weight of extract; per mL m (g)**	**Absorbance**	**QC, C (µg/mL)**	**QC, C (mg/mL)**	**TFC as QE $= \frac{c \times v}{m}$ (mg/g)**
1	0.001	1.109	49.252	0.049252	49.252

Table 17: Total flavonoid content in the essential oil of *A. adenophora*

Sample Concentration (mg/mL)	**Weight of extract; per mL m (g)**	**Absorbance**	**QC, C (µg/mL)**	**QC, C (mg/mL)**	**TFC as QE** $= \frac{c \times v}{m}$ **(mg/g)**
1	0.001	0.003	3.372	0.003372	3.372

Based on table 16, table 17 and figure 41 the total flavonoid content of chloroform extract and essential oil was tested as quercetin equivalent by reference to a standard curve (y = 0.025x – 0.0813, R^2 = 0.9981). The total flavonoid content in the chloroform extract and essential oil of *A. adenophora* was found to be 49.252 mg quercetin; equivalent/g of dry extract and 3.372 mg quercetin; equivalent/g of dry extract respectively which is shown in table 16 and 17 respectively. This indicates that chloroform extract is more significant than essential oil to perform biological activity.

4.9. Brine Shrimp Lethality Analysis

The lethality of a test sample in a simple zoological organism such as the shrimp (*Artemia salina*) has been utilized by Meyer *et al*. (1982) in the Brine Shrimp Cytotoxicity Test (BSCT). The brine shrimp bioassay has been established as a safe, practical, and economical method for the determination of bioactivities of the synthetic compound as well as plant products[126–129]. National Cancer Institute (NCI, USA) demonstrated the significant correlation between *in vitro* growth inhibition of human solid tumor cell lines and the Brine shrimp assay and showed the value of this bioassay as a prescreening tool for antitumor drug research[66,103]. LC_{50} values lower than 1000 µg/mL are considered bioactive in toxicity evaluation of plant extracts by Brine shrimp lethality bioassay[130].

The results of the Brine Shrimp Lethality assay are given in the table below:

Table 18: Effect of chloroform extract of *A. adenophora* in brine shrimp

Concentration (C) (µg/mL)	Log C	Percentage Mortality
800	2.903	100
400	2.602	93.19
200	2.301	82.98
100	2	28.57
50	1.698	14.95

Table 19: Effects of essential oil of *A. adenophora* in brine shrimp

Concentration (C) (µg/mL)	Log C	Percentage Mortality
500	2.698	100
250	2.397	90
125	2.096	70
62.5	1.1795	50
31.25	1.494	30
15.625	1.193	12.65

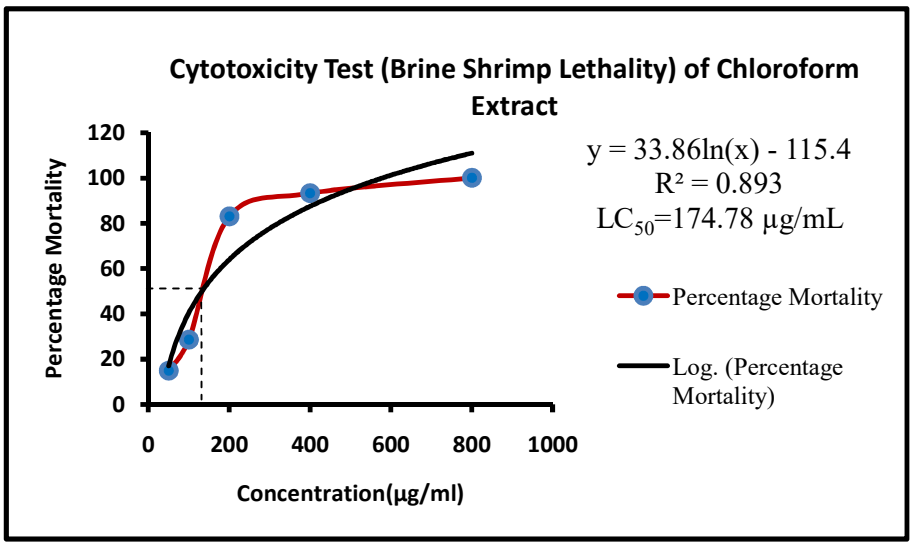


Figure 42: Plot of the concentration of chloroform extract of *A. adenophora* versus percent shrimp mortality after 24 hours of exposure

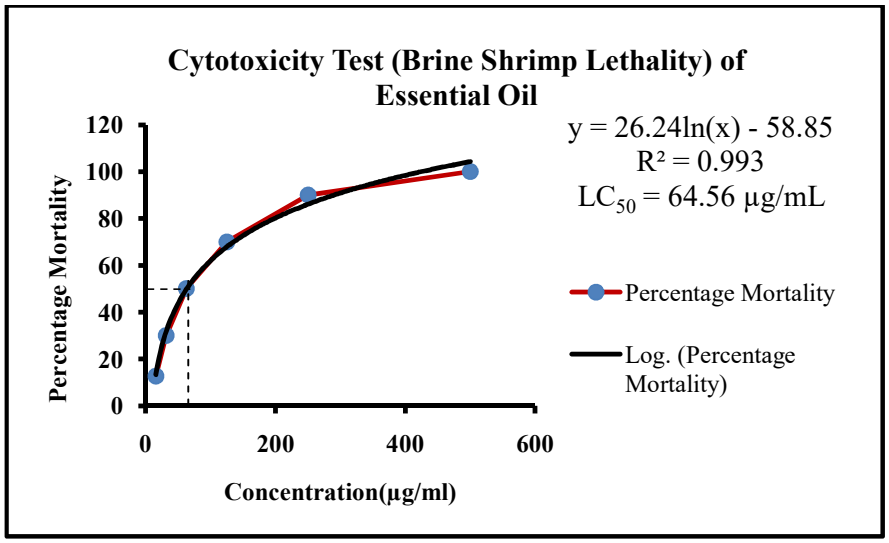


Figure 43: Plot of the concentration of essential oil of *A. adenophora* percent shrimp mortality after 24 hours of exposure

Tables 18 and 19 show the lethality of chloroform extract and essential oil of *A. adenophora* respectively to the Brine Shrimp nauplii. The degree of lethality shown by the extracts was found to be directly proportional to the concentration of the extracts ranging from the lowest concentration (50 µg/mL) to the highest concentration (800 µg/mL). Similarly, the lethality of essential oil was also found to be directly proportional to the concentration of essential oil ranging from the lowest concentration (15.625 µg/mL) to the highest concentration (500 µg/mL). This concentration-dependent increment in percent mortality of Brine Shrimp nauplii produced by the *A. adenophora* indicates the presence of cytotoxic principles in these extractives and essential oil. This study shows that the LC_{50} value of chloroform extracts and essential oil of *A. adenophora* was found to be 174.78 µg/mL and 64.56 µg/mL respectively which reveals that essential oil is more toxic than that of chloroform extract.

4.10. FTIR spectrum of chloroform extract of *A. adenophora* leaves

Table 20: FTIR peaks values and functional groups of chloroform extracts of *A. adenophora* leaves

Extracts	Absorption (Cm^{-1})	Types of Stretching	Appearance	Functional group	Frequency Range
Chloroform of *A. adenophora*	748.38	C-H bending	Strong	Monosubstitued,1,2-disubstituted	750±20
	1033.85	S=O stretching	Strong	Sulfoxide	1070-1030
	1226.73	C-O stretching	Strong	Alkyl Aryl Ether	1275-1200
	1373.32	O-H bending	Medium	Phenol	1390-1310
	1458.18	CH_2, CH_3, C–H bending	Medium	Alkanes	1480-1440
	1612.49	C=C stretching	Medium	Conjugate alkene, Cyclic alkene	1650-1600
	1728.22	C=O stretching	Strong	Aldehyde, Saturated aliphatic, α,β-unsaturated ester	1740-1720
	2854.65	C-H stretching	Medium	Alkane	3000-2840
	2916.37	C-H stretching	Strong	Carboxylic acid	3300-2500
	3371.57	N-H Stretching	Medium	Aliphatic primary (1°)amine	3400-3300

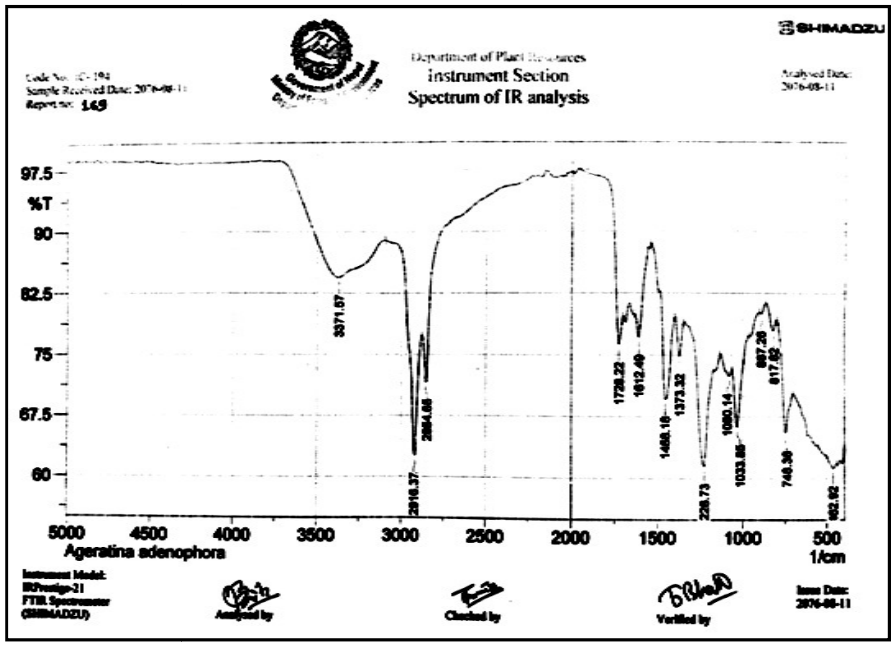


Figure 44: FTIR Spectrum analysis of chloroform extract of *A. adenophora* leaves

The FT-IR spectrum was used to identify and detect the characteristic peaks and functional groups of the active components present in the extract; based on the peak value in the region of infrared radiation. The figure shows ten

different absorption peaks at wave numbers which correspond to functional molecules of the chloroform extract of *A. adenophora* leaves Table20. The results revealed the presence of various phytochemicals which are formed during the plant's normal metabolic processes. When the chloroform extract of *A. adenophora* leaves was subjected to FT-IR analysis and the functional groups of the components were separated based on the ratio of its peak.

Results were compared using the infrared chart. The results were compared using an infrared chart of FTIR analysis to confirm the presence of functional groups such as the medium band was observed at 3371.57 cm^{-1} corresponds to N-H stretching aliphatic primary (1°) amine. The Strong band at 2916.37 cm^{-1} indicates the presence of O-H stretching Carboxylic acid. A medium band was observed at 2854.65 cm^{-1} corresponds to C-H stretching alkane. The strong band at 1728.22 cm^{-1}assigned to C=O stretching aldehyde, saturated aliphatic. The medium band at 1612.49 cm^{-1} was assigned to the conjugate alkene, cyclic alkenes. Then the medium band was observed at 1458.18 corresponding CH_2, CH_3, C–H bond alkanes. A medium band at 1373.32 cm^{-1} indicates the presence of O-H bending phenol. The Strong band at 1226.73 cm^{-1} was the corresponding C-O group alkyl aryl ether. The Strong band was observed at 1033.85 cm^{-1} is assigned to S=O stretching Sulfoxide groups, and the Strong band at 748.38 cm^{-1} indicates C-H Bending aliphatic Monosubstitued, respectively. The Strong band shown at absorption peak 748.38 cm^{-1} is designated to C-H Bending means Monosubstituted, etc. These functional groups are associated with the bioactive phytochemicals in the leaf extract.

4.11. TLC Analysis

The TLC analysis of various extracts of methanol, chloroform, hexane, ethyl acetate, and essential oil of *A. adenophora* was carried out.

Table 21: R_f values of the different solvent systems such as essential oil, methanol, hexane, and chloroform leaf crude extracts of *A. adenophora*

Different Extracts	**Solvent system Ratio(%)**	**Number of spots**	**R_f value**	**Stained TLC colour**
Essential oil	Ethyl acetate: Hexane (10:90)	9	0.56, 0.45,0.32, 0.71, 0.58,0.53, 0.50, 0.46, 0.36	Blue

Essential oil	Ethyl acetate: Hexane (30:70)	7	0.55, 0.58, 0.60, 0.62, 0.68,0.79, 0.85	Blue
Essential oil	Chloroform: Hexane (5:95)	4	0.60,0.58,0.51,0.49	Blue
Essential oil	Hexane: Ether: (10:90, 20:80, 30:70, 40:60)	3	0.58, 0.55,0.48	Blue
Methanol	Ethyl acetate: Hexane (10:90)	4	0.23, 0.35, 0.26, 0.20	Blue, Dark blue
Methanol	Ethyl acetate : Hexane (30:70)	17	0.73, 0.68, 0.65, 0.61, 0.58, 0.55, 0.52,0.45,0.42	Blue, Red, black
Methanol	Ethyl acetate: Hexane (20:80)	8	0.57, 0.55,0.50, 0.48,0.45, 0.43, 0.42, 0.39	Blue, Red, Dark blue,
Methanol	Ethyl acetate: Hexane: (80:40)	15	0.71,0.70, 0.69, 0.65,0.62, 0.59, 0.57, 0.56,0.55, 0.52,0.45, 0.42 0.39,0.37, 0.35	Blue, Dark blue, Red
Chloroform	Ethyl acetate: Hexane (20:80)	6	0.61, 0.58, 0.55, 0.45, 0.42, 0.39	Dark blue, white, Red
Chloroform	Ethyl acetate: Hexane (10:90)	4	0.43, 0.40, 0.35 0.26	Dark blue, white, Red
Hexane	Ethyl acetate: Hexane (20:80)	6	0.54, 0.52, 0.49, 0.44, 0.42, 0.40	Blue, Dark blue, Red

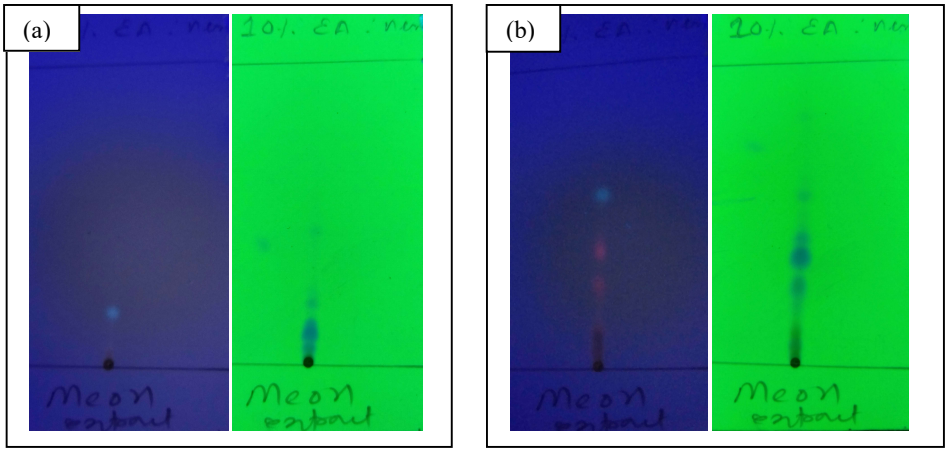


Figure 45: TLC showing spots of methanol extracts of *A. adenophora* under UV 254nm and UV 366nm (a) 10% (b) 20% ethyl acetate in hexane solvent systems

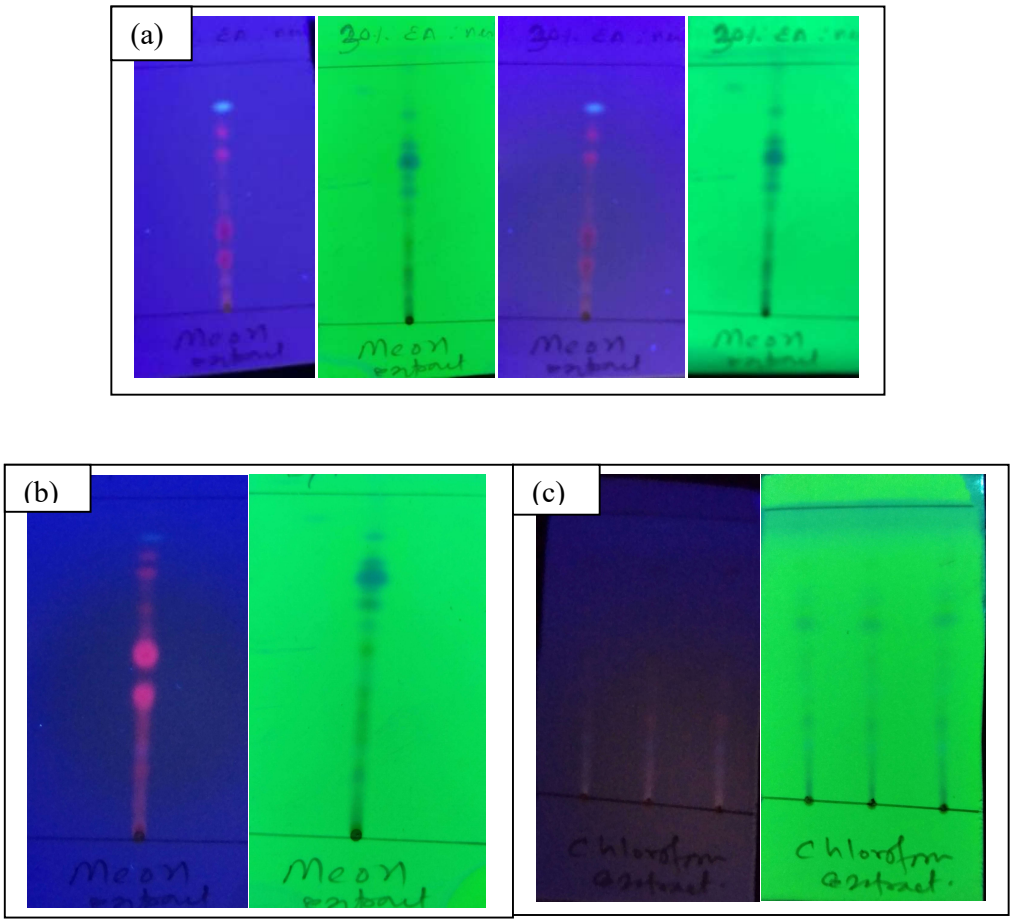


Figure 46: TLC showing spots of methanol and chloroform extracts of *A. adenophora* under UV 254nm and UV 366nm (a) 30% (b) 40% and (c) 20% ethyl acetate in hexane solvent systems

4.12. Column Chromatography of Methanol Extract of *A. adenophora* leaves.

The shade dried and powdered leaf of *A. adenophora* was extracted exhaustively with methanol by the cold percolation method. About 1 kg of the powdered plant material was used for the methanol extract in two percolators, each of 1-liter capacity. The yield obtained was approximately 8.0 g. This methanol extract was adsorbed in about 16.0 g of silica gel and loaded onto a silica gel (60-120 mesh) packed in the column having an internal diameter of 28mm with an adsorbent height of 50cm. The column was eluted with hexane at first and then with the gradual increase in polarity with ethyl acetate to obtain several fractions as shown in the figure. After receiving each fraction, TLC was checked, and with the results of TLC, the polarity of the mobile

phase was increased. The selection of solvents in a systematic order proves the effect of polarity on the extraction. About 181 fractions with different R_f values were separated during the column chromatography procedure (Table 5).

Figure 47: Column chromatography of *A. adenophora* leaves methanol extract.

Table 22: Elution with Column, TLC, and R_f values of Fractions collected from methanol leaf crude extract of *A. adenophora*.

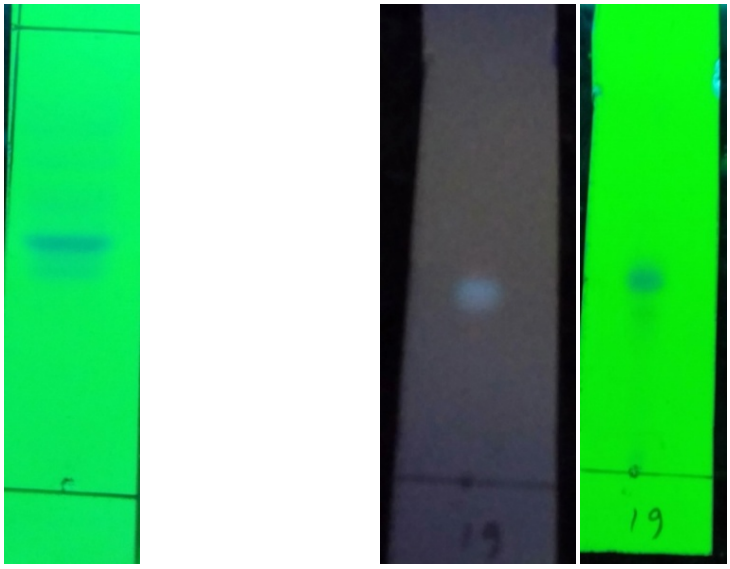
S.N	**Elution Solvent system**	**Fractions number**	**Volume of eluents (mL)**	**TLC solvent system**	**R_f values**	**TLC Report** (UV active)
1	100% Hexane	(A, B, C)	180	1% EtOAc in hexane	0	No spots
2	2% EtOAc in hexane	1-4	200	2% EtOAc in hexane	0.42, 0.44, 0.50	3 spots
3	5% EtOAc in hexane	5-9	250	5% EtOAc in hexane	0.45, 0.52,	2 spots
4	5% EtOAc in hexane	9-13	250	5% EtOAc in hexane	0.57	Single spot
5	10% EtOAc	14-19	300	10% EtOAc	0.55	Single

	in hexane			in hexane		spot
6	10% EtOAc in hexane	19-33	450	10% EtOAc in hexane	0.54, 0.56	2 sports
7	20% EtOAc in hexane	34-42	270	20% EtOAc in hexane	0.54, 0.61, 0.63	3 sports
8	20% EtOAc in hexane	43-53	440	30% EtOAc in hexane	0.62	Single spot
9	30% EtOAc in hexane	53-60	400	30% EtOAc in hexane	0.44, 0.45, 0.52, 0.56, 0.58, 0.52	6 spots
10	30% EtOAc in hexane	60-68	240	40% EtOAc in hexane	0.45, 0.45, 0.56, 0.59	4 spots
11	40% EtOAc in hexane	69-79	330	40% EtOAc in hexane	0.46, 0.46, 0.54	3 sports
12	50% EtOAc in hexane	80-93	420	50% EtOAc in hexane	0.33, 0.41, 0.50, 0.52, 0.56, 0.59, 0.59	7 spots
13	60% EtOAc in hexane	94-115	440	60% EtOAc in hexane	0.32, 0.44, 0.50, 0.54	4 spots
14	70% EtOAc in hexane	116-127	240	70% EtOAc in hexane	0	Tailing
15	80% EtOAc in hexane	128-143	320	80% EtOAc in hexane	0	Tailing
16	90% EtOAc in hexane	144-150	210	90% EtOAc in hexane	0.40, 0.45, 0.54, 0.57, 0.57	5 spots
17	100% Ethyl acetate	151-167	340	100% EtOAc in hexane	0	Tailing
18	10% Methanol	167-171	250	10% EtOAc in hexane	0	Tailing
19	100% Methanol	172-178	350	100% Methanol	0	Tailing

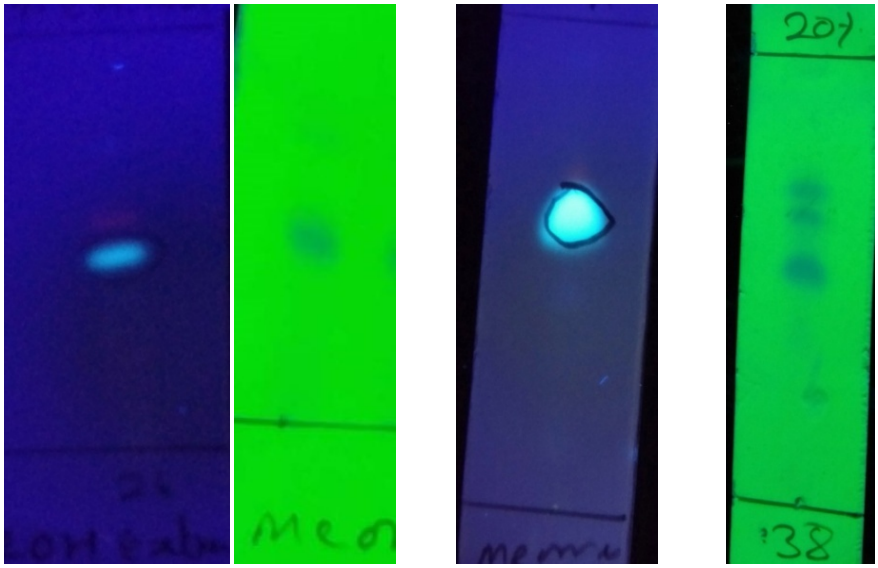
Table 23: TLC and R_f value of fractions (A, B, C, D, and E) of the different solvent systems under UV light.

Fractions	**Solvent system Ratio(%)**	**Number of spots**	**R_f value**	**Stained TLC colour**
A	Ethyl acetate: Hexane (05:95)	1	0.57	Blue
B	Ethyl acetate: Hexane (10:90)	1	0.55	Dark Blue

C	Ethyl acetate: Hexane (10:90)	2	0.54, 0.56	Blue, Dark Blue
D	Ethyl acetate: Hexane (20:80)	1	0.62	Sky Blue
E	Ethyl acetate: Hexane (20:80)	3	0.54, 0.61, 0.63	Blue, Dark Blue



Fraction A Fraction B



Fraction C Fraction D Fraction E

Figure 48: TLC plate showing the spot of fractions A, B, C, D, and E under UV 254nm and UV 366nm

CHAPTER 5: CONCLUSION AND RECOMMENDATION

The presented results revealed that leaves of *A. adenophora* from Ribdikot, Palpa district of Nepal was collected and different extracts, as well as essential oils from the collected plant materials, have been extracted. Based on the results of the study, the preliminary phytochemical screening of methanol, hexane, chloroform, and ethyl acetate extracts showed the various phytoconstituents alkaloids, flavonoids, phenols, steroids, quinones, saponins, tannins, cardiac glycosides, carbohydrate, terpenoids, proteins, and amino acid in different solvent extracts were identified.

The essential oil was isolated using the hydro distillation method and the essential oils of *A. adenophora* were analyzed by Gas chromatography-mass spectrometry (GCMS). GC-MS analysis of chloroform extract showed the presence of 10 possible compounds whereas essential oil showed 14 possible compounds and the major compound 1-napthalenol (24.56%) was found most abunadantly.

Antibacterial activities of the chloroform extract and essential oil showed antibacterial activity towards different tested bacteria using the disc diffusion method, however, antibacterial activity of both extract and oils varies according to the types of microorganism. The chemical constituents present in the chloroform extract as well as essential oils could be responsible for antibacterial susceptibility activity. The maximum zone of inhibition 12mm against *Escherichia coli, Proteus vulgari* and *Staphylococcus subsp. aureus* respectively.

The plant also contains phenols in a significant amount which acts as a powerful antioxidant. The antioxidant activity of chloroform extract and essential oils were investigated using DPPH radical scavenging method and the IC_{50} value of both was found to be 1.46 mg/mL and 17.21 mg/mL respectively. From their IC_{50} value chloroform extract was found to be more effective than that of essential oils in inhibition of specific biological or biochemical functions.

Total phenolic content (TPC) and total flavonoid content (TFC) were estimated using Folin-Ciocalteu and aluminium chloride colorimetric methods respectively. The TPC content of the essential oils and extract was found to be 53.42 mg gallic acid/g of equivalent and 89.75 mg gallic acid/g of equivalent. The TFC content in essential oil and chloroform extract was found to be 3.372 mg quercetin equivalent/g of dry extract and 49.252 mg quercetin equivalent/g of dry extract respectively.

The essential oil as well as chloroform extract also showed higher cytotoxic activity against brine shrimp with LC_{50} value of 64.56 µg/mL and 174.78 µg/mL respectively and considered as containing active components. Although Brine Shrimp Lethality Assay is insufficient in determining the mechanism of action of the bioactive substances in the plant, it is very useful by providing a preliminary screen that can be supported by a more specific bioassay, once the active compound has been isolated. Thus, some useful drugs of therapeutic importance may develop out of the research work. This study on *A. adenophora* also recommends further extensive study on effective, safe, cheap, and toxic drug formulations, which would only increase value to our resources but also generates a rational approach to exploit our resources.

In FTIR analysis of Chloroform leaf extracts of *A. adenophora* studies revealed the presence of these functional groups: 1° amine (N-H), the carboxylic acid (O-H), alkane (C-H), aldehyde (-CHO), saturated aliphatic (C=O), Conjugate alkene, cyclic alkene (C=C), alkanes (CH_2, CH_3, C–H), Phenol (O-H), Alkyl Aryl Ether (C-O), Sulfoxide (S=O), and monosubstituted (C-H).

For the isolation and identification of factors in addition evaluation had been carried out. Thin layer Chromatography used to be carried out in methanol, hexane, chloroform and ethyl acetate extract in the extraordinary solvent system. The excellent TLC was once confirmed with the aid of methanol extract in the hexane and ethyl acetate solvent system. Thus, column chromatography used to be run by way of hexane and ethyl acetate in unique concentration for the methanol extract. Around 181 fractions with a different solvent run ratio of hexane: EtOAc (9:1, 8:2, 6:4, 5:5, 4:6, 2:8) were collected

sequentially and recorded with different R_f values. Similarly, A single spot was seen in the fractions at 5%, 10%, and 20% ethyl acetate in hexane.

Further *in vitro* study to determine detailed essential oil composition and antibacterial activity of this plant on different microorganisms might be helpful to discover a new class of antibiotic agents that could serve as a selective substance for infections, chemotherapy, and control of the various diseases. This approach may open a new point of view to the possibility of the usage of plants in drug development for further utilization. The more significant antibacterial activity of chloroform extract and essential oil may be due to the presence of flavonoids may be due to the combined effect of glycoside, saponin, alkaloid, tannin, and flavonoid. However, this claim demands a further study of isolation of individual components and observing their effect in the protection against the various bacterial and fungal organisms.

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APPENDICES

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 volume 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 in the solution and stirred properly.

Hager's Reagent: 6 g of picric acid was dissolved in 100 mL of hot water to form a saturated solution.

Molish's Reagent: 1 g of 1-Naphthol, 6 g of sodium hydroxide, and 16 g of sodium carbonate were dissolved in 100 mL of water and stirred.

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 hot distilled water.

Solution II: 4.5 g of CuSO4 was dissolved in 25 mL water.

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

Finally, 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 tartarate and 77 g of sodium hydroxide were dissolved in water to produce a 500 mL solution.
Finally, an equal volume of solutions I and II were mixed to prepare Fehling's solution.

Gelatin Solution (1%): 1 g of gelatin was dissolved in 100 mL of hot water.

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 a dilute sulfuric acid solution.

Concentrated Hydrochloric Acid: 36 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 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 100 mL volumetric flask and diluted to the mark by adding distilled water.

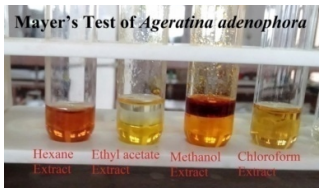
Hydrodistillation process by Clevenger apparatus and essential oil of *A. adenophora*

Grinding the leaf of *A. adenophora*

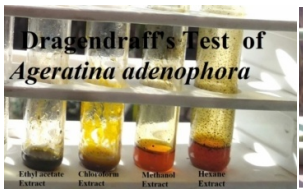
Cold percolation in methanol extract crude methanol extract by rota vapor

Collected Methanol Extracts

Test results for alkaloids



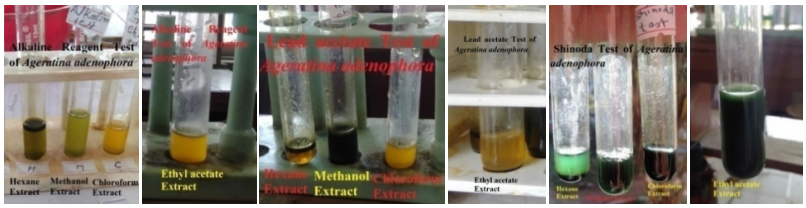
Mayer's Test



Dragendorff's Test

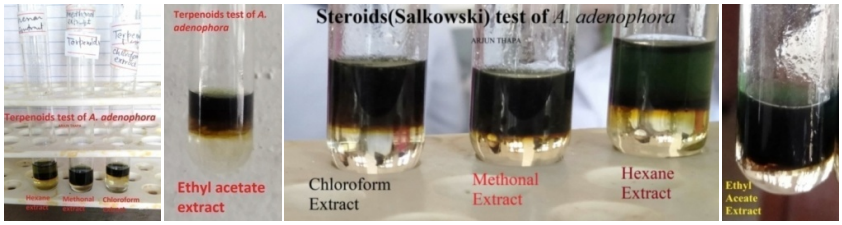
Wagner's Test

Test for Flavonoids



Alkaline Reagent Test **Lead acetate Test** **Shinoda Tests**

Test results for Terpenoids **Test of Steroids (Salkowski test)**



Test for Phenols **Test for Saponin**

Ferric Chloride Test

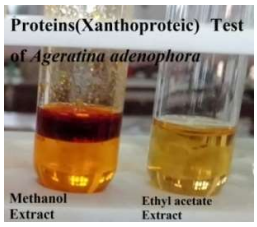
Test for Quinone **Test for Tannins**

Gelatin Test

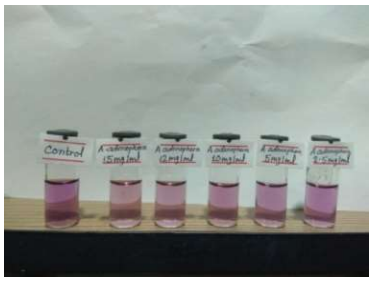
Test for Carbohydrates

Fehling's Test **Molisch's Test**

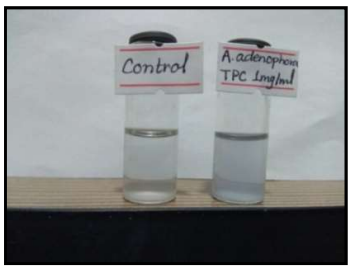
Test of proteins

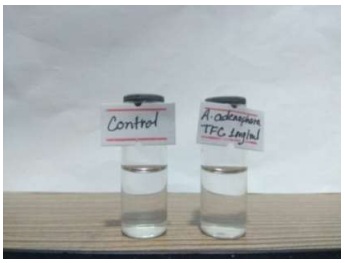


Xanthoproteic Test **Soxhlet extraction**



Antioxidant activity of essential oil and chloroform extract by DPPH Assay





TPC and TFC of essential oil and chloroform extract

Column Chromatography and different fraction

Research activities in Amrit Campus Laboratory