PHYTOCHEMICAL SCREENING, GC-MS ANALYSIS, ANTIOXIDANT ACTIVITY, AND FTIR ANALYSIS OF *Catharanthus roseus* (L.) G. Don

A DISSERTATION

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

BY

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October 2021

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This dissertation is entitled "**Phytochemical Screening, GC-MS Analysis, Antioxidant Activity and FTIR Analysis of *Catharanthus roseus* (L.) G. Don**" by Rakesh Kumar Yadav, under the supervision of Asst. Prof. Dr. R.L. (Swagat) Shrestha Department of Chemistry, Amrit Campus is hereby submitted for the partial fulfillment of the Master of Science (M.Sc.) Degree in Chemistry. This dissertation has been accepted for the award of a master's degree.

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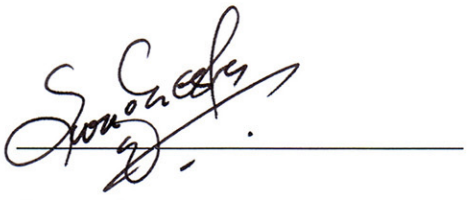
RECOMMENDATION LETTER

This is to recommend that **Mr. Rakesh Kumar Yadav** has carried out dissertation work entitled "**Phytochemical Screening, GC-MS Analysis, Antioxidant Activity and FTIR Analysis of *Catharanthus roseus* (L.) G. Don**" for the partial fulfillment for the requirement of 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, Lainchour for the submission of the dissertation for the partial fulfillment of the requirement for the Master of Science Degree in Chemistry.



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DECLARATION

I, **Rakesh Kumar Yadav**, hereby declare that the dissertation entitled "**Phytochemical Screening, GC-MS Analysis, Antioxidant Activity and FTIR Analysis of *Catharanthus roseus* (L.) G. Don**" 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.

Rakesh

Rakesh Kumar Yadav

October 2021

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Rakesh Kumar Yadav

LIST OF ACRONYMS AND ABREVIATIONS

µg	Microgram
µL	Microliter
AgNPs	Silver nanoparticles
C	Concentration
Cm	Centimeter
CO_2	Carbondioxide
Conc.	Concentrated
$CuSO_4$	Copper sulphate
DMSO	Dimethyl Sulfoxide
DPPH	2,2-diphenyl -1- picrylhydrazyl
EDX	Energy- Dispersive X-ray
$FeCl_3$	Ferric Chloride
$FeCl_3.6H_2o$	Ferric Chloride Hexa Hydrate
FTIR	Fourier Transform Infrared
Fw	Fresh weight
G	Gram
GC-MS	Gas Chromatography- Mass Spectrometry
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
$HgCl_2$	Mercuric Chloride
IC_{50}	Inhibitory Concentration of drug for killing 50% cells
IR	Infrared
Kg	Kilogram
KI	Potassium Iodide
m	Meter
M	Molarity
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
N	Normality

Na_2CO_3	Sodium Carbonate
NaOH	Sodium Hydroxide
NH_4OH	Ammonium Hydroxide
NMR	Nuclear Magnetic Resonance
rpm	Rotation per minute
RSA	Radical Scavenging Activity
Sec	Second
Sp.	*Species*
UV	Ultra-Violet
XRD	X- ray diffraction
$ZnCl_2$	Zinc Chloride
ZOI	Zone of Inhibition

LIST OF SYMBOLS

°C	Degree Celsius
A	Alpha
β	Beta
µ	Mu
%	percentage

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ABSTRACT

Cathranthus roseus (Family Apocynaceae), commonly known as periwinkle, is widely distributed in many tropical and subtropical regions worldwide. It is commonly found in mountains and the Terai region of Nepal. *C. roseus* which is named on the basis of their flower colour that is the pink flowered "Rosea" and the white flowers "Alba". It is ever green shrub with 1 m tall, erect and short stem, green hair-less leaves. It bears pink flowers with non-edible fruits. It is used traditionally for the treatment of different disease like diabetes, tranquilizer, blood pressure etc. The objective of this research is to study the phytochemical analysis of methanol extract, hexane extract and chloroform extract and to study antioxidant properties, antibacterial activity, GC-MS analysis, and FTIR analysis of chloroform extract of *C. roseus*. Whole plants of fresh *C. roseus* were collected from Balwa, Mahottari, Nepal. The collected plant materials were shaded dried and subjected to cold percolation extraction process. The *C. roseus* plants were extracted using organic solvents Methanol, Hexane, and Chloroform consecutively. Qualitative phytochemical analysis of methanol and chloroform extracts of *C. roseus* plant showed the presence of alkaloids, tannins, proteins, phenols, and flavonoids. GC-MS analysis of crude chloroform extract showed 7 different major compounds such as diethyl phthalate (40.96%), cis-9-hexadecenal (10.78%), n- hexadecanoic acid(9.28%) etc. Free radical scavenging activity was evaluated using 2,2- diphenyl- 1-picrylhydrazyl (DPPH) free radical method. The IC_{50} value of the chloroform extract was found to be 1391.74 µg/mL. Antibacterial tests against different pathogens did not show any activity. FTIR analysis of crude chloroform extract showed the presence of hydroxyl functional (-OH), methyl ($-CH_3$) asymmetric bond, (-C-H) symmetric bond, the simple carbonyl functional group, and Alkenyl (-C=C-) stretched bonds.

Keywords: *Catharanthus roseus*, Phytochemical Screening, Antioxidant, Antimicrobial, GC-MS, FTIR

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CHAPTER 1: INTRODUCTION

1.1. About Nepal

Nepal is a hilly country in the central Himalayas that stretches for roughly a third of the length of the Himalayan mountain range (800 km). Eight of the world's top ten highest mountains are found in Nepal, including Mount Everest (8,848.86 m). In addition to mountains, steep gorges, river basins, and flat regions, it offers a unique mix of incredibly different habitats and significant biodiversity within a tiny geographical area. Despite the fact that Nepal's area 147516 km^2 account for less than 0.1 percent of the world's geographical area, it is home to a disproportionately diversified array of flora and animals.

Nepal's vast biodiversity is a result of the country's unique geographical location, as well as altitude and climatic changes. Nepal lies at the crossroads of two biogeographical realms: the Palaearctic to the north and the Palaeotropic to the south. The country is encircled by six Asian floristic provinces and is situated at the meeting point of the west and east Himalayan floristic provinces (Kindlmann, 2012).

1.2 Medicinal plants in Nepal

Because the phyto-compounds present in plant extracts have no negative effects, herbal medications are safer than manufactured drugs. Medicinal herbs have long been utilized to cure and prevent a variety of ailments around the world, particularly in developing countries (Shoba et al., 2018).

Natural ingredients derived from plants can come from any part of the plant, including the bark, leaves, flowers, roots, fruits, seeds, and so on. The therapeutic characteristics of plants that are unique to specific plant species or groups are congruent with the idea that a plant's secondary products are taxonomically distinct. There is an increasing understanding of the relationship between a medicinal plant's phytochemical elements and its pharmacological activity. A number of pharmacological properties of this plant, including anti-inflammatory, diuretic, expectorant, hepatoprotective, and nephroprotective effects (Shoba et al., 2018).

Medicinal plants are responsible for maintaining the health of about 70-80 % of 23.1 million people in Nepal. There is a discrepancy in the reports of the total number of medicinal plants in Nepal. A total of 1950 species of medicinal plants are found in Nepal and, out of this list 1906 species were represented by vascular groups (angiosperms, gymnosperms and, pteridophytes). There are more than 1064 ethnobotanical studies related to documentation population biology experimental bioassays chemical screening etc. of medicinal plants from Nepal. Apart from the above-mentioned studies, it is important to describe distribution patterns of medicinal plants along the elevational gradient in Nepal. Understanding an elevational distribution of medicinal plants, uses and, their types may help in detecting the hot-spots of resources in the region and thus contribute to their effective protection (Rokaya et al., 2012).

1.3 About *Catharanthus roseus* (L.) G. Don

Catharanthus roseus (L.) G. Don (*C. roseus*) is one of the most important medicinal plants from an Apocynaceae family. *C. roseus* is also known by the name *Vinca rosea*, *Ammocallis roseus*, and *Lovhnera roseus.* The common name of this plant is based on the color of their flower, i.e. pink flower "Rosea" and the white flower "Alba". other English names occasionally used for the plant include Cape Periwinkle, Rose Periwinkle, Rosy Periwinkle, and "Old Maid".

Catharanthus roseus which is proudly known as the Madagascar periwinkle is found to be a species of Catharanthus native and also endemic to Madagascar. *C. roseus* is rich in alkaloids which are useful in diabetes, blood pressure, asthma, constipation, menstrual problems, and mostly in cancer treatment. (Santhosh Aruna et al., 2015)

Figure 1*: Catharanthus roseus*

1.4 Morphology of *C. roseus*

C. roseus is an ever-green herb growing up to 1 meter tall. The leaves are hairless, oval and 2.5 - 9.0 cm long and, 1 - 3.5 cm board. It has a short petiole of about 1- 1.8 cm long arranged in opposite pairs. The color of the flower varies from white to dark pink with a dark red center, having a basal tube about 2.5 - 3 cm long and about 2 – 5 cm long in diameter, corolla with five petals like lobes. Fruit is a pair of follicles about 2 – 3 cm long and 3mm diameter (Santhosh Aruna et al., 2015).

1.5 Geographical Distribution

Catharanthus roseus is a flowering plant native to Madagascar, an island in the Indian Ocean. It is an endangered plant in the world, and habitat destruction by slash and burns agriculture is the main cause of its decline. However, it is now abundant in many tropical and subtropical locations around the world, including the Southern United States. (Santhosh Aruna et al., 2015).

1.6 Scientific classification

Kingdom: Plantae

Division: Magnoliophyta

Class: Manoliopsida

Order: Gentianales

Family: Apocynaceae

Genus: *Catharanthus*

Species: *C.roseus*

Binomial name: *Catharanthus roseus* (L.) G. Don

1.7 Common name of *Catharanthus roseus*

Nepali: Barhamase

English: Periwinkle, old maid

1.8 Medicinal use of *Catharanthus roseus*

C. roseus was identified in Europe during the mid-1700s. It was cultivated for ornamental purposes. This plant has been used for treating various diseases. Some of them are described in the following figure:

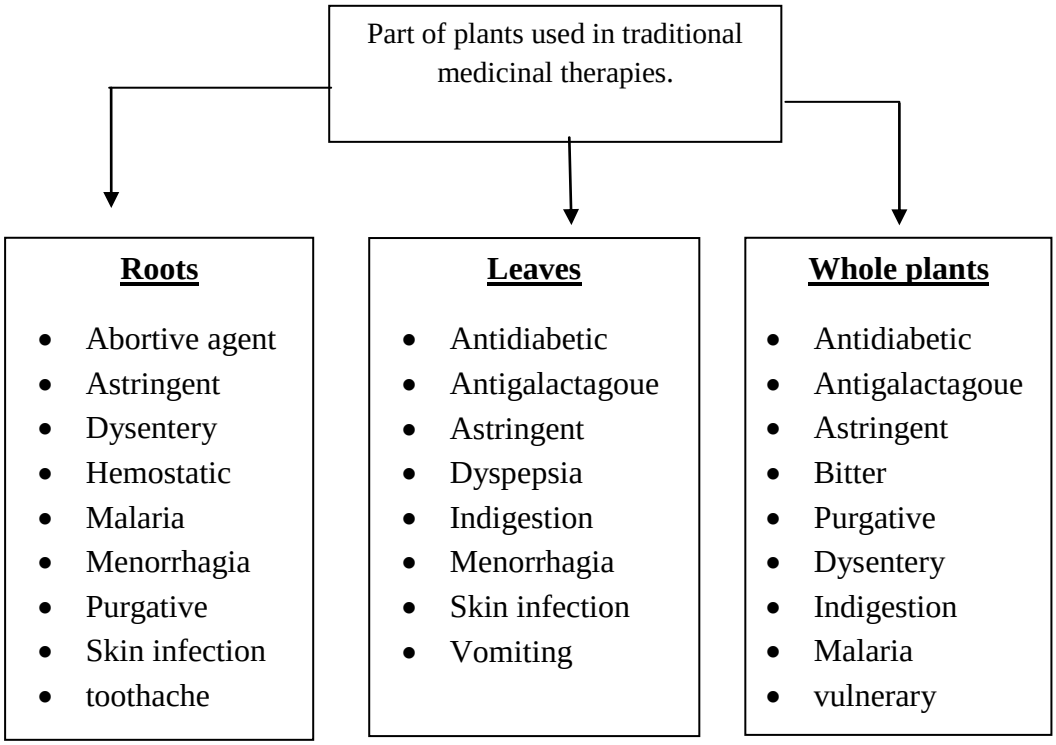


Figure 2: Medicinal uses of *C.roseus*

It is used to treat many fatal diseases as it contains useful alkaloids, used in diabetes, blood pressure, asthma, constipation, cancer, and menstrual problem (Santhosh Aruna et al., 2015).

For hundreds of years, *C. roseus* extracts have been used to treat diabetes, as hemostatics and tranquilizers, to decrease blood pressure, and as disinfectants. Hair loss was a side effect of utilizing the extracts. The juice from the plant's leaves was used to treat wasp stings in India. The herb was boiled and used to stop bleeding in Hawaii. It is used as an astringent, a diuretic, and a cough treatment in China. It was used to treat lung congestion, inflammation, and sore throats in Central and South America. Flower extract is used to treat eye discomfort and infections in the Caribbean (Arora et al., 2010).

1.8 Objective of the study

The objectives of this study are as follows:

1.8.1 General Objectives

- To find out the phytochemical constituent and medicinal value of plant extract of *C. roseus*

1.8.2 Specific objectives

- To extract constituents in the plant by using different solvents (Methanol, Hexane, and Chloroform)
- To perform phytochemical screening of extracts
- To conduct GC-MS Chromatography and study the GC- MS chromatogram
- To study the antimicrobial activity of the plant
- To study the antioxidant activity of the plant by using DPPH free radical scavenging assay.
- To study the FTIR spectrum

CHAPTER 2: LITERATURE REVIEW

2.1 Hisory of *C. roseus*

In 1910, Peckolt recorded the usage of a leaf infusion in Brazil to treat bleeding and scurvy, as a mouthwash for toothaches, and to heal and clean chronic wounds. Related species have been utilized in Europe for the proprietary reduction of milk flow. It has been used to treat diabetic ulcers in the British West Indies, and it has been described as an effective oral hypoglycemic medication in the Philippines. Recently discovered that whole alkaloids have limited antibacterial activity as well as a considerable and long-lasting hypotensive effect. Although one of the hypoglycemic and antibacterial actions has been validated, the other has not (Santhosh Aruna et al., 2015).

2.2 Chemical constituents of *C. roseus*

Phytochemical screening of the ethanolic extract of *C. roseus* leaves revealed that the leaf extract contains alkaloids, Terpenoids, flavonoids, tannins, saponins, protein and carbohydrate, Terpenes or terpenoids(Shoba et al., 2018).

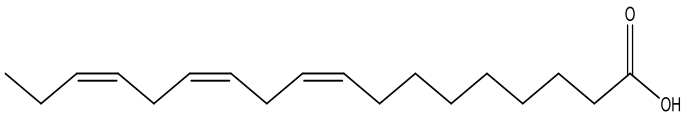
More than 400 alkaloids are present in the plant, which are used as pharmaceuticals, agrochemicals, flavor and fragrance, ingredients, food additives and pesticides. The alkaloids like actineo plastidemeric, Vinblastin, Vincrestine, Vindesine, Vindeline Tabersonine etc. are mainly present in aerial parts whereas ajmalicine, vinceine, vineamine, raubasin, reserpine, catharanthine etc are present in roots and basal stem. Rosindin is an anthocyanin pigment found in the flower of *C. roseus* (Santhosh Aruna et al., 2015).

Studies showed that the presence of the chemical constituents in *C. roseus* leaf extract is responsible for the synergic mosquito larvicidal action. Ethanolic extract showed the presence of fifteen different compounds and four of them are N- Hexadecanoic acid, Methyl 7,111,14 eicosatrienoate, Hexatriacontane and, Vitamin E which possess different pharmacological activities (Shoba et al., 2018).

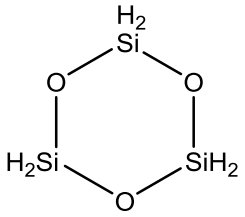
Carbohydrate, flavonoids, saponin, and alkaloids are all present in *C. roseus*. *C. roseus* contains the most potentially active chemical elements, alkaloids. The plant contains about 400 alkaloids, which are utilized as medications, agrochemicals, flavor and fragrance, food additives, and pesticides. Actineo plastidemeric, Vinblastin, Vincrestine, Vindesine, Vindeline Tabersonine, and other alkaloids are mostly found in the aerial sections, whereas ajmalicine, vinceine, vineamine, raubasin, reserpine, catharanthine, and other alkaloids are mostly found in the roots and basal stem. The anthocyanin pigment rosindin is present in the flower of *C. roseus* (Santhosh Aruna et al., 2015).

From the ethanolic leaf extract of *C. roseus* different compounds were detected. The compound N-Hexadecanoic acid showed Anti-inflammatory, Rheumatic symptoms, Methyl 7,11,14 icosatrienoate showed Anti-inflammatory, Hentriacontane showed antifungal, Anti- inflammatory activity and Vitamin E present showed Antioxidant and Anti-inflammatory (Das et al., 2017).

The ethanolic leaves extract of *C. roseus* showed 15 peaks indicating the presence of 15 compounds. GC-MS analysis revealed that the presence of Dodecanedioic acid, Bis (Trimethylsilyl) ester (17.299), Methyl-19-methyl-Eicosanate (18.140), N- Hexadecanoic acid (18.575), (1S,15S)-Bicyclo (13.1.0) Hexadecan-2-one (19.600), Methyl 7,11,14 Eicosatrienoate (19.665), Alpha-Linolenic acid, trimethylsilyl ester (20.371), 1-Methylene, 2B-hydroxymerthyl-3, 3-Dimethyl- 4B-(3-methylbut-2-ethyl)-cyclohexane (20.441), Alpha linolenic acid, trimethylsilyl ester (20.601), Hentriacontane (22.191), Sulfurous acid, octadecyl 2-Propyl ester (22.972), butyl tridecyl ester (23.702), Vitamin E (26.783), Octasiloxane, 1, 1, 3, 3, 5, 5, 7, 7, 9, 9, 11, 11, 13, 13, 15, 15- hexadecamethyl (30.360), Cyclotrisiloxane, Hexamethyl (30.665) (Shoba et al., 2018).



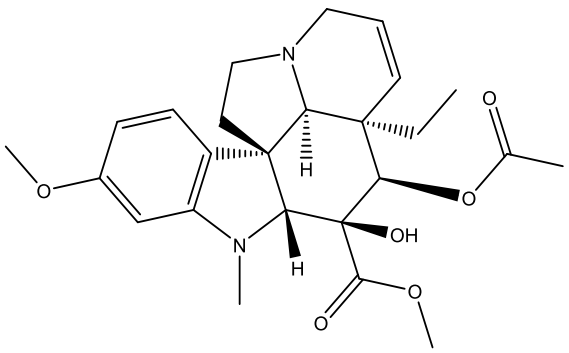
Alpha-Linolenic acid



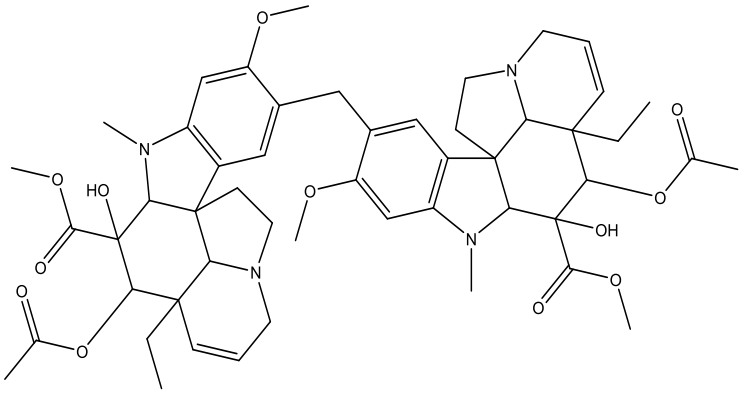
Cyclotrisiloxane

The alkaloids isolated from *C. roseus* that were vindoline, vindolidine, vindolicine, and vindolinine induced relatively high glucose uptake in β- T C_6 or C_2C_{12} cells at low dosages, these alkaloids have good antioxidant potential by alleviating H_2O_2 induced oxidative damage in β- TC6 cells. Vindolicine alkaloids showed potent activity in PTP- 1B inhibition which supports that these alkaloids as a novel one. PTP- 1B inhibitor that may serve as "Insulin sensitizer" management of type 2 diabetes (Tiong et al., 2013).

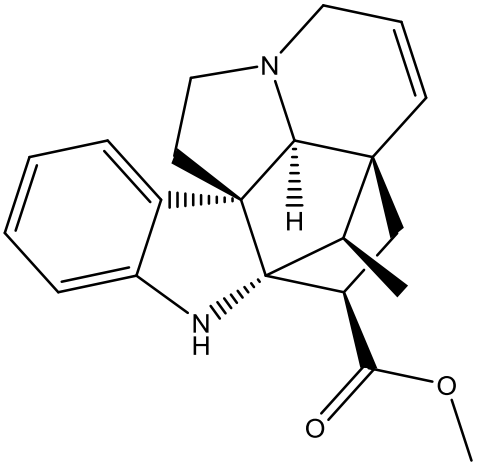
CuO Nps can also be synthesized from the leaf of *C. roseus* by green synthesis. This synthesis is applicable to control the size of nanoparticles (20-30nm) rod-like structure. Leaf extract of *C. roseus* helps to control the morphology and size of the products. The structure of Nps was crystalline with a monocyclic structure explained by XRD analysis. It is also applicable in the development of new combination semiconductors metal oxide nanocomposites (Anbuvannan Mari et al ., 2020).



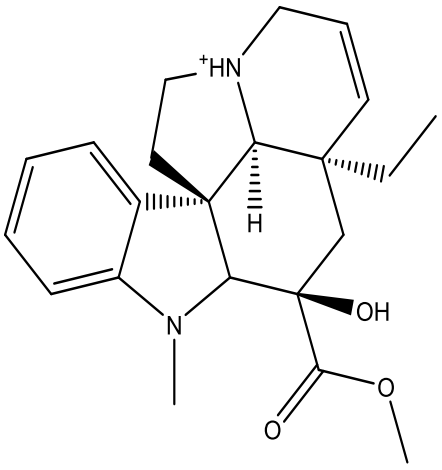
Vindoline



Vindolicine



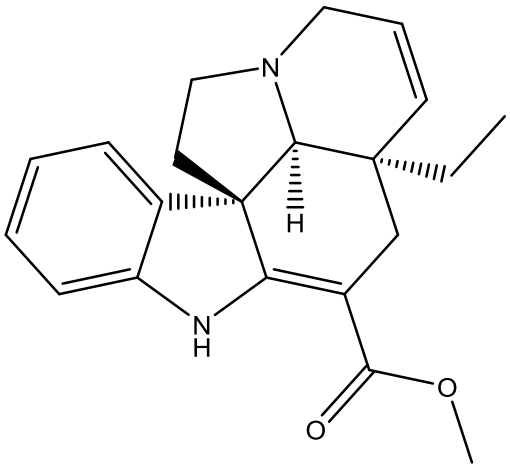
Vindolinine



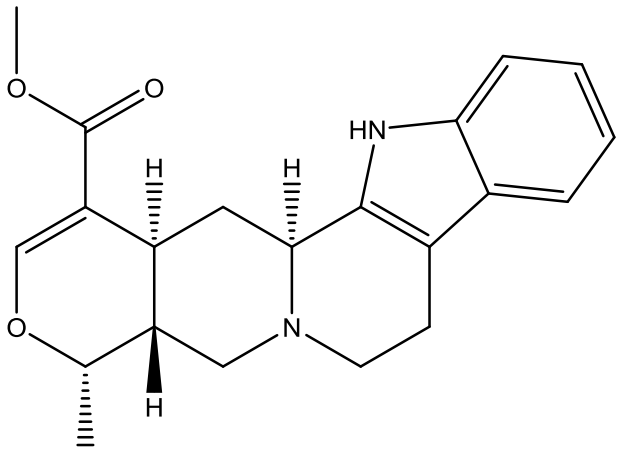
Vindolidine

The use of elicitors to activate genes involved in the TIA metabolic pathway is good to increase the biotechnological production of antitumor TIAS compounds. Elicitors such as MeJA and CDS supplying the medium with or

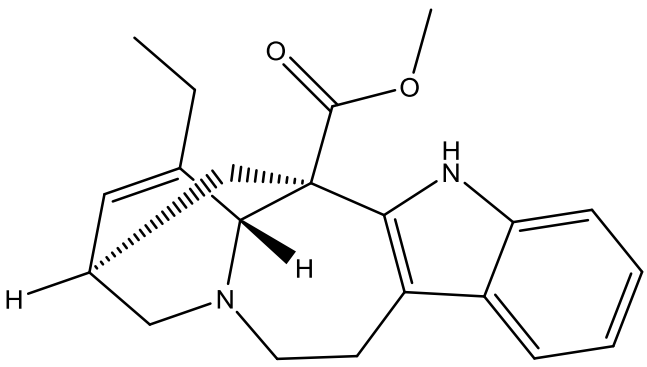
without the help of UV light, induce an important reprogramming of gene expression in *C. roseus* cell cultures that result in the high production of some TIAS, specially tabersonine, ajmalicine and, catharathine (Almagro et al., 2015).



Tabersonine



ajmalicine



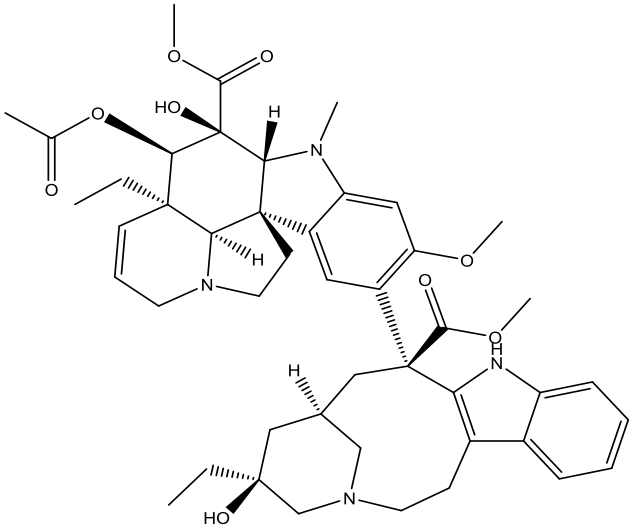
catharanthine

The plant surface has been well characterized for a diversity of their small molecules chemistry that usually contain fatty acid, terpenes and, phenols. The alkaloids were found in all of the *C. roseus* on their leaf surface. The presence of such alkaloids on the plant surface chemistry that includes MIAS may expand the range of metabolites with the biological activity present on the surface that is likely to protect plants against the majority of animals and insects (Yu & De Luca, 2013).

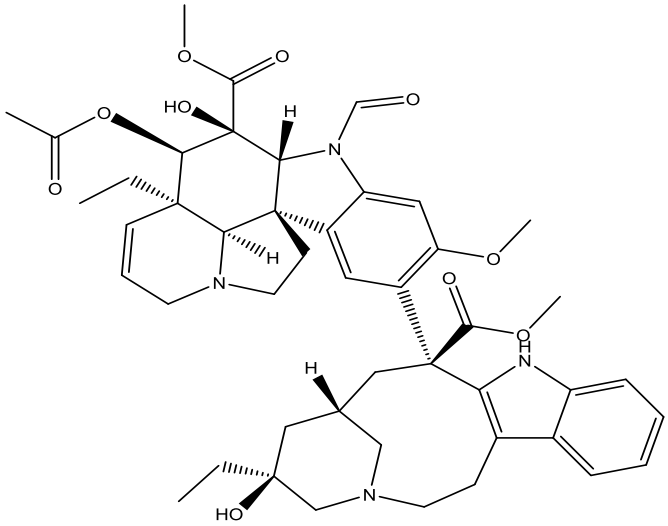
The addition of $CaCl_2$ to drought-stressed *C. roseus* plants appears to play a key role in the partial relief of water shortage stress. Thus, it is obvious that components of osmoregulation and secondary metabolite concentration are highly controlled in plants under water stress, and that these components can be affected by $CaCl_2$ treatment (C. Abdul Jaleel et al., 2007)

Vindoline biosynthesis is boosted in seedlings by light, but not by NMT activity. Light boosts the activity of the cytoplasmic enzymes 4-hydroxylase and DAT by 6- and 10-fold in seedlings, respectively; NMT activity is only increased by 30%. 17 Because our roots were grown in the dark, the vindoline levels in this study may reflect a starting point for vindoline production at later phases. Plant tissue culture-based production of catharanthine and vindoline, followed by catalytic coupling in vitro, could be a viable alternative to tissue culture-based production of vinblastine and vincristine(Bhadra et al., 1993).

Antineoplastic alkaloids like Vinblastin and Vincristine were isolated from *C. roseus* by the use of liquid column chromatography. In this chromatography silica gel and aluminium oxide were used 1:1 ratio and the charcoal column was finally purified by centrifugally accelerated radial chromatography(Shams et al., 2009).



vinblastin



Vincristine

2.3 Biological Activities *C. roseus*

In comparison to placebo controls, an ethanol extract of *C. roseus* flower exhibits features that make it capable of encouraging rapid wound healing activity. Wound contraction, enhanced tensile strength, higher hydroxyproline content, and antibacterial activity all support the use of *C. roseus* as a topical wound therapy (Nayak & Pinto Pereira, 2006).

C. roseus was investigated from ancient times for its phytochemical components and their therapeutic effect. The plants contain enormous phytochemical constituents of various medicinal applications. These plants

have medicinal properties like anti-cancerous, anti-diabetic, antihelmintic, anti-diarrheal, anti-microbial properties, etc. (Sain & Sharma, 2013)

The effectiveness of *C. roseus* on various diseases is scientifically proven. It contains more than 130 alkaloids, some of which successfully marked for cancer treatment like Vinblastin and Vincristine. The *C. roseus* contains alkaloids like Vinacula also used for the treatment of diabetes patients. Vasodilatory alkaloids are known for analgesic treatment. Ajmalicine and serpentine alkaloids found in the root of *C. roseus* are effective in cardiovascular diseases. Uses of *C. roseus* are wide in a traditional way in many countries, many of them proven scientifically by bioactive analysis of compounds (Barik et al., 2016).

Ethyl acetate extract of *C. roseus* has the best antibiogram due to the high solubility of active compound varieties with ethyl acetate during the extraction process. Root, stem, leaf and, flower extract with different solvents show best antimicrobial activity towards *B. subtilis* followed by *Klebsiella sp.* but shows less antimicrobial activity in *Streptococcus sp.* (Jaleel et al., 2008).

It may be established that antioxidative system components and secondary metabolite content are extensively regulated in drought-stressed plants. Cultivation of medicinal plants such as *C. roseus* in water-scarce places would boost antioxidant metabolism and active principle levels. The facts reported here, however, emphasize the need for a physiological examination of plant response, which must be conducted in conjunction with field trials and evaluation (Jaleel et al., 2008).

Catharanthus have high medicinal properties which were proven by different studies and were continuously being used in the treatment of several diseases. Monomers of various alkaloids were successfully identified in culture media with high yields (Aslam et al., 2010).

Improvement of alkaloids production by *C. roseus* cultures affected by the genetic modification overexpression of key genes in the terpenoid indole alkaloid pathway may also represent a way of improving alkaloid production (Moreno et al., 1995).

An increase in y-glutamyl transpeptidase activity in plasma indicates that impairment in liver function. *C. roseus* treated animal activity of y- glutamyl transpeptidase shown in plasma was close to normal activity. DCM extract of *C. roseus* has no side effect on liver function shown by recovery of plasma AST levels of diabatic. An increase in alkaline phosphate activity in testes and protestants at 300mg/kg for 24 days by ethanolic extract of *C. roseus* reports normal in animals (Suri et al., 2001).

The aqueous extract of *C.roseus* leaves was used to make environmentally friendly Pd-NP from [$Pd(OAc)_2$]. The average particle size was found to be 38 nanometers. Palladium (II) valent ions were discovered to be reduced to Covalent ions by phenolic chemicals found in secondary metabolites. The yield of biosynthesized Pd-NP was shown to be greater at 2 hours of the procedure in a time-dependent investigation. In addition, Pd-NP was employed to degrade phenol red. At an ideal pH of 8.0, Pd-NP could successfully destroy the dye (Kalaiselvi et al., 2015).

C. roseus is rich in sources of phenolic organic acids and amino acids with chemical compositions of alkaloid directed. Aqueous extract of leaves and stems of *C. roseus* inhibits acetylcholinesterase that opens another possibility for the medicinal use of these plants. *C. roseus* is a good source for the food and cosmetic industries (Pereira et al., 2009).

The potential of *C. roseus* is good to remediate radioactivity contamination by ^{137}Cs. The remediation of ^{137}Cs by *C. roseus* at various concentrations is eco-friendly, solar energy-driven in situ remediation technology that utilizes the inherent abilities to live plants to clean up the environment (Fulekar et al., 2010)

C. roseus plants have a unique attractive character that this plant can cultivate in submerged culture using a bioreactor. The reactor help in the high production of a compound in high concentration in a short time under a fully sterile condition with full compliance CGMP for drug manufacturing (Taher et al., 2019).

Different pharmacological activities were observed from the different parts of the *C. roseus* like

Anti cancer activity: Vinblastine and Vincristine are anticancer alkaloids produced from the stem and leaves of *C. roseus*. Some human cancers respond to these alkaloids by slowing down their growth. Vinblastine is suggested for Hodgkins disease and chorio carcinoma and is used experimentally to treat neoplasmas. Another alkaloid, vincristine, is used to treat childhood leukemia. Different percentages of methanolic crude extracts of *C. roseus* were discovered to have considerable anticancer activity against a variety of cell types, with the greatest activity against multidrug resistant tumor types.

Anti diabetic activity: The ethanolic extracts of *C. roseus* leaves and flowers demonstrated a dose-dependent reduction in blood sugar levels that was comparable to the standard medication. Blood sugar control is equivalent to that of the common medication glibenclamide. Because of the increased glucose utilization in the liver, a hypoglycemic effect has developed. When compared to dichloromethane and methanol extracts, which lowered blood glucose levels by 49-58 percent in diabetic rats, the aqueous extract was found to lower blood glucose by roughly 20% in diabetic rats.

Anti microbial activity: Antibacterial activity was tested on crude extracts from several regions of the plant. The effectiveness of a leaf extract was much higher. The antibacterial activity of the plant's leaf extract was tested against bacteria such as *Pseudomonas aeruginosa* NCIM2036, *Salmonella typhimurium* NCIM2501, and *Staphylococcus aureus* NCIM5021, and it was discovered that the extracts could be used as a prophylactic agent in the treatment of a variety of diseases.

Anti oxidant property : The anti-oxidant potential of the ethanolic extract of the roots of two *C. roseus* varieties, rosea (pink flower) and alba (white flower), was determined using a variety of assays, including hydroxyl radical scavenging activity, uperoxide radical scavenging activity, DPPH radical scavenging activity, and nitric oxide radical inhibition method. The results showed that the ethanolic extract of Periwinkle varieties' roots had an acceptable scavenging effect in the entire assay in a concentration-dependent

manner, while *C. roseus* was shown to have higher antioxidant activity than *C. alba.*

Anti helminthic activity: Helminthes infections are a chronic disease that affects both humans and cattle. *Catharanthus roseus* has been utilized as an anti-helminthic agent from the ancient times. *C. roseus* anti-helminthic properties were assessed using *Pherithema postuma* as an experimental model and Piperazine citrate as a standard reference. The anti-helminthic activity of the ethanolic extract at a concentration of 250 mg/ml was discovered to be substantial.

Anti ulcer property: The plant's vincamine and vindoline alkaloids have anti-ulcer properties. Vincamine, an alkaloid found in plant leaves, has cerebrovasodilatory and neuroprotective properties. The plant leaves were found to have anti-ulcer effects in rats who had been exposed to experimentally induced stomach injury.

Hypotensive property: The hypotensive effect of the plant's leaves extract was significant. Among other pharmacologically active chemicals, the leaves are reported to contain 150 valuable alkaloids. Leaf extracts (hydroalcoholic or dichloromethane-methanol) have been shown to have significant antihyperglycemic and hypotensive activity in laboratory animals.(Santhosh Aruna et al., 2015).

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Solvents

Methanol, Hexane, and Chloroform were the solvents used in the extraction procedure. All the solvents were of analytical grade, hexane manufactured by SD fine- chem limited. Methanol and Chloroform manufactured by Fisher chemical company India.

3.1.2 Chemicals, plants material, and Test Organisms

- TLC Aluminium sheets silica gel 60 F_{254}
- Concentrated HCL
- Concentrated H_2SO_4
- 2,2- FDiphenyl-1- Picrylhydrazyl (DPPH)
- Mercuric chloride
- Potassium iodide
- NaOH
- Dimethyl Sulfoxide (DMSO)

Plant Material: Plant part of *C. roseus*

Test organism: The test microorganism used for this research were four gram-positive bacteria *Bacillus subtilis* ATCC 6051, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC6538P, and *Staphylococcus epidermidis* ATCC 1228, six gram-negative bacteria; *Escherichia coli* ATCC 8739, *Proteus vulgaris* ATCC 6380, *pseudomonas aeruginosa* ATCC 9027, *Salmonella enterica* ATCC 29630, *Shigella dysenteriae* ATCC 13313 and, *Klebsiella pneumoniae* ATCC 700603.

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
- Double beam UV spectrophotometer
- Separating funnel

3.2 Methods

3.2.1 Collection of the plant materials

About 15 kg of the plant of *C. roseus* were collected from Balwa Mahottari, Nepal at about 2500m of altitude in April 2019.

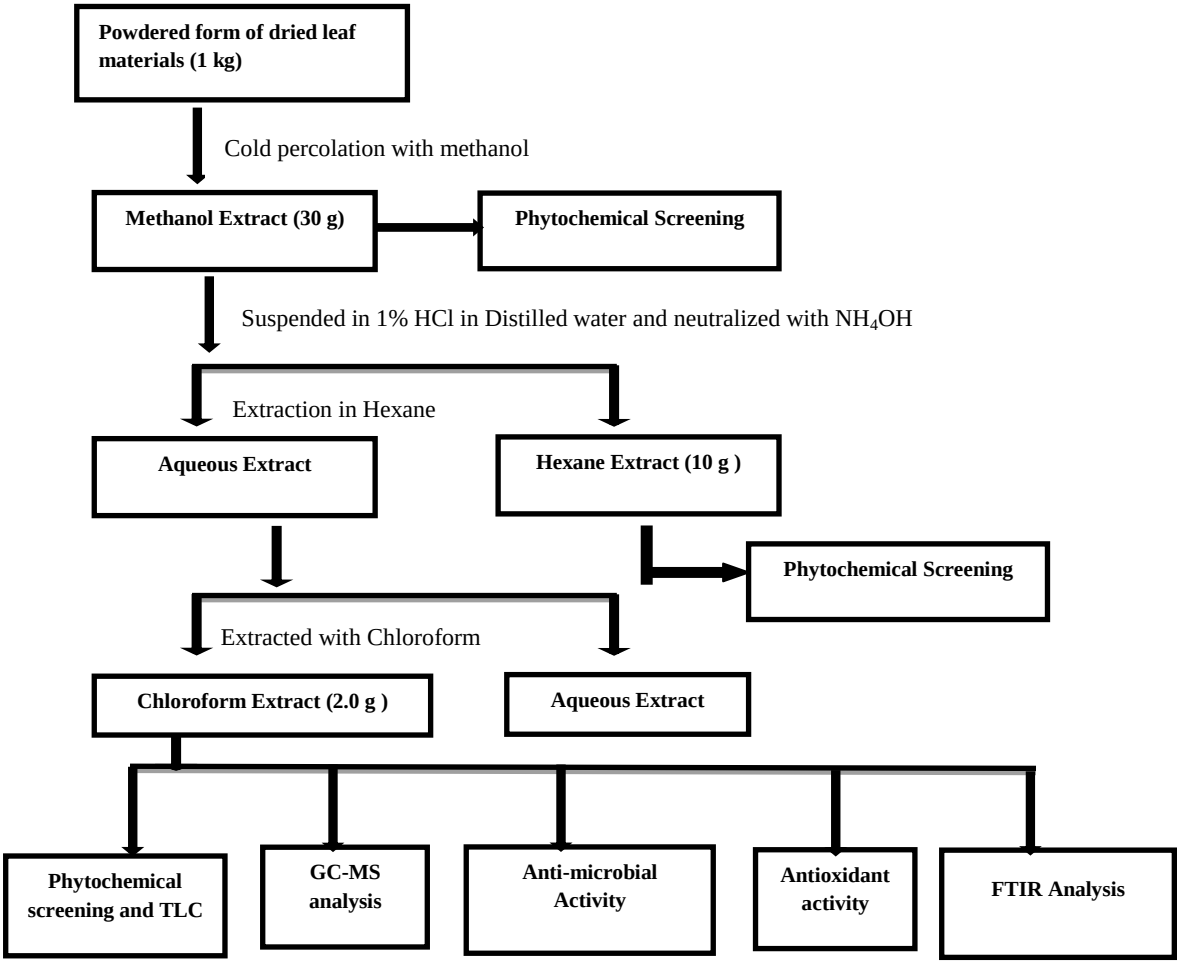
3.2.2 Drying and Grinding

The plant part of *C. roseus* was collected and cleaned with water and air-dried for 15 days. The dried leaves were grinded to powder form.

Figure 3: Dried plant parts

3.2.3 Extraction procedure

The powder form of plants material was first exhaustively extracted with 4L methanol by cold percolation process for 30 days for 2 times. The content was filtered using filter paper. The filtrate i.e, methanol extract was concentrated using a Rota evaporator. The small portion of concentrated methanol extract was subjected to various phytochemical tests. The remaining portion was dissolved in 1% HCl then this solution further proceeded for successive extraction with n-Hexane in a separating funnel. It was shaken vigorously with a continuous release of air in a certain interval of time. The light Hexane fraction remain at the top and the heavy aqueous fraction decant at the bottom was separated and collected in a separate beaker. The crude Hexane extract was obtained from concentrated hexane fraction using a rota evaporator and subjected to various phytochemical tests. The aqueous fraction was mixed with chloroform for further extraction in a separating funnel with a similar extraction process as of Hexane. The mixture was shaken vigorously with continuous release of air with high care. The mixture separated as chloroform extract and aqueous extract. The heavy Chloroform fraction was concentrated using a rota-evaporator and subjected to various phytochemical tests. The qualitative analysis of constituents in Chloroform extract was carried out through thin layer chromatography. Then after the chloroform extract was subjected to GC-MS analysis, anti- microbial activity, anti-oxidant activity, and FTIR analysis.



Scheme 1: Research Process

3.2.4 Phytochemical screening

3.2.4.1 Test for Alkaloids

Extracts were dissolved individually in dilute Hydrochloric acid and filtered.

Mayer's Test: 1mL of the filtrate was treated with Mayer's reagent. The formation of a yellow-colored precipitate indicates the presence of alkaloids.

Dragendroff's Test: To 2-3 mL of Filtrate, few drops of Dragendroff's reagent were added. The formation of an orange-brown precipitate indicates the presence of alkaloids.

Wagner's Test: Few drops of Wagner's reagent were added to 2-3 mL of extract. The formation of a reddish-brown precipitate indicates the presence of alkaloids.

3.2.4.2 Test for Flavonoids

Alkaline Reagent test: Extracts were treated with few drops of sodium hydroxide solution and shake well. The formation of intense yellow color, which becomes colorless on the addition of dilute HCl, indicates the presence of Flavonoids.

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

Pew's Test: 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 missed with 2-3 ml extract followed by drop-wise addition of conc. HCl and boiled for 5 minutes. The formation of magenta color indicates the presence of flavonoids.

3.2.4.3 Test for Glycosides

The extracts were hydrolyzed with dil. HCl, and then subjected to test for glycosides treating them with modified Borntrager's reagent and Legal's reagent.

Modified Borntrager's Test: Extract were treated with Ferric Chloride solution and heated by immersing in boiling water for about 5 minutes, cooled and, shaken with an equal volume of benzene. The resultant solution was treated with ammonia solution. The formation of rose-pink color in the ammonical layer indicates the presence of anthranol glycosides.

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

3.2.4.4 Test for Phenols

Ferric Chloride Test: Extracts were treated with 3-4 drops of ferric chloride solution. The formation of bluish-black color indicates the presence of phenols.

3.2.4.5 Test for Quinone

2 ml of extract was added in few drops of Conc. H_2SO_4 or aqueous NaOH solution. Color formation indicates the presence of the quinoid compound.

3.2.4.6 Test for Tannins

Lead acetate test: Extracts were mixed with few drops of 10% lead acetate solution. The formation of white precipitate indicated the presence of tannins.

3.2.4.7 Test for Saponins

About 1 ml of plant extract was diluted with 2 ml of distilled water, vigorously shaken and, left to stand for few minutes during which time, the development of foam indicates the presence of saponins.

3.2.4.8 Test for Proteins

The extract was diluted in 10ml of distilled water and filtered with Whattman filter paper. 2ml of the filtrate was heated with 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 occur in nearly in all protein.

3.2.4.9 Test for carbohydrates

An equal volume of Fehling A and Fehling B mixture was heated gently with the extract. The formation of red color indicates the presence of carbohydrates.

3.2.4.10 Test for Emolin

An extract was vigorously shaken with 1ml of 25% NH_4OH. The formation of red color indicates the presence of Emolin.

3.2.5 Thin Layer Chromatography

Chromatography is the collective term for a set of laboratory techniques for the separation of mixtures into their components. All chromatography works on the same principle and has stationary and mobile phases. The stationary phase is solid or liquid supported on a solid and the mobile phase may be liquid or gas. The mobile phase should be freshly prepared for each run. The mixture to be separated is dissolved in a liquid is called the mobile phase, the stationary phase carries it through a structure holding another material. Silica can be used for the stationary phase in normal conditions, it is also considered polar (Bele and Khale, 2011).

The word chromatography was obtained from the Greek word in greek, 'Chroma' means color and 'graphein' means writing, hence the word chromatography means 'color writing'. In 1903; Mikhail Tswett the Russian botanist first developed chromatography. He used to separate plant pigments through the calcium carbonate column.

Adsorption chromatography; Mobile phase is absorbed into the surface of the stationary phase. The difference in the affinity towards the stationary phase help to separate the compounds. The compound which has more affinity with the stationary phase will be eluted slowly and compounds with less affinity with the stationary phase will be eluted fast. Eg. Column chromatography, TLC, etc. (Parasuraman et al., 2014)

Thin-layer chromatography (TLC) is the most useful tool for identifying the purity of organic compounds in phytochemistry and the development of the organic chemical reaction. TLC takes the advantage of different affinities of an analyte with mobile phase and stationary phase to achieve the separation of a complex mixture of organic molecules like all chromatographic techniques. TLC technique is used to determine the best solvent system for column chromatography, monitor the progress of a reaction, identification and, purity of compound present in a given substance (Kumar S. *et al* 2013).

The chloroform extract of *C. roseus* was carried out through thin layer chromatography to observe the quality analysis of constituents present in that

extract. Here TLC has performed on TLC aluminium sheets silica gel 60 F_{254} pre-coated TLC plate 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 acetone to hexane gradually. The plates were visualized in a UV fluorescence lamp.

The concentration of the solvent system for TLC is shown in the table

Table 1: The concentration of solvent system for TLC

S.N.	Solvent system of TLC
1.	10% Acetone in Hexane
2.	20% Acetone in Hexane
3.	30% Acetone in Hexane
4.	40% Acetone in Hexane
5.	50% Acetone in Hexane
6.	60% Acetone in Hexane
7.	70% Acetone in Hexane
8.	80% Acetone in Hexane
9.	90% Acetone in Hexane
10.	100% Acetone

3.2.6 Gas Chromatography-Mass spectrometry

This technique is known as the hyphenated technique in which gas chromatography is coupled with mass spectrometry. This method identifies the composition of the mixture of organic compounds based on their molecular mass and volatility.

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 constituent based on boiling points (Volatility) and the molecular weight. 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 pass 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.

3.2.6.1 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 15.0; Helium as carrier gas with an Rtx- 5MS column of dimension 30m x 0.25mm x 0.25 µm, temperature-programmed at 50°C and 300°C with a hold time of 2.00 and 5.00 min while the ion source temperature and interface temperature maintain 200°C and 250°C respectively. Identification was accompanied by a comparison of Mass.

The analytical line for GC-MS Analysis

Column oven temperature	: 80.0°C
Injection Temperature	: 220.00°C
Injection Mode	: Split
Flow Control Mode	: Linear Velocity
Pressure	: 67.7 kPa
Total flow	: 18.5mL/min
Column Flow	: 1.03mL/min
Linear Velocity	: 37.4cm/sec
Purge flow	: 2.0mL/min
Split ratio	: 15.0
High pressure injection	: OFF
Carrier Gas Saver	: OFF
Splitter Hold	: OFF
Ion Source Temperature	: 200.00°C

Interface Temperature : 250.00°C

Solvent Cut Time : 4.00 min

Detector gain mode : Relative

Detector Gain : 0.99kV +0.00kV

Threshold : 0

Analytical Line for Mass

Start Time : 4.00min

End Time : 40.33 min

ACQ mode : Scan

Event Time : 0.50 sec

Scan Speed : 1000

Start m/z : 50.00

End m/z : 500.00

3.2.6.2 Analysis of Chloroform Extract

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.

3.2.7 Antibacterial Activity

Those compounds that can kill or slow down the growth of bacteria without being in a general toxic effect on surrounding tissue are termed antibacterial activity. Antibacterial agents are more important to fight against infectious diseases. The antibacterial screening of the plant was carried out by the agar well diffusion method based on the procedure given by the chemist. In this method, the average diameter of zone inhibition (ZOI) produced by plant extract 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 an antibacterial test. They are the diffusion method and dilution method. Of these, diffusion based method is commonly known as Kirby- Bauer's method. It is a quite suitable test of antibacterial activity. This method follows the following procedures:

3.2.7.1 Preparation of Stock/ Working Solution

25 mg of plant extract (Chloroform extract) was dissolved in 500µL methanol to make the concentration of 50mg/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 stock/ working solution the tubes were sealed and stored in a refrigerator at 40°C until use. The tube was capped, sealed and, stored in cool until use.

3.2.7.2 Collection of Standard Culture

Active culture of ten standard strains of bacteria and two fungi were provided by the Government of Nepal, Department of Plant Resources, Biological department, Banaspati Marg, Kathmandu, Nepal. The following organism was included in the study; four gram-positive bacteria *Bacillus subtilis* ATCC 6051, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC6538P, and *Staphylococcus epidermidis* ATCC 1228, six gram-negative bacteria; *Escherichia coli* ATCC 8739, P*roteus vulgaris* ATCC 6380, P*seudomonas aeruginosa* ATCC 9027, *Salmonella enterica* ATCC 29630, *Shigella dysenteriae* ATCC 13313 and, *Klebsiella pneumoniae* ATCC 700603. All micro-organism was cultured in Nutrient Broth and kept viable by subculturing in Nutrient Agar. The purity of the organism was maintained by subculturing using the streak plate technique.

3.2.7.3 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 9mL of sterile nutrient broth and incubated for 24 hours at 37°C.

A) Nutrient Agar

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

B) Nutrient Broth

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

C) Muller Hinton Agar

3.42g 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-plate in 15mL per plate and the plates were left as such for solidification.

3.2.7.4 Screening and Evaluation of Antibacterial Activity

Already prepared sterile Muller- Hinton Agar 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 wall of the tube above the liquid level and then swabbed carefully all over the plates. The plate was rotated at an angle of 60° after each swabbing. Finally, the swab was passing around the edges of the Agar surface. The inoculated plates were left to dry for minutes by closing with a lid.

The wells were made in the incubated media plates with the help of a sterile cork borer (4 mm) and labeled properly. Then 15µL of the working solution of the plant extract was loaded into the respective wells with the help of a micropipette. The solvent (Methanol) was tested for its activity as a control at the same time in the separate well. The plates were then left for half an hour with the lid closed so that extracts were diffused to the media. The plates were incubated for six hours at 37°C. After proper incubation, the plates were 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.

3.2.8 Antioxidant Activity

Antioxidants are substances that can either slow or stop the oxidation processes that occur when oxygen or reactive oxygen species are present in the environment. Polymeric products, petrochemicals, foodstuffs, cosmetics, and medications are all stabilized with them. Antioxidants have a role in the body's defense mechanism against diseases caused by free radicals.

Antioxidants have received a lot of interest in the areas of radicals and oxidative stress, cancer prevention and treatment, and lifespan. In many of these circumstances, the target analytes are phenols and polyphenols, which can be identified using enzymes like tyrosinase or other phenol oxidases, or even plant tissues that contain these enzymes (Pisoschi & Negulescu, 2012).

Free radical, Reactive oxygen and, nitrogen species (ROS and RNS) are known to damage lipids, proteins, enzymes, and nucleic acids resulting in cell or tissue injury, and have been linked to aging and a variety of degenerative diseases, including inflammation, cancer, atherosclerosis, diabetes, liver injury, Alzheimer's, Parkinson's, and coronary heart disease, among others.

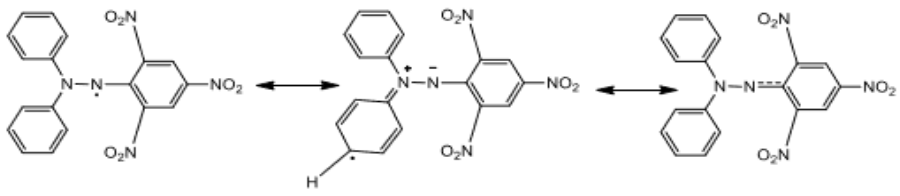
Superoxide (O_2 •), hydroxyl (OH•), peroxyl (ROO•), peroxynitrite (• ONOO-), and nitric oxide (NO•) radicals, as well as non-free radicals such as hydrogen peroxide (H_2O_2), nitrous acid (HNO_2), and hypochlorous acid (HNO_2), are all examples of ROS and RNS (HOCl)(Mosquera et al., 2007).

Enzymes like superoxide dismutase, catalase, and glutathione peroxidase, as well as non-enzymatic substances such uric acid, bilirubin, albumin, and metallothioneins, are examples of endogenous antioxidants. When endogenous factors are unable to maintain strict control and total protection of the organism against reactive oxygen species, exogenous antioxidants, such as nutritional supplements or pharmaceutical medicines containing an antioxidant molecule as the active principle, are required.

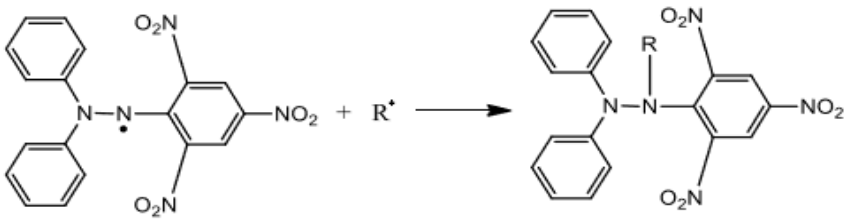
Vitamin E, vitamin C, -carotene, vitamin E, flavonoids, and mineral Se are well-known exogenous antioxidants, although vitamin D and vitamin K3 are also important (Pisoschi & Negulescu, 2012).

3.2.8.1 Principal of DPPH Assay

In methanol, DPPH is a stable radical that absorbs at 515 nm and has a purple color. This assay is based on the idea that when DPPH accepts a hydrogen (H) atom from a scavenger molecule, such as an antioxidant, it is reduced to DPPH2, resulting in a change in color from purple to yellow and a decrease in absorbance at 515 nm. The color change is measured spectrophotometrically, and the parameters for antioxidant qualities are calculated (Ojha, 2011).



Stable DPPH free radical



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

The reaction of DPPH free radical

3.2.8.2 Preparation of DPPH solution

2,2- Diphenyl -1- picrylhydrazyl (DPPH) has a molecular weight of 394.32 g/ml. Thus, 100 mL of 0.1mM solution of DPPH was prepared to weight 4 mg of the DPPH carefully weighing and dissolving it with methanol and finally maintaining the volume to 100 mL.

3.2.8.3 Measurement of DPPH Free Radical Scavenging Activity

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

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

where Ao = Absorbance of the DPPH

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

The 50% inhibitory concentration value IC_{50} is indicated as the effective concentration of the sample 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.

3.2.8.4 General protocol for Antioxidant Assay.

1 mg sample was dissolved in 1 ml to solvent gives the solution of concentration of 1mg/ml and 2 mg in 1ml solvent gives the solution 2mg/ml. so 10 mg of sample (Chloroform extract of *C. roseus*) to be tested was dissolved in 5 ml methanol to get stock solution of concentration 2mg/ml (2000µg/mL). Different concentration (1500, 1000, 500, 250 and 125 µg/mL) of the 1000 µL (1mL) extract were prepared by two-fold dilution method using stock solution.

Table 2: 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

500 µL (0.5 mL) of these solutions were added to 1500 µL (1.5 mL) of 0.1 mM DPPH (4 mg DPPH in 100 mL methanol) differently. The solutions were prevented from light by covering with aluminum foil and the solution was shaken vigorously for about 2 minutes. The solutions were left for 30 minutes in the darkroom at room temperature. After 30 minutes, their absorbance was taken at 517 nm against methanol as a blank. The solution prepared by 0.5 mL methanol and 1.5 mL DPPH solution were taken as control and its absorbance was also taken spectrophotometrically at 517 nm. And a calibration curve was prepared.

3.2.9 FTIR Analysis

Fourier transform infrared spectrometry is one of the widely used physic-chemical analytical techniques to identify the chemical constituent. FTIR is used to identify the structure of an unknown composition compound. FTIR measures the vibration of bonds associated with a functional group and generates the spectra that help to detect the minor and concrete structure of plant secondary metabolites (Bobby et al., 2012).

3.2.9.1 Principle of FTIR

To circumvent the limitations of dispersive equipment, FTIR spectrometry was created. The delayed scanning process was the main issue. A method for simultaneously sensing all infrared frequencies. rather than on an individual

basis, was required. A solution has been devised. An interferometer was used, which is a fairly simple optical apparatus. The interferometer created a one-of-a-kind signal that contained all of the IR information. It has frequencies "programmed" in it. The time element of each sample was lowered because the signal was measured in seconds.

The beam splitter in most interferometers accepts the incoming IR beam and separates it into two optical beams. A flat mirror that is set in situ reflects one beam. A flat mirror reflects the other beam. This mirror features a mechanism that allows it to travel a short distance (usually a few millimeters) from the beam splitter. The signal that exits the interferometer is the consequence of these two beams "interfering" with each other because one beam's path is fixed while the other's is continually changing as its mirror travels. An interferogram is a resultant signal. Near-IR (12,500 to 4000 cm^{-1}), mid-IR (4000 to 400 cm^{-1}), and far-IR (400 to 10 cm^{-1}) are the three sub-regions of the IR spectrum. The energy of IR photons is sufficient to induce groupings of atoms to vibrate concerning the bonds that bind them. Vibrational transitions, like electronic transitions, have distinct energies, and molecules absorb IR light only at specific wavelengths and frequencies. When exposed to IR radiation, chemical bonds vibrate at specific frequencies, and they absorb the radiation at frequencies that match their vibration modes. When the frequency of light absorption is measured, a spectrum is produced that can be used to identify functional groups and compounds. In the infrared region, some contaminants produce distinct bands. The amounts of contaminants and their bonding with the host material are determined by spectral measurements of these bands. The observed interferogram signal cannot be evaluated directly because the analyst requires a frequency spectrum, which is a visualization of the strength at each frequency for identification. It is decided to use a well-known mathematical technique called Fourier transformation to “decode” the various frequencies (FT). A computer performs the transformation and then offers the user the desired spectral information for analysis (Dutta, 2017).

CHAPTER 4: RESULT AND DISCUSSIONS

4.1 Plant Extract

C. roseus extracts were produced in Methanol, Hexane, and Chloroform, crudified using a Rota-evaporator and, dried in a water bath. The following table shows the weight of several plant extracts:

Table 3: Weight of Plant Extract

Plant extract	Weight (g)
Methanol	30
Hexane	10
Chloroform	2

4.2 Phytochemical Screening Analysis

The biological or pharmacological activity of the majority of medications may be traced back to specific chemical ingredients.

Biomarkers should be used to assess the active ingredient's qualitative and quantitative characterization. Before announcing any particular molecule, the biomarker must be defined extremely precisely and with a great deal of thought. In addition, the mixture should be examined to create a fingerprint profile(Pandey & Tripathi, 2014). The results of phytochemical screening of *C. roseus* are shown below:

Table 4: Phytochemical screening of plant extract

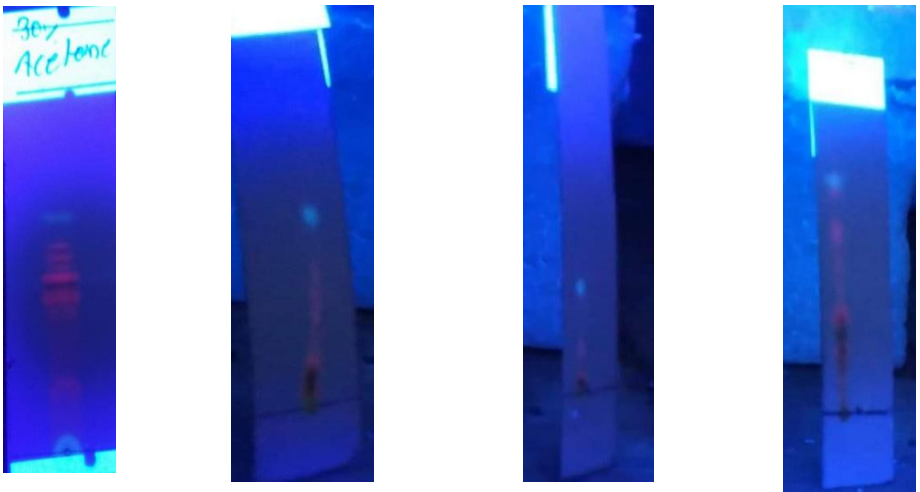
Phytochemical Constituents	Qualitative analysis			Conclusion
	Methanol	Hexane	Chloroform	
Alkaloids	+	+	+	Alkaloids were present in all extract
Flavonoids	+	-	-	Flavonoids present in methanol and absent in Hexene and chloroform extract

Glycosides	+	+	+	Glycosides were present in all extracts
Phenols	+	+	-	Phenols were present in methanol and hexane extract and absent in chloroform extract
Quinines	-	-	-	Quinines were absent in all extracts
Tannins	+	+	+	Tannins were present in all three extracts
Saponins	+	-	+	Saponins were present in Methanol and chloroform and absent in hexane extract
Carbohydrates	+	-	+	Carbohydrates were present in methanol and chloroform extract and absent in hexane extract
Proteins	+	+	+	Proteins were present in all extract
Emolin	+	+	+	Emolin were present in all extract

'+' indicates presence and '-' indicates absences

4.3 TLC Analysis

TLC was used to perform a qualitative analysis of a chloroform extract of *C. roseus*, The polarity of hexane to acetone was gradually increased. to obtained proper spots. The plates were produced in several solvents and observed under a UV-florescence light. The visualized plates were shown in the following figures.



(a) (b) (c) (d)

Figure 4: TLC Showing spots of extract of C. roseus

The TLC reports are illustrated in the following table:

Table 5: TLC of different fractions of chloroform extract of *C. roseus*

S.N.	Solvent system of TLC	TLC report
1	10%acetone	Tailing
2	20% acetone	2 spots with tailing
3	30% acetone	6 clear spots
4	40% acetone	6 spots with tailing
5	50% acetone	4 spots with tailing
6	60% acetone	Tailing
7	70% acetone	2 spots with tailing
8	80% acetone	No movement
9	90% acetone	No movement
10	100% acetone	No movement

4.4 GC-MS Spectra Analysis

GC-MS chromatogram of the chloroform extract of *C. roseus* showed the presence of 7 major possible compounds. The GC-MS chromatogram of chloroform extract of *C. roseus is* presented below.

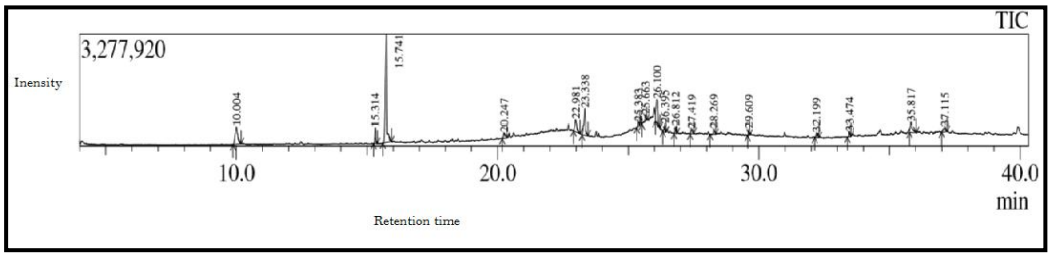


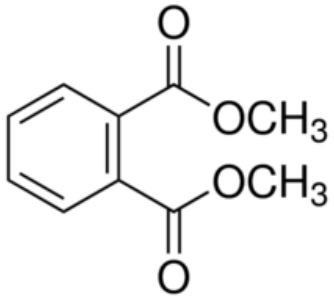
Figure 5: Spectra of GC-MS analysis

The composition of chloroform extract of *C. roseus* was analyzed by GC-MS coupled with mass library search revealed the presence of 7 major compounds. Major compounds analyzed by GC-MS analysis are shown below:

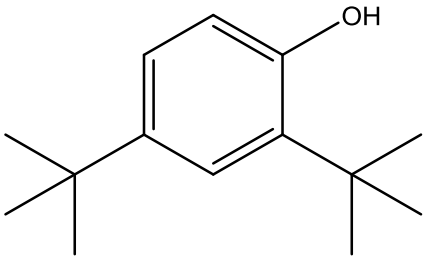
Table 6: List of compounds detected in Chloroform Extract

S.N.	Name of the compound	Retention time	Molecular formula	Molecular weight	Area (%)
1.	Dimethyl phthalate	10.004	$C_{10}H_{10}O_4$	194	9.76
2.	Phenol,2,4-bis(1,1-dimethylethyl)	15.314	$C_{14}H_{22}O$	206	3.08
3.	Diethyl phthalate	15.741	$C_{12}H_{14}O_4$	222	40.69
4.	Oxacyclotetradecan-2-one	22.981	$C_{13}H_{24}O_2$	212	4.93
5.	n-Hexadecanoic acid	23.338	$C_{16}H_{32}O_2$	256	9.28
6.	Cis-9- Hexadecenal	26.100	$C_{16}H_{30}O$	238	10.78
7.	Octadecane, 1- bromo	35.817	$C_{18}H_{37}Br$	332	4.67

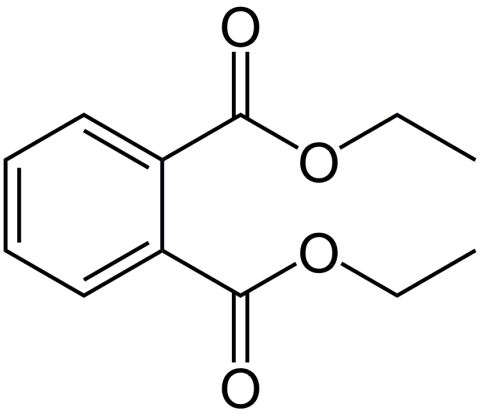
4.4.1 Structure of Compounds Detected from GC-MS Analysis of Chloroform Extract of *C. roseus*



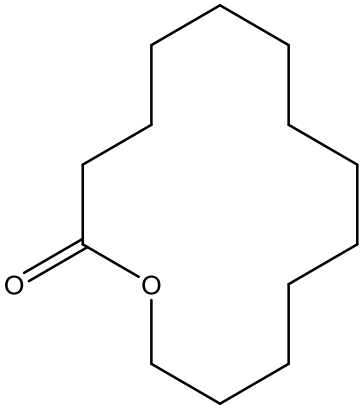
Dimethyl phthalate



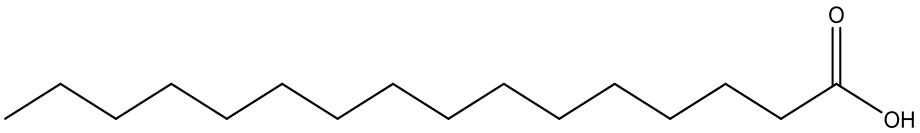
Phenol,2,4-bis(1,1-dimethylethyl)



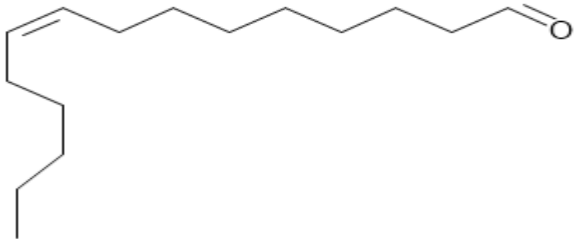
Diethyl phthalate



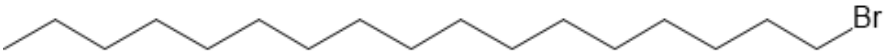
Oxacyclotetradecane-2-one



n-Hexadecanoic acid



Cis-9-Hexadecenal



Octadecane, 1-bromo

4.4.2 Mass spectral Data of Constituents Identified by GC-MS

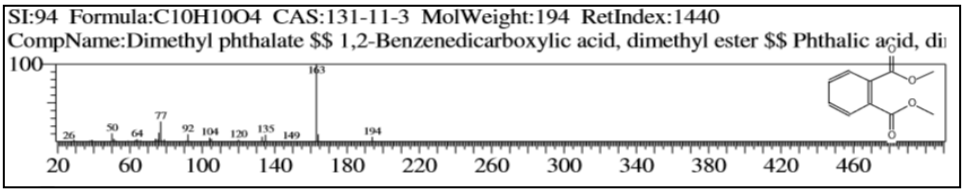


Figure 6: Mass spectral data of Dimethyl phthalate

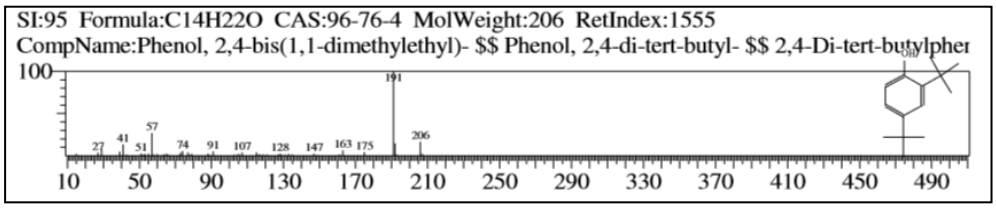


Figure 7: Mass spectral data of Phenol, 2,4-bis(1,1- dimethyl ethyl)

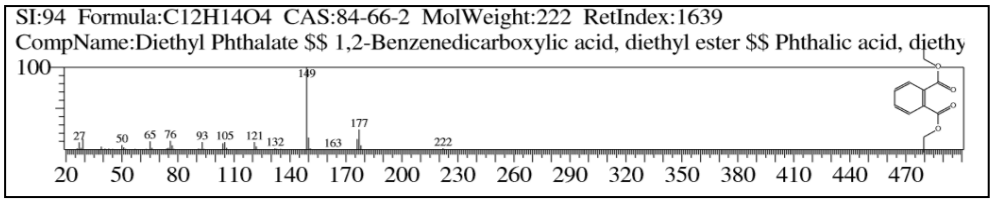


Figure 8: Mass spectral data of Diethyl phthalate

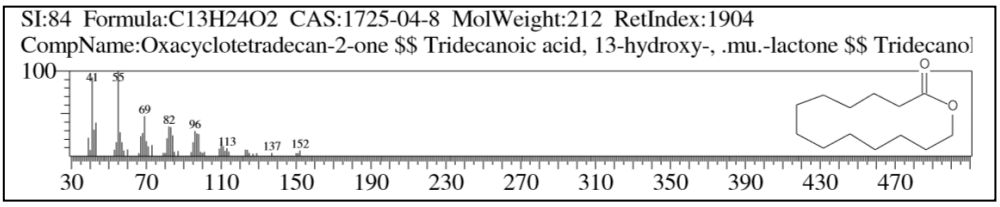


Figure 9: Mass spectral data of Oxacyclotetradecane-2-one

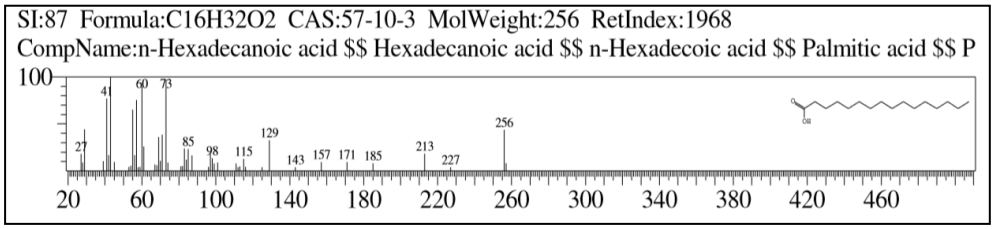


Figure 10: Mass spectral data of Hexadecanoic acid

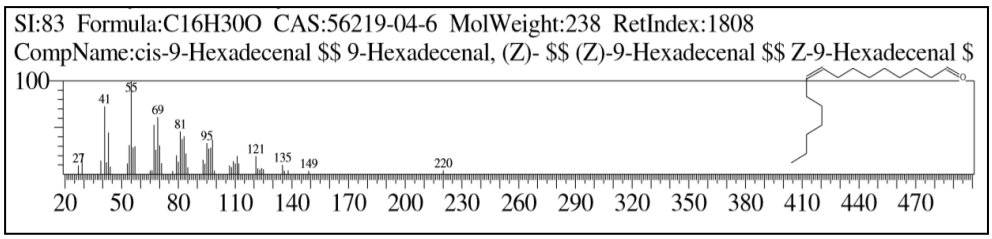


Figure 11: Mass spectral data of cis-9- Hexadecenal

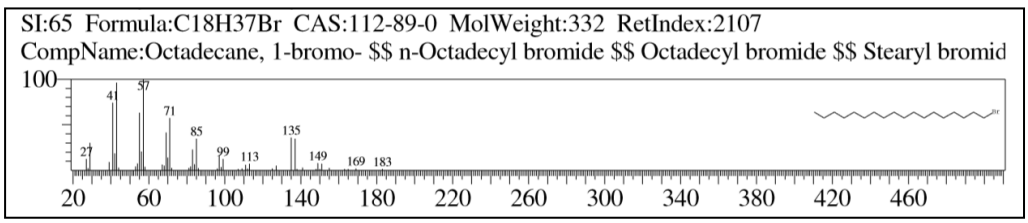


Figure 12: Mass spectral data of Octadecane,1-Bromo

4.5 Antibacterial and antifungal screening analysis

The chloroform extract of *C. roseus* was examined against 10 bacteria samples; *Bacillus subtilis, Enterococcus faecalis, Escherichia coli, Klebsiella pneumonia, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella enteric, Shigellady senteriae, Staphylococcus aureus* and, *Staphylococcus epidermidis* and 2 fungi samples; *Candida albicans* and *Saccharomyces cerevisiae* for the antimicrobial potential of the chloroform extract. The plant extract did not show any antimicrobial activity.

4.6 Antioxidant Screening Analysis

The activity of "free radical scavenging enzymes" (superoxide dismutase, catalase, peroxidase, etc.) and the concentration of antioxidant chemicals, namely phenolic compounds, carotenoids, tocopherol, and ascorbic acid, are clearly linked to plant antioxidant capacity. The antioxidant potential is inversely proportional to the IC_{50} value, which may be estimated using linear regression of percent inhibition against the antioxidant activity. High antioxidant activity is indicated by a lower IC_{50} value. The conventional approach is used for all calculations (Brand-Williams et al., 1995).

The absorbance of each solution was measured and recorded as follows

Table 7: Result of DPPH assay

Sample	**Concentration (µg/mL)**	**Absorbance(nm)**			**Average Absorbance (nm)**	**Percentage Scaveged**
Control		0.921	0.927	0.923	0.923	
C. roseus (Chloroform Extract)	2000	0.311	0.345	0.345	0.33	63.875
	1500	0.491	0.488	0.489	0.489	47.022
	1000	0.540	0.541	0.538	0.539	41.573
	500	0.703	0.700	0.700	0.701	24.106
	250	0.781	0.778	0.779	0.779	15.626

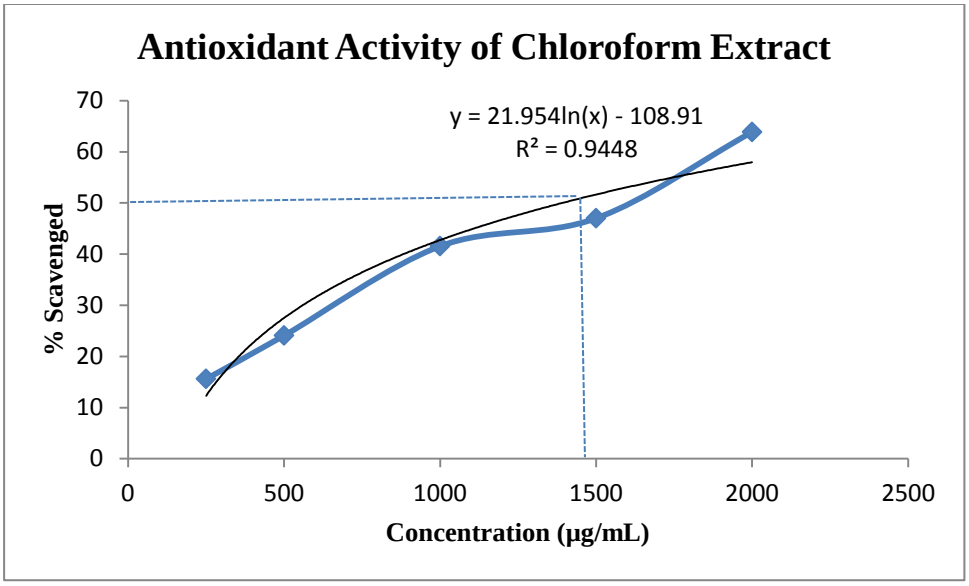


Figure 13: Graphical representation of DPPH assay of the extract

The half-maximum inhibitory concentration (IC_{50}) is a measure of a substance's ability to inhibit a certain biological or metabolic function. This numerical value specifies how much of a medicine or other substance (inhibitor) is required to block a biological process (or component of a process, such as an enzyme, cell, cell receptor, or microbe) by half. Molar concentration is the most common unit of measurement. In pharmacological research, it is widely employed as a measure of antagonist drug potency. According to the FDA, the IC_{50} denotes the medication concentration required for 50% inhibition in *vitro.*

This study shows that the IC_{50} value of chloroform extract of *C.roseus* was 1391.74 µg/mL. This shows that the chloroform extract of *C.roseus* was average towards antioxidant activity.

4.7 FTIR Spectra Analysis

FTIR Chromatogram of the Chloroform extract of *C. roseus* showed the presence of a different compound having a different functional group. The FTIR Chromatogram of chloroform extract of *C. roseus* is presented below.

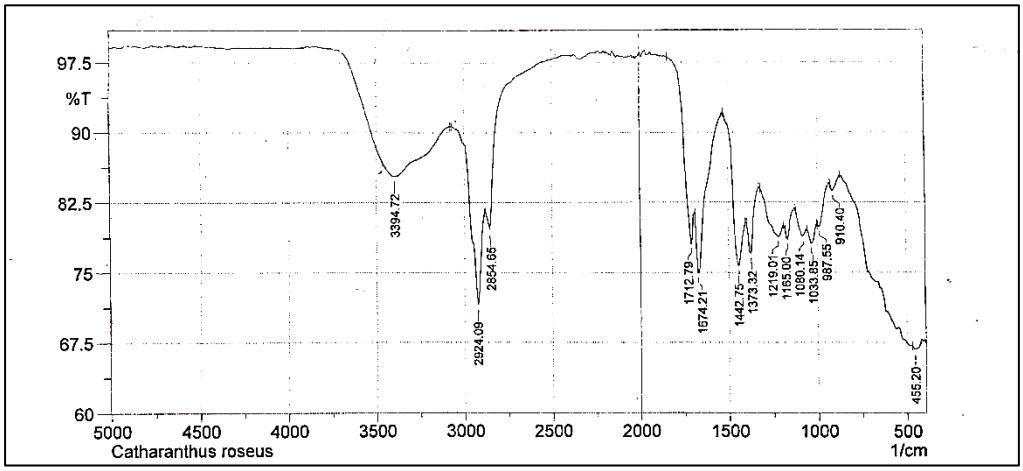


Figure 14: Spectra of FTIR Analysis

Table 8: FTIR peaks values and functional group present in chloroform extract

Extract	**Absorption**	**Types of stretching**	**Appearance**	**Funtional group**
Chloroform extract of *C. roseus*	455	s-s strech		Aryl disulphate
	910	C-H out of plane	Weak	Vinyl
	1033		Weak	Pheloro
	1165	C-O stretch	Medium	Ether
	1219	-o stretch	Weak	Ether
	1373		Medium	Nitrate ion
	1442		Strong	Carbonate ion
	1674	C=C stretch	Strong	Alkenyl
	1712		Strong	-CO
	2854	Assymetri stretch	Strong	-C-H
	2924	Symmetry strech	Strong	-C-H
	3394		Strong	-OH

The broad absorption peak at the range from 3500 and 3250cm^{-1} indicates that the presence of a hydroxyl (-OH)functional group that contains oxygen-related functional groups e.g alcohol or phenols.

The sharp absorption peak at 2924.09cm^{-1} indicates the presence of a methyl C-H asymmetric bond. Similarly, the sharp absorption peak at 2854.65cm^{-1} indicates the presence of a C-H symmetric bond of a methyl group.

There is no absorption peak at the region of 2500 to 2000cm^{-1} that indicates the absence of a triple bond in the compound.

The peak at 1712.79cm^{-1} indicates that the presence of simple carbonyl compounds such as ketones, aldehydes, esters, or carboxyl.

The peak at 1674.21cm^{-1} indicates that the presence of Alkenyl C=C stretched bond. This indicates unsaturated olefinic compounds.

CHAPTER 4: CONCLUSION AND RECOMMENDATION

From the result of this study, it can be concluded that the methanol extract, hexane extract and chloroform extract of leaves of *C. roseus* contain a variety of phytochemicals, including alkaloids, glycosides, tannins, proteins and emolin in all extract where as phenols were present in the methanol and hexane extract, Saponins, carbohydrates were present in methanol and chloroform extract. GC-MS spectra analysis showed the presence of 7 different compounds. Major compounds analyzed in the chloroform extract by GC-MS analysis were Diethyl phthalate (40.69%) , Cis-9- Hexadecenal (10.78%) , and Dimethyl phthalate (7.76%). Using the DPPH radical scavenging method, the antioxidant activity of chloroform extracts of *C. roseus* showed average inhibition with an IC_{50} value of 1391.74 g/mL.

Chloroform extract of *C. roseus* did not show any antibacterial and antifungal activity. However, the extract in another solvent may have antimicrobial properties, which can be determined with more research in literature. FTIR analysis of Chloroform extract showed the presence of a different compound having a different functional group. The FTIR chromatogragm showed broad absorption peak from 3500 and 3250 cm^{-1} which indicates hydroxyl (-OH) group, absorption peak at 1712.79 cm^{-1} indicates simple carbonyl compounds like ketones, aldehydes, esters or carboxyl, as no absorption peak at 2500 to 2000 cm^{-1} indicates a triple bond and sharp absorption peak at 2924.09 cm^{-1} indicates presence of methyl C-H asymmetric bond and absorption peak at 2854.65 cm^{-1} indicates the presence of C-H symmetric bond of methyl group.

To separate and identify the active components in the extracts, more research is needed. This study also demonstrates that in any drug discovery process, it is preferable to use a variety of solvents (from nonpolar to polar) to extract different bioactive compounds within them, as using only one solvent system in this study resulted in significant differences in the extracts' pharmacologic activity. The study also suggests that a comprehensive study on effective, safe, inexpensive, and nontoxic medicinal formulations be conducted, which would not only add value to our resources but also offer a reasonable method to exploiting them.

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APPENDICES

Reagents used for Phytochemical Screening

Mayer's Reagent: 1.358 g of HgCl2 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 few crystals of iodine the solution and stirred properly.

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

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

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

Benedict's Reagent:

Solution I: 50 g of crystalline sodium carbonate, 50 g of crystalline sodium citrate and 31.25 g of potassium thiocyanate were dissolved in 200 mL 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 100mL water.

Finally, Benidict‟s eagent 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 500 mL solution.

Fehling B: 176 g of sodium potassium tartarate and 77 g of sodium hydroxide was dissolved in water to produce 500 mL solution.

Finally, equal volume of solution 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 dilute sulfuric acid solution.

Concentrated Hydrochloric Acid: 36 N concentrated hydrochloric acid solution was 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 hexa hydrate (FeCl3.6H2O) was dissolved in 100 mL of distilled water.

Ammonia Solution: 25% of 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 CO2 free water.

1 M Na2CO3 Solution: 10.6 g of Na2CO3 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 AlCl3 crystals was dissolved in little distilled water in 100 mL volumetric flask and diluted to the mark by adding distilled water.

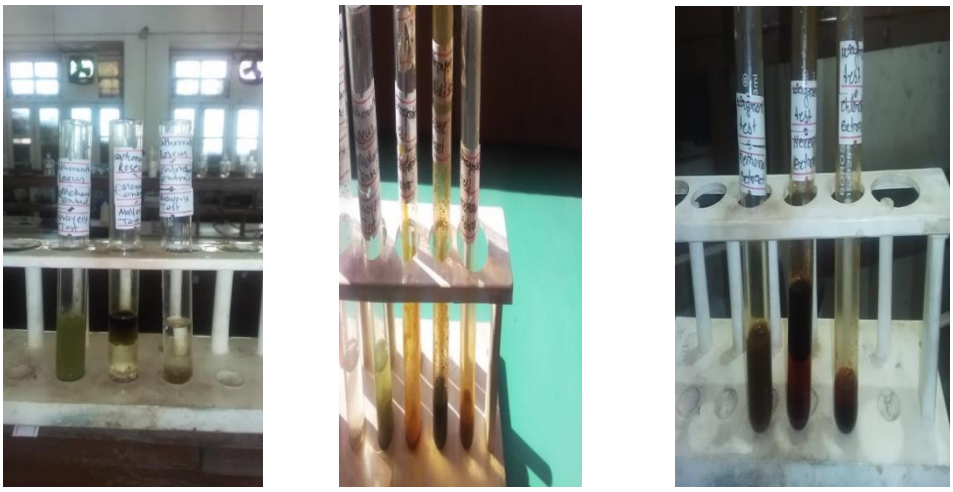
Photos

Extraction by soxhlet

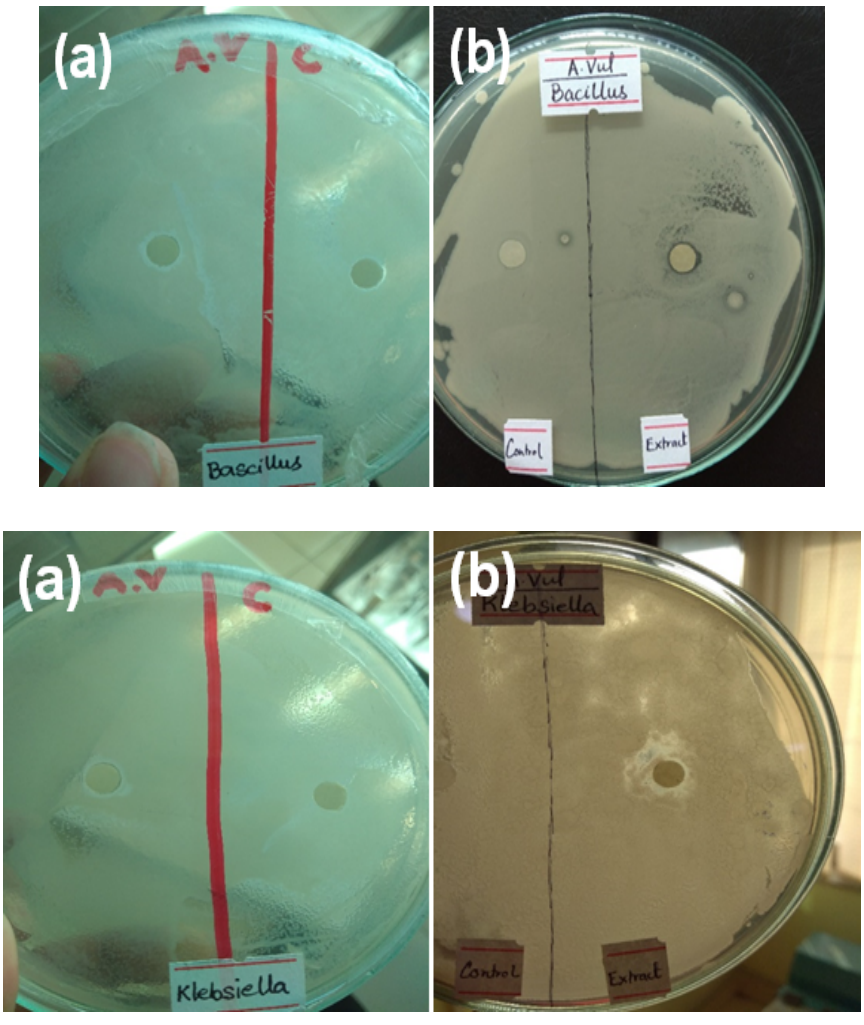
separation of chloroform extract

Methanol extract

dragendroff test of methanol extract



Phytochemical analysis of Methanol, Hexane and Choloform extrac



Antibacterial analysis of plant extract