BIOLOGICAL ACTIVITIES *OF CALLICARPA MACROPHYLLA* VAHL. LEAVES AND HRLCMS BASED PROFILING

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BY

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DECLARATION

Thesis entitled "**BIOLOGICAL ACTIVITIES *OF CALLICARPA MACROPHYLLA* VAHL. LEAVES AND HRLCMS BASED PROFILING**" which is being submitted to the Department of Chemistry, Birendra Multiple Campus, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the partial fulfilment of the requirements for the Master of Science Degree in Chemistry, is a research work carried out by me, Sabi Thapa (Exam roll no. 1654/075, TU registration no. 5-2-19-504-2014), under the supervision of Asst. Prof. Dr. Bodh Babu Bhattarai, Department of Chemistry, Birendra Multiple Campus, Tribhuvan University and co-supervised by Assoc. Prof. Dr. Ganga Raj Pokhrel, Department of Chemistry, Birendra Multiple Campus, Tribhuvan University.

This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

Sabi Thapa

RECOMMENDATION

This is to recommend that **Sabi Thapa** has carried out research entitled **"BIOLOGICAL ACTIVITIES *OF CALLICARPA MACROPHYLLA* VAHL. LEAVES AND HRLCMS BASED PROFILING"** for the partial fulfilment of the requirements of Master of Science Degree in Chemistry under our supervision. To our knowledge, this work has not been submitted for any other degree.

She has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Masters of Science Degree in Chemistry.

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BOARD OF EXAMINERS AND CERTIFICATE OF APPROVAL

This is to certify that the thesis entitled "**BIOLOGICAL ACTIVITIES OF *CALLICARPA MACROPHYLLA* VAHL. LEAVES AND HRLCMS BASED PROFILING**" by **Sabi Thapa**, under supervision of **Assistant Professor Dr. Bodh Babu Bhattarai** and **Associate Professor Dr. Ganga Raj Pokhrel**, Birendra Multiple Campus, Tribhuvan University, Nepal, for the partial fulfillment of the Master of Science Degree in Chemistry has been accepted after appropriate assessment and standards successfully met in the final examination in the Department of Chemistry, Birendra Multiple Campus, Tribhuvan University.

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ABSTRACT

Callicarpa macrophylla Vahl. is a traditional medicinal plant that belongs to Verbaceaceae family growing in Nepal, India, Bhutan and Sri Lanka.

The leaves of *Callicarpa macrophylla* were collected from Tanahun district in the month of October. The leaves were then air dried, crushed into powder using kitchen mixer. The powdered sample was extracted with methanol by maceration, concentrated using Rota vapor and analyzed the concentrated extract for the presence of different bioactive compounds, total phenolic content (TPC), total flavonoid content (TFC), total antioxidant activity (TAC), effectiveness against microbes along with HR-LCMS analysis. The antibacterial activity was tested against 6 bacterial strains like *B.subtilis, S.aureus* (gram positive strains) and *K.pneumonia, E.coli, S.typhi, P.aureginosa (*gram negative strain). The TAC was quantitated using Phosphomolybdenum assay. TPC and TFC were evaluated using Folin- Ciocalteu reagent assay and Aluminum Chloride assay methods, respectively.

The semi-solid methanol extract of leaves of the *C.macrophylla* obtained was 10.8% w/w of the dried powder. The qualitative analysis showed the presence of alkaloids, flavonoids, phenols, saponins, amino acid, coumarins, gums and resins, fixed oils and fats. The anti-bacterial test showed the following inhibition zones: *B.subtilis*- 17.333 mm, *S.aureus*- 14.167 mm and *K.pneumonia*- 9.667 mm but no activity was shown in *E.coli*, *S.typhi, and P.aeruginosa.* The TPC was observed to be 43.489±0.175 mg GAE/g of dry material while TFC was 189.8287±0.429 mg RE/g of dry material. The TAC was found to be 63.953±0.271 mg AAE/g of dry material.

The HR-LCMS analysis showed total of 70 known compounds and 5 unknown compounds. Some of the useful phytochemicals observed were Trimeprazine, Methotrimeprazine, Flurandrenolide Oxazepan, Sakacin, Antimycin A, Ritterazine A, Pleuromutilins etc. The most abundant compound was Trimeprazine, an antipruritic and analgesic agent. These compounds are of high medicinal value and employed in the treatment of various cancers

(colon, prostate, breast), antibacterial diseases, multiple sclerosis, seizure disorder, inflammation, swelling and also used as sedative.

Since ancient, the plant has been used as remedy by local people to treat different disorders. The HR-LCMS data provided the evidence of the presence of different medicinal bioactive constituents in the plants, which was further assisted by antibacterial activity, TPC, TFC and TAC values. However, it also contained some unknown compounds that may have remedial properties suggesting the further analysis of these compounds.

Keywords: phytochemical, antioxidant, antimicrobial, phenolic, flavonoid, HR-LCMS

LIST OF ACRONYMS AND ABBREVIATIONS

AAC	Ascorbic acid Concentration
AAE	Ascorbic acid Equivalent
C.macrophylla	Callicarpa macrophylla
C	Concentration
Conc.	Concentrated
HCl	Hydrochloric acid
Dil.	Dilute
DPPH	2, 2-diphenyl-1-picrylhydrazyl
ME	Methanolic Extract
$FeCl_3$	Ferric chloride
GAC	Gallic acid concentration
GAE	Gallic acid equivalent
H_2SO_4	Hydrochloric acid
LC-MS	Liquid Chromatography- Mass Spectrometry
mg AAE/G	Milligram Ascorbic acid equivalent per gram
mg GAE/g	Milligram Gallic acid equivalent per gram
mg RE/g	Milligram Rutin equivalent per gram
mg / mL	Microgram per milliliter
Nm	Nanometer
RE	Rutin equivalent
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RSA	Radical Scavenging Activity
S	Second
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
UV	Ultra-violet
ZOI	Zone of Inhibition

LIST OF SYMBOLS

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

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CHAPTER 1
INTRODUCTION

1.1. Background of the Study

From the beginning of civilization, people used herbal medicines in the treatment of complex diseases without adverse effects as the plants have natural phytochemicals called secondary metabolites. Scientific research has revealed that medicinal plants contain phytochemical compounds that exhibit a variety of physiological activities that are used as prophylactic agents (Afrin, et al., 2021).

Finding active principles in nature is the elemental phase for advancing pharmaceutical drugs. A novel method for locating therapeutically effective secondary metabolites in diverse plant species is the screening of plant extracts. The biological features of phytochemicals, such as those found in flavonoids, tannins, saponins, alkaloids, and terpenoids include inhibition of oxidation, inflammation, prevention or control of cancer and diabetes (Olivia, et al., 2021).

Despite the fact that synthetic organic chemistry has expanded significantly over the past century, plants still produce about a quarter of all drugs that have been authorized by drug administration boards (Bandyopadhyay & Dey, 2022). Antioxidant, antibacterial, and anti-inflammatory phytochemicals are becoming more and more desired for their potential use in treating a range of chronic illnesses and infections (Bandopadhyay, et al., 2022). Plants, as a viable option for source of antimicrobial compounds, may exhibit strong bioactivity towards pathogenic bacteria while posing lower risks. Plant secondary metabolites are recognized to have antimicrobial and antioxidant effects, and several of them have received universal safety approval (Bandyopadhyay & Dey, 2022).

Modern pharmacopoeias provide genuine information about herbal medicines and their analeptic efficiency. The pharmacological value of plants is owing to the presence of a huge number of labile plant derived beneficial biochemical compounds present in them. The distant plant parts bear these analeptic bio-chemicals during their different stages of

the life cycle (Evans, 2009) (Mishra, et al.,2021). They further benefit by facilitating efficient pollination and seeds dispersal and reproduction and by protecting plants from biotic and abiotic stresses (Mishra, et al., 2021). The continuous change in climate affects plant life cycle patterns, distribution, and phyto-social behavior along with the changes in the phytochemical constituents of individual plant species**.** These diverse secondary metabolites produced by plants can induce specific pharmacological responses, making certain plant species potential sources of new drugs (Cragg & Newman, 2013) (Mishra, et al., 2021).

Phytochemical constituents from medicinal plants require precise selection of appropriate extraction methods and solvents for recognizing, segregation and fingerprinting/ specialization. Practical, inexpensive, effective and adaptable extraction techniques are desirable for small- and medium-scale bioactive compound research operations. The extraction method requires the application of suitable solvent that increases the phytochemical content of the extract, which affects its antioxidant activity (Vongsak, et al., 2013) (Akinmoladun, et al., 2022).

1.1.1 Nepal

Nepal is small mountainous landlocked country with 147,181 km^2 area. It is located at 26° 22′ to 30° 27′ N and from 80° 04′ to 88° 12′ E. The elevation vary between 60 m in the south to 8,848 m in the north (Kansakar, et al., 2004) (Karki, et al., 2015). In accordance with increasing altitude, the country in categorized / grouped into five regions: i) low land Terai with 14% of total area with altitude <500 m above sea level, ii) Siwaliks covers 12% of area with altitude range of 500- 1,000 m above sea level, iii) Mid mountains covers 20% of area with altitude range 1,000- 3,000 m above sea level, iv) high mountains covers 20% of area with altitude range 3,000- 5,000 m above sea level and v) snowcapped high Himalayas covers 24% of area with altitude range >5,000 m above sea level (Kunwar , et al., 2022).

Nepal is rich in flora due to its distinct topography and climatic changes together with altitude, slope and ecosystem variation. Out of the countries in Asia, Nepal is positioned

9th with approximately 9,000 species of angiosperms. Medicines, fragrance and food products are produced from different parts of plants. Initially, Pandey (1961) stated 73 medicinal and aromatic plants (Map's). In 1970, DMP (Department of Medicinal Plants) recorded 483 species then in 1984 Malla and Shakya stated 690 species of MPA's. In 2002, Manandhar reported 1,500 plant species in Nepal (Bhattarai & Ghimire , 2007).

In 2010 Kunwar et.al, reported 6,653 angiosperm species in Nepal (Kunwar R, et al., 2010) (Kunwar, et al., 2013). Of these, almost 50% are useful and has ethno botanical value while 25%-50% have ethno medicinal value (Uprety, et al., 2010) (Kunwar, et al., 2013). Rokaya in 2010 reported about 1,792 to 2,331 medicinal and aromatic plants in Nepal that relieve illness through subsistence, classical therapies and domestic cure (Baral & Kurmi, 2006) (Manandhar, 2002) (Rokaya, et al., 2010) (Kunwar, et al., 2013). In 2020 Chaudhary, et.al., updated the record to 13,000 plant species (Chaudhary, et al.,2020) while in 2018 the number of angiosperms recorded was 7,000 species (Kunwar R, et al., 2010) (Shrestha, et al., 2018) (Kunwar , et al., 2022).

1.1.2. Phytochemicals

Plant chemicals with different form and functions are commonly called phytochemicals which assist in defense and reproduction (Huang, et al., 2016).

Due to the high antioxidant ability and extensive health benefits, phytochemicals are of great appeal. The natural antioxidants analyses in the last twenty years support valuable vision with lesser side effects by its framework function (Thakur, et al., 2020). Phytochemicals are categorized into two classes as in-vitro and in-vivo antioxidants. Hydrogen donors, electron donors, peroxide decomposers, singlet oxygen quenchers, enzyme inhibitors, synergists, and metal-chelating agents are all functions of free radical scavengers. Polyphenols, flavonoids, iso-flavonoids, anthocyanins, phytoestrogens, terpenoids, carotenoids, limonoids, phytosterols, glucosinolates, and fibers are a few phytochemicals that have been linked to possible health benefits (Huang, et al., 2016) (Thakur, et al., 2020).

Naturally found bioactive compounds in the plants are generally divided into primary and secondary metabolites (Baladrin, et al., 1985). The framework and retention are the vital functions of the primary metabolites which are present in each organism (Alqetham & Aldhebiani, 2020). On the contrary, secondary metabolites have medicinal value and are derived from the primary metabolites. They play vital role in the protection of plants and chemical defenses from predators such as microbes, insects or even superior predators (Bernhoft, et al., 2010) (Alqetham & Aldhebiani, 2020).

1.1.3. Medicinal plants

The classical medicinal system such as Ayurvedic, Western, Chinese, Cambodian, Greco-Arabic, Islamic and Unani/Tibb has herbal medicine as the classical form of remedy, which is also called as botanical medicine or herbalism phyto-therapy (Saad & Said, 2011).

In the progression of traditional medicine, ethno-pharmacology had a major role and will hold significant part in upcoming generations. Herbs, mushrooms, honey, bee wax, fibers, proteins from stocks along with other byproducts portray ancient structure of organic health products well-known to people. One-fourth of the common medicines are more or less of any phytochemical active ingredients obtained from plants. A part are created using plant decoction and rest in use are synthetic materials that resemble real plant compounds (Saad B, et al., 2017).

According to WHO, indigenous medicine is defined as the complete blend of knowledge and exercise that is used to avoid and eradicate the ailments, passed down to inheritor either verbally or by manuscript (Agidew, 2022). As mentioned by WHO, more than 80% of the people in developing nations use conventional and herbal remedy to heal their common disorder (Ghazanfar & Al, 1993).

1.1.4. Antimicrobial activity

According to figures, there are around 250,000 to 500,000 different kinds of plants on Earth; of those, only 1%-10% have been studied for their use till date. Plant compounds, more commonly crude extracts, were utilized as antimicrobial agents for the cure of infectious disorders throughout much of human history, according to ancient texts (Prakash, et al., 2020).

An antimicrobial agent either eliminates or prevents the development of bacteria. Antimicrobial drugs can be categorized based on the effects that microorganisms have on humans. As a result of presently available cure and the evolution of drug-resistant microbes, it paved way to escalate/ boost the search for novel anti-microbial and antioxidant drugs (Jaiswal, et al., 2010) (Noumedem, et al., 2013). The wide range of bioactive phytochemicals present in different parts of plants are promising medicinal drugs. Presently, people around the world prefer herbal remedies as the first choice to protect their health and treat the health ailments (Govindarajan, et al., 2008) (Noumedem, et al., 2013).

The hunt for beneficial compounds via plants utilizing different pathways able to combat infectious microorganisms and antioxidant rich components that are specialized in shielding human systems from infectious microorganisms and antioxidant rich substances have been sparked by growing patterns of microbes immune to antimicrobial agents as well as varied persistent and destructive disorders of humans resulting from reactive oxygen molecules (Buffet-Bataillon, et al.,2012) (Ndam, et al., 2016).

1.1.5. Antioxidant:

Free radicals are any atoms or molecules with unpaired elections. Reactive oxygen species include oxygen-derived free radicals like superoxide anion ($O2^-$), hydroxyl (OH*), hydroperoxyl (OOH*), peroxyl (ROO*), alkoxyl radical (RO*), and non-free radicals like hydrogen peroxide (H_2O2), hypochlorous acid (HOCl), ozone (O3), and singlet oxygen (O2) (Senguttuvan, et al., 2014).

The substance that slows or inhibits oxidative stress to a target molecule is called an antioxidant. Antioxidants are characterized by their capability to scavenge free radical

through their redox hydrogen donors and the singlet oxygen restorator (Wu, et al., 2011) (Anokwuru, et al., 2011) (Senguttuvan, et al., 2014).

Antioxidants can respond to free radicals and stop the chain reactions that damage vital organelles. At present, vitamin E (α-tocopherol), vitamin C (ascorbic acid), β-carotene, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and tert-butylhydroquinone (TBHQ) are utilized as antioxidants within the nourishment framework. However, significant artificial antioxidant such as BHA, BHT, and PG have been detailed to have toxicity, malignant consequences along with limited solubility. (Chanda & Nagani, 2010) (Adamez, et al., 2012) (Prakash, et al., 2020). Therefore, the pharmacological sector is searching for natural successor for synthetic artificial antioxidants. In this setting, the primitive utilized phytochemicals (non-nutritive components, which are usually present in exceptionally little amounts), namely flavonoids, carotenoids, phenolic acids, alcohols, tocopherols, lignans, stilbenes, tannins and ascorbic acid, have considerable amount of anti-oxidative capacity and may be favored alternatives to synthetic antioxidants with low toxicity and suppression of reactive oxygen species (ROS) (Ahmad & Aqil, 2007) (Kasote, et al., 2015) (Prakash, et al., 2020).

Phenols are secondary metabolites with simple or complex polymerized complexes having benzene ring with one or more hydroxyl groups (Velderrain-Rodríguez, et al., 2014) (Derong , et al., 2016). The phenols are determined based on size of phenol groups and structural component that links the rings together. The phenols provide the functions in plants by helping them in reproduction, in defense against the predators such as insects and pests in addition to providing the color to the plants while it has various human benefits including antioxidant properties, anti-inflammation, anti-diabetic, anti-mutagenesis, anti-cancer properties and reduces the risk of heart ailments (Liu R. , 2004) (Khatiwora, et al., 2010) (Khoddami et al.,2013) (Malta & Lui, 2014).

Flavonoids are 15C skeleton compounds with aromatic A and aromatic B rings linked to 3C of heterocyclic oxygenated ring or C ring (Liu R. , 2004). Based on the heterocyclic linked C ring structure they are different types such as flavanols, flavonones, isoflavonoids,

flavononols and anthocyanidins. The flavonoids are claimed to have antioxidant properties, anti-inflammation, anti-bacterial and anti-viral activity, antiallergenic, anti-mutagen and anticancer activities (Malta & Lui, 2014).

1.2. Introduction of the selected plant

Callicarpa macrophylla is a woody shrub growing up to 1.5-2.5 m in height. Leaves are oval-lance late, 10-35 cm long, and 2-18 cm wide. When mature, leaves have white haired beneath, petioles are extended to 4-12 mm long and densely tomentose. Flowers are on short gland dotted tomentose pedicle. Fruits are white, smooth, and globular, with nearly 2 mm in diameter. Fruits are sweet-bitter in taste with fragrant odor and are edible (Goel, et al., 2007) (Dangol, 2008). Flowering season is during May to August and fruiting is during September to February (Leeratiwond, et al., 2009) (Mehta, et al., 2010).

1.2.1. Systematic classification of *C.macrophylla*

Kingdom	Plantae
Division	Angiosperm
Order	Lamiales
Family	Verbanaceae
Class	Dicotyledons
Genus	*Callicarpa*
Species	*macrophylla*

1.2.2 Habit and Habitat

Callicarpa macrophylla Vahl is the member of Verbanaceae family. *C.macrophylla* is commonly habitat to Asian countries like Thailand, China, Vietnam, Myanmar, Nepal and India (Ban, 2003) (Chi, 2012) (Lam, et al., 2019).

1.2.3. Ethno botany and Pharmacological Properties

Callicarpa macrophylla leaves are employed in the rheumatism treatment while fragrance oil from roots is used for treatment of stomach related ailments (Goel, et al., 2007). *C.macrophylla* heals the bleeding by stopping it along with its detumescence and analgesic

properties (Zhen-Hui, et al., 2017). From ancient times, *C.microphylla* leaves are used to properties (Zhen-Hui,et al.,2017). From ancient times, C.macrophylla leaves are used to cure hemorrhage in digestive tract, epistaxis, hematemesis, and cough (Lam, et al., 2019). Natives of India heat the leaves and use to heal osteoarthritis (Lam, et al., 2019).

1.3. Objectives of the study

1.3.1 General Objective

The primary objective of the research was to conduct phytochemical screening, metabolite profiling and evaluate the antioxidant and antimicrobial effects of the *C.macrophylla* leaves.

1.3.2 Specific Objectives

- To prepare the methanolic extract of the leaves of *C.macrophylla* through maceration method.
- To analyze different classes of phytochemicals present in the methanolic extract of the leaves of *C.macrophylla*.
- To evaluate the total phenolic and total flavonoid content of the extract.
- To determine antioxidant activity of the extract.
- To determine the antibacterial activity of the extract.
- To identify the bioactive constituents in the extract by HR-LCMS profiling.

CHAPTER 2
LITERATURE REVIEW

Singh and Agrawal isolated 16 α, 17 isopropylideno-3-oxo-phyllocladane, calliterpenone monoacetate and calliterpenone from the essential oil of *C.macrophylla* leaves (Anil & Pawan, 1994)

Singh et.al. isolated β-selinene (41.6), α-selinene (6%), dendrolasin (2.5%) from the fruit oil while cedr-8(15)-en-9α-ol (12.6% and 14.5%) and dendrolasin (6.2% and 5.7%) from leaf oil of *C.macrophylla* (Singh, et al., 2009).

Y. Shi.et.al., 2013 compared the three *Callicarpa* species namely, *Callicarpa nudiflora* Hook.et Am, *Callicarpa macrophylla* Vahl and *Callicarpa* using HPLC with diode array detector and ESI-MS technique. Out of 33 compounds identified in these three species Citric acid, Cinnamic acid, Decaffeoylacteoside, Cistanoside F, beta-OH-forsythoside B, Campneoside II, β-OCH_3-forsythoside B, Campneoside, Forsythoside B, Acteoside, Apigenin-7-O-diglucuronide, 2-acetyl-campneoside II, Poliumoside, Isocampneoside I, Isoacteoside, Eukovoside, 2'-acetylacteoside, Martinoside, Acacetin-diglucuronide, Acacetin-7-O-β-glucuronide were found in *C. macrophylla* (Yatao, et al., 2013).

Jing Xu, 2015 analysed the phytochemicals of *C.macrophylla* and isolated five new compounds: Macrophypene A, Macrophypene B, Macrophypene C, Macrophypene D and Macrophypene E as well as nine familiar compounds namely, calliphyllin, 14α,18-dihydroxy-7,15-isopimaradiene, 8α,9α,13α,14α-diepoxyabietan-18-oic acid, 7α-hydroxydehydroabietic acid, 7-oxodehydroabietic acid, 47 abieta-8,11,13,15-tetraen-18-oic acid, 45 17-acetoxy-16 β-hydroxy-3-oxo-ent-kaurane , ent-16α,17-dihydroxykauran-3-one and 3-oxoanticopalic acid from the leaves of *C.macrophylla*. The structural configuration was determined by NMR, X-ray diffraction and ECD. As 3-oxoanticopalic acid exhibiting the most remarkable stimulation of NGF-mediated outgrowth of neurite from PC12 cells, these Macrophypene A, Macrophypene E, calliphyllin, 14α,18-dihydroxy-7,15-isopimaradiene, 47 abieta-8,11,13,15-tetraen-18-oic acid, 45 17-acetoxy-

16-β-hydroxy-3-oxo-ent-kaurane, ent-16α,17-dihydroxykauran-3-one compounds shown potentiating the effects of NGF-mediated neurite outgrowth from PC12 cells. So, these compounds can be used in the rehabilitation of neurological ailments like Alzheimer's (Xu, et al., 2015).

Wanga et al. isolated callicapene M1, callicapene M2, and callicapene M3 (three new isopimarane-type diterpenoids) along with four isopimarane-type diterpenoids (isopimaradien-3β, 18-diol, 14α-hydroxyisopimaric acid, 7α-hydroxysandaracopimaric acid and 8(14),15-sandaracopimaradiene-7α,18-diol). Of these isolated compounds 7α-hydroxysandaracopimaric acid and 8(14),15-sandaracopimaradiene-7α,18-diol showed 40.23-46.78% of inhibition on NO development LPS activated RAW 264.7 macrophage cells by MTT test technique (Zhen-Hui, et al., 2017).

Wanga et al., 2018 isolated three previously undescribed labdane-type diterpenoids and identified their structure by spectroscopic method from *Callicarpa macrophylla*. The callicarpene M3, Callicarpene M4 and Callicarpene M5 exhibited inhibition of IC50 value of 48.15, 46.31 and 38.72 IM respectively by MTT assay on NO production in LPS-ACTIVATED RAW 264.7 macrophages cells (Zhen-Hui, et al., 2018).

Wang et.al 2022 in addition to Isopimarane diterpenoids named callicapene M1, callicapene M2 and callicapene M3 also isolated four previously studied isopimarane diterpenoids from *C.macrophylla* (Wang, et al., 2019).

Niu et.al., 2020 isolated and used the spectroscopic method for structural elucidation of eight known phenylethanoid glycosides jionoside C ,forsythoside B ,alyssonoside, acteoside, isoacteoside , martinoside , isomartinoside and leucosceptoside B and two novel phenylethanoid compounds , macrophylloside E and macrophylloside F from ethanolic extract of whole parts of *C.macrophylla* (Nui, et al., 2020).

Niu et.al., 2020 isolated three unknown labdane-type diterpenoids termed as callicarpoic acids M1 and M2 and callicarpol M3 along with three published labdane type diterpenoids, labda-8(17),13(*Z*)-diene-15,16-diol, 17-hydroxy-13-methyl-labda-7,13*Z*-diene-15-oic

acid, 6-hydroxynidorellol , and six recognized isopimarane-type diterpenoids callicapene M2, callicapene M3, isopimaradien-3*β*,18-diol, 14α-hydroxyisopimaric acid, 7*α*-hydroxysandaracopimaric acid and 8(14),15-sandaracopimaradiene-7*α*,18-diol from *C. macrophylla* whole plant. Among the isolated diterpenoids, callicapoic acids M1, callicapene M2, 14α-hydroxyisopimaric acid, 7*α*-hydroxysandaracopimaric acid showed strong antitumor properties (Niu, et al., 2020).

Du et.al. isolated and elucidated the structure of formerly known five abietane type diterpene derivatives: jiadfenoic acid B, ~~8~~,11,13-abietatriene-16,18-diol, 4-epi-triptobenzene L, pseudosinin F, and holophyllin F and new abietane-type namely Macrophypene F, Macrophypene G, Macrophypene H, Macrophypene I and Macrophypene J through HRESIMS, 1D and 2D NMR spectroscopy from *C.macrophylla*. Consequently, on analyzing all the derivatives for inhibitory action on extracellular PCSK9 protein levels by PCSK9 ALISA screening showed 56.80% and 43.18% inhibition by Jiadfenoic acid B and holophyllin, respectively at 20 μM (Du, et al., 2023).

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials:

Solvents:

Methanol was used for the extraction of *Callicarpa macrophylla* leaves. The solvent used was analytical grade manufactured by Merck.

Chemicals

- Aluminium Chloride
- Rutin
- Gallic acid
- Folin Ciocalteu reagent
- Ascorbic acid

Test organisms:

S. No.	**Name of the organism**
	Gram negative bacteria
1	*Escherichia coli*
2	*Klebsiella pneumonia*
3	*Salmonella typhi*
4	*Pseudomonas aeruginosa*
	Gram positive bacteria
5	*Bacillus subtilis*
6	*Staphylococcus aureus*

Instruments

- UV spectroscopy
- HRLCMS

3.2. Methods

3.2.1. Methods of collection of plants:

The plant studied was collected from Ramjakot, Tanahun from Gandaki province according to its ethno botanical aspects at the altitude of 637 m in the month of October.

3.2.2. Herbarium preparation

Collected plant specimen along with flowers and fruits were pressed dried between the newspapers. The newspaper was replaced to avoid fungal growth until completely dried. The dried specimen was mounted carefully on the herbarium sheet, labelled and submitted for its identification.

3.2.3. Identification of plant

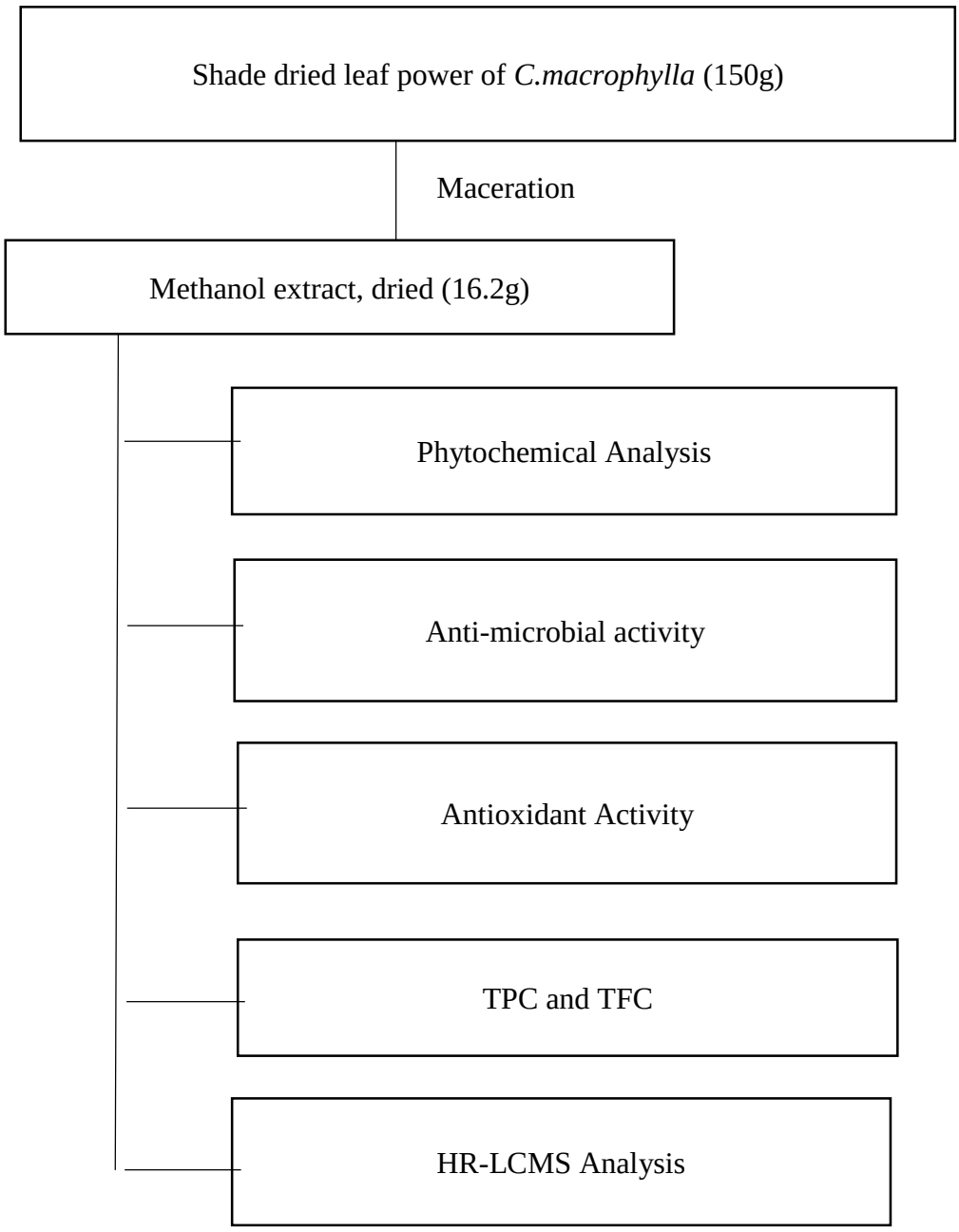
Plant specimen was dissected by National Herbarium and Plant Laboratories (NHPL), Godawari-5, Lalitpur, Nepal.

3.2.4. Drying and Grinding

Leaves were separated from the branches and healthy and disease free leaves were chosen. The separated leaves were cleaned in running water followed by distilled water to get rid of the contaminants, dust and other dirt. The foliage was aerated in the clean room away from light until it was completely dry. The dried leaves were grinded into fine powder and passed through the sieve to maintain the uniform size of the sample. The powdered sample was stored in clean plastic zipper bag (doubled) and kept in shade.

3.2.5. Extraction procedure

The extraction was done by maceration with methanol at room temperature. 150 g of the powdered leaves was dispersed in 750 mL of methanol in a 2,000 mL breaker at room temperature with occasional stirring. After 3 days, the methanol with phytochemical constituents was decanted and stored in air tight container. The process of extraction was repeated three times using fresh methanol each time in the already used powder for maximum extraction. The decanted extract was filtered and concentrated by rotary evaporator. The concentrated extract from rotavapor was finally evaporated with the help of a digital water bath maintaining the temperature at 40^0C.



Scheme 1: *Research Process*

3.2.6. Storage of the extract:

The semi-solid extract after evaporation of methanol was sealed in vials after weighing and kept in the refrigerator at -4^0C for further use.

3.2.7. Phytochemical Analysis

The phytochemical analysis of the extract was done by following the protocol of (Iqbal, et al., 2015) (Abdullahi, et al., 2013) (Alqetham & Aldhebiani, 2020) (Silva, et al., 2017) (Basumatary, 2016) (Bandiola, 2018) (Pandey & Tripathi , 2014) (Theis & Lerdau, 2003) (Ayoola, et al., 2008) (Majid, et al., 2015) (Dwivedi, et al., 2020).

3.2.8. Anti-microbiological activity:

Agar well diffusion approach was employed to investigate the antibacterial effectiveness of the extract. Inhibition of the bacterial growth was measured as the zone of inhibition (ZOI). Antibacterial susceptibility is the method to determine the ability of anti-bacterial agent. The following are the procedure for antimicrobial susceptibility test.

3.2.8.1. Preparation of working solution:

1,000 mg of leaves extract was dissolved in 10 mL of methanol to obtain 100 mg/mL concentration. The stock solution was then sealed and stored in refrigerator until further use.

3.2.8.2. Collection of Standard Culture:

The test micro-organisms taken were gram positive bacilli *B.subtilis* and *S.aureus* as well as gram negative strains *E.coli, K.pneumonia, S.typhi* and *P.aeruginosa* from Bharatpur Hospital, Chitwan. All the micro-organisms were sub-cultured in the nutrient agar for keeping the micro-organisms viable and pure.

Preparation of media:

Nutrient Agar:

5.6 g of nutrient agar was mixed with 200 mL of distilled water to make its ratio 28g/L in a 250 mL Erlenmeyer flask. It was shaken and boiled until complete mix and sterilized at

121°C for 15 minutes. The suspension was given time to drop to 50°C and distributed evenly in 90 mm sterile petri plates. The media was left to solidify.

Muller Hilton Agar

9.5 g of agar was taken in a suitable conical flask, shaken with water and boiled until no lumps were observed. It was then autoclaved at 121°C for 15 minutes to sterilize and allowed to cool down to 50°C. 15 mL of the cooled mixture was poured uniformly in each plate uniformly and allowed to solidify.

Preparation of standard culture:

Inoculum: The isolated colonies of the micro-organism from the sub-culture were transferred in a test tube containing 7 mL of sterile water and turbidity was checked with 0.5 McFarland turbidity standard.

3.2.8.3. Screening and Evaluation of Antibacterial Activity

Earlier prepared petri dish with Muller-Hilton agar medium was dried to remove excess moisture. The disposable cotton swabs was dipped in the ready inoculum and surplus inoculum was eliminated gently squeezing the swap around the inner wall surface of the test tubes. The bacterial swab was rotated at 60° angle for 3 times for 180° complete straining of the agar medium in the petri dish followed by swabbing it at the corners of the petri dish. With the cork borer no. 4, five wells were made in the lawn of about 6 mm of diameter having 25 mm distance from each other. 60µL of the extract was poured into the well with the help of micropipette. The suitable antimicrobial agent was employed as positive agent while solvent was employed as negative control. The culture plates were let to sit for 2 hours so as to permit maximum media dispersion. The zone of inhibition was measured by using ruler after petri plates were incubated at upright position at 37°C for 18-24 hours for bacterial growth. (Erin, et al., 2008) (Baskaran, et al., 2012)

Total Phenol Content Assay

The assessment of total phenol content was done using Folin-Ciocalteu reagent assay method.

3.2.8.4. Preparation of Folin-Ciocalteu Reagent

10 mL of Folin-Ciocalteu reagent was diluted with distilled water to make 100 mL in a volumetric flask.

3.2.8.5. Preparation of Standard Gallic Acid Solution

25 mg Gallic acid was dissolved in 25 mL solvent (methanol) to achieve 1mg/mL (1000µg/mL) stock solution. The various concentrations of 120- 20 µg/mL of Gallic acid solution were made by double dilution method from stock solution.

3.2.8.6. Measurement of Total Phenolic Content (TPC)

The TPC of *C.macrophylla* leaves methanol extract was calculated as mg GAE by the formula given below:

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

Where,

C= Concentration of Gallic acid obtained from calibration curve (mg/mL)

V= Volume of extract (mL)

m= Weight of plant extract (g)

3.2.8.7. General Protocol for Total Phenolic Content

1 mL of the plant extract (5 mg/mL) was infused with 5 mL FCR (1:10 v/v with distilled water), stirred and let stand for 4 minutes. 2 mL saturated sodium carbonate (Na_2CO_3) solution (75g/L) was then add to the above mixture and mixed. The mixture was kept in dark for 120 minutes at ambient temperature before observing absorbance of the reaction products at 760 nm with methanol as blank. Gallic acid was used as standard reference. TPC was calculated in milligram of GAE/g of dried plant sample (Singleton & Rossi, , 1965) (Song, et al., 2010) (Fu, et al., 2011).

3.2.9. Total Flavonoid Content Assay

Total flavonoid content was observed with Aluminium chloride colorimetric method, which is based on color formation at the end of the reaction.

3.2.9.1. Preparation of Standard Rutin Solution.

The stock solution of 2 mg/mL or 2,000 µg/mL was obtained by mixing 50 mg Rutin in 25 ml Methanol. Other lower concentrations of rutin standard solution (1500, 1000, 750, 500 & 250 µg/mL) were prepared by double dilution from the stock solution.

3.2.9.2. Measurement of Total Flavonoid Content (TFC):

The total phenol content was estimated as mg RE using the equation given below:

$$TFC = \frac{(C * V)}{m}$$

Where,

C= concentration of Rutin from curve (µg/mL)

V= volume of extract (mL)

m = weight of plant extract (g)

3.2.9.3. General Protocol for Total Flavonoid Content

1 mL aliquot (2.5 mg/mL extract in methanol) was taken in 10 mL volumetric flask. 0.3 mL of 5% $NaNO_2$ solution and 1.5 mL of distilled water was added to it and let it untouched for 5 minutes. 0.6 mL of 10% $AlCl_3.H_2O$ was then added to the above mixture and again let it stand for 6 minutes. The resultant mixture was then alkalized with 2 mL of 1 M NaOH and volume was made up with distilled water. Absorbance of the resultant solution was taken at 510 nm taking methanol as a blank and reference as Rutin. TFC of the *C.macrophylla* extract was calculated as mg of RE/g of dry plant extract (Ti, et al., 2O14) (Gu, et al., 2021).

3.2.11. Total antioxidant capacity

The Phosphomolybdenum assay is one of the quantitative methods for the total antioxidant analysis. The fundamental concept of Phosphomolybdenum method is the conversion of molybdenum (VI) to molybdenum (V) by reduction reaction either by SET (single electron

transfer) or HAT (hydrogen atom transfer) mechanism. The ammonium molybdate is reduced to greenish blue Phosphomolybdenum oxide complex, Keginn ion [$H_3PO_4(MoO_3)_{12}$] in acidic environment. With Antioxidant the former Keginn ion converts to [H_4PMo_8(VI) Mo_4(V)O40]$^{3-}$ through reduction (Sadeer, et al., 2020).

3.2.11.1 Preparation of Standard Ascorbic Acid Solution.

The stock solution was prepared by mixing 50 mg Ascorbic acid in 50 mL methanol resulting in 1 mg/mL (1000 µg/mL) concentration. Then, different concentrations (264, 396, 528, 660, 792 and 924 µg/mL) of Ascorbic acid were prepared by double dilution method from the stock solution.

3.2.10.2. Measurement of Total Antioxidant Capacity

The total antioxidant capacity of the sample extract was estimated as mg of Ascorbic acid equivalent/g of dried sample extract by applying the formula below:

$$TAC = \frac{(C * V)}{m}$$

Where,

C= concentration of Ascorbic acid from curve (µg/mL)

V= volume of extract (mL)

m= weight of plant extract (g)

3.2.10.3. General Protocol for Total Antioxidant Capacity

0.6 mL of the crude sample (6.6 mg/mL methanol extract) was mixed with 6 mL of reagent solution (0.6M H_2SO_4, 28mM Sodium phosphate and 4mM ammonium molybdate). The resultant mixture was heated in a water bath at 95°C until green color appears (about 90 min.). The solution was then cooled down to ambient temperature. Reagent blank was prepared replacing the sample/ standard with methanol and absorbance of sample/ standard was taken against the reagent blank at 695 nm. Total antioxidant capacity (TAC) of the extract was calculated as mg AAE/g of dried material (Saeed, et al., 2012) (Shahid-Ud-Daula, et al., 2019).

3.2.11. HRLCMS method

About 2.0 g of the methanolic extract of leaves of *C.macrophylla was* packed in 5 mL capped borosilicate tube and sent to "Sophisticated Analytical Instrument Facility (SAIF), IIT, Mumbai, India by DTDC Express Ltd. for HR-LCMS with database for plant extract metabolite identification. The HR-LCMS used was Agilent 1290 Infinity UHPLC system (Agilent Technologies, USA) equipped with an Agilent G6550A Accurate-Mass Q-TOF LC/ MS system (Agilent Technologies, USA). The details of acquisition method is given in Appendix-II.

3.2.12. Statistical analysis:

All the analyses were carried out successfully in triplicates and the results of all the samples are expressed in Mean ± SD.

CHAPTER-4
RESULTS AND DISCUSSION

4.1. Percentage yield of plant sample:

The percentage yield of methanol crude extract of *Callicarpa macrophylla* leaves through maceration was found to be 10.8%.

Table 1: Percentage yeild of methanolic extract of C.macrophylla

Plant	Dry weight of plant leaves, g	Weight of methanolic extract, g	Color	% yield
C.macrophylla	150.0	16.2	Brownish green	10.8%

4.2. Qualitative analysis

4.2.1. Phytochemical screening:

The methanolic extract of *C.macrophylla* leaves was screened for different phytochemicals with suitable reagents. The phytochemical results of methanolic extract is tabulated below in table 2.

The screening results show that the methanolic leaf extract of *C.macrophylla* contains secondary metabolites alkaloids, flavonoids, phenols, saponins, cardiac glycosides, coumarins, gums & resins and fixed oils & fats. On the other hand, tannins, carbohydrates, terpenoids, aminoacids and phlobatannins are absent in the extract. The crude extract need to be dry, so for making concentrated form of extract, rotary evaporator was used, which required heating during concentration process and after this extract was further dried by using water bath, because of this heating process some phyto-constituents having low boiling point might have been destroyed. Due to this reason, some secondary metabolites might have been absent in some extracts.

The literature data and the data that was listed in the table above sometimes don't match on same plants. There are many reasons such as, method, place and time of sample collection, method of extraction, laboratory condition, chemical quality and quantity etc that might have influenced the results of phytochemical screening.

Table 2: Phytochemical Screening of the methanolic leaf extract of C.macrophylla Vahl

S. no	Phytochemical	Test	Reference	Observation	Results		
					R_1	R_2	R_3
1.	Alkaloids	Mayer's	Cream color ppt	Turbid ppt	+	+	+
		Wagner's	Yellow ppt	Yellow ppt	+	+	+
		Hager's	Brown ppt	Brown ppt	+	+	+
2.	Flavonoids	Alkaline reagent	Yellow color	Yellow color	+	+	+
		Lead acetate	Yellow ppt	Yellow ppt	+	+	+
		Pew's	Red ppt	Rosy-red ppt	+	+	+
3.	Phenols	$FeCl_3$	Blue or green color	Green color	+	+	+
		Liebermann's			+	+	+
		Lead acetate	White ppt	White ppt	+	+	+
4.	Saponins	Froth	Froth formation	Froth appeared	+	+	+
		Foam	Foam formation	Foam appeared	+	+	+
5.	Tannins	$FeCl_3$	Black/ blue color	Bluish green	-	-	-
		Potassium dichromate	Yellowish brown ppt	No ppt	-	-	-
		Gelatin	White ppt	No ppt	-	-	-
6.	Cardiac glycosides	Modified Borntrager's	Rose-pink color	Dark rose color	+	+	+
		Keller-Killani	Brown ring at interface	Brown ring at interface	+	+	+
		Legal's	Pink color	Light pink color	+	+	+
7.	Carbohydrates	Molisch	Violet ring	Yellow	-	-	-
		Benedict's	Orange-red ppt	No color changed	-	-	-
		Fehling's	Red ppt	No color changed	-	-	-
8.	Terpenoids	Chloroform / Salkowski	Reddish brown color	No color changed	-	-	-
9.	Amino acid	Millon's	Flesh red ppt	White ppt	-	-	-
		Ninhydrin	Purple color	White	-	-	-
		Xanthoproteic	Yellow ppt	Yellow color	-	-	-
10.	Coumarins		Yellow color	Yellow color	+	+	+
11.	Phlobatannins		Red ppt	No ppt	-	-	-
12.	Quinones		Color formation	No color formed	-	-	-
13.	Gums and resins		Ppt formation	Ppt formed	+	+	+
14.	Fixed oils and fats	Spot test	Oil strain	Oil strain	+	+	+

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

4.2.2. HRLC-MS data analysis

HR-LCMS analysis of the methanol extract of *C.macrophylla* Vahl leaves showed the presence of 70 known compounds and 5 unknown compounds. The HR-LCMS chromatogram of the methanol extract of *C. macrophylla Vahl* is presented below:

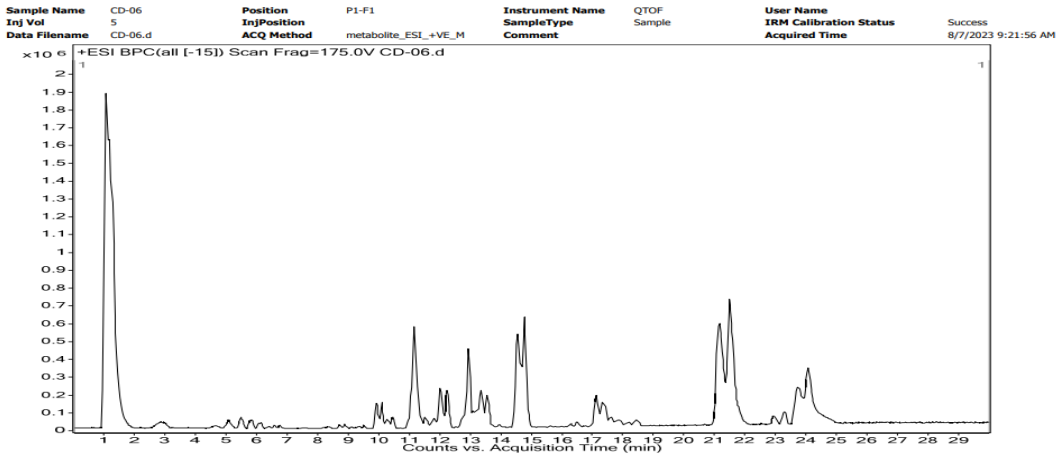


Figure 1: HR-LCMS chromatograph of the methanolic extract of C.macrophylla leaves in positive ESI mode

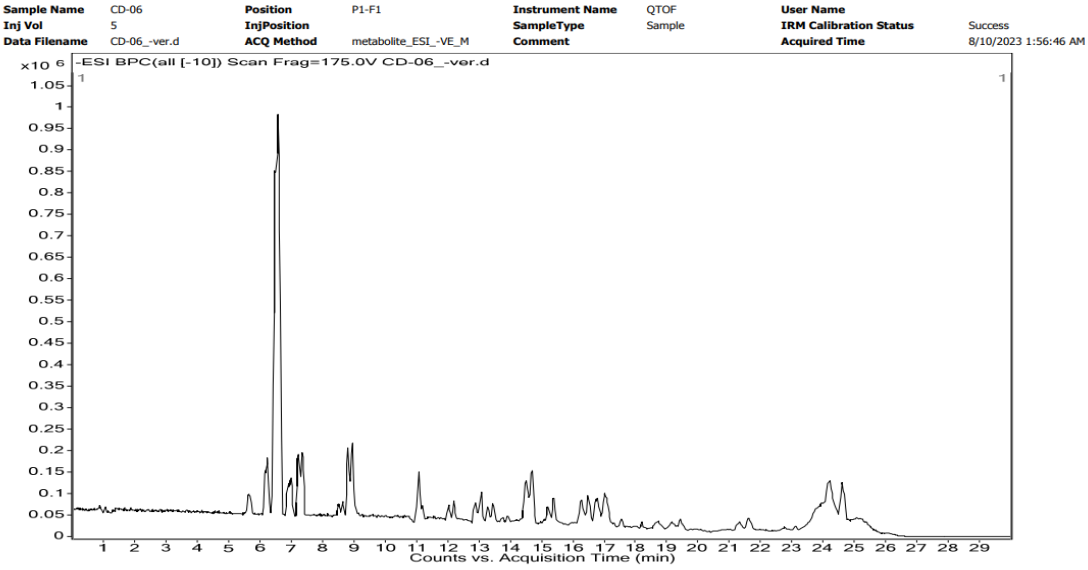


Figure 2: HR-LCMS chromatograph of the methanolic extract of C.macrophylla leaves in negative ESI mode

The analysis of methanolic extract of leaves of *C.macrophylla* in HR-LCMS with library search resulted in the 21 important compounds. Major compounds analyzed by HR-LCMS is listed in table 3 below: All the compounds, known and unknown are listed in Appendices VI and VII

Table 3: List of some compounds of methanol extract of C.macrophylla leaves in HRLCMS analysis

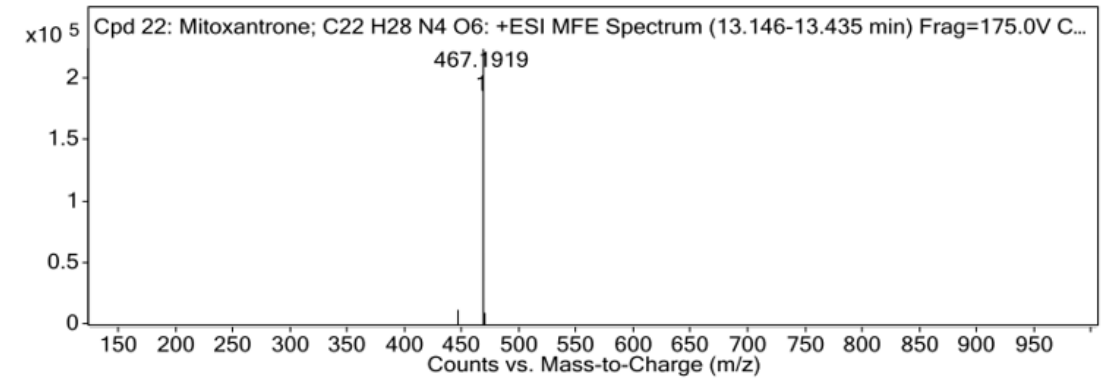
S.no	Name of the compound	Molecular formulae	Molecular mass	Retention time	DB diff. (ppm)	Class of compound
1.	Mitoxantrone	$C_{22}H_{28}N_4O$	444.2027	13.356	-4.02	Anthraquinones
2.	Methotrimeprazine	$C_{19}H_{24}N_2OS$	328.1624	11.763	-4.46	Phenothiazine
3.	Flurandrenolide	$C_{24}H_{33}FO_6$	436.226	5.501	0.23	Steroids
4.	Trimeprazine	$C_{18}H_{22}N_2S$	298.1484	1.105	6.61	Phenothiazine
5.	Citalopram-N-Oxide	$C_{20}H_{21}FN_2O_2$	340.1592	1.092	-1.42	Amino group
6.	Oxazepan	$C_{15}H_{11}ClN_2O_2$	286.0511	9.006	-0.68	Chloro-phenyl group

a. Mitoxantrone

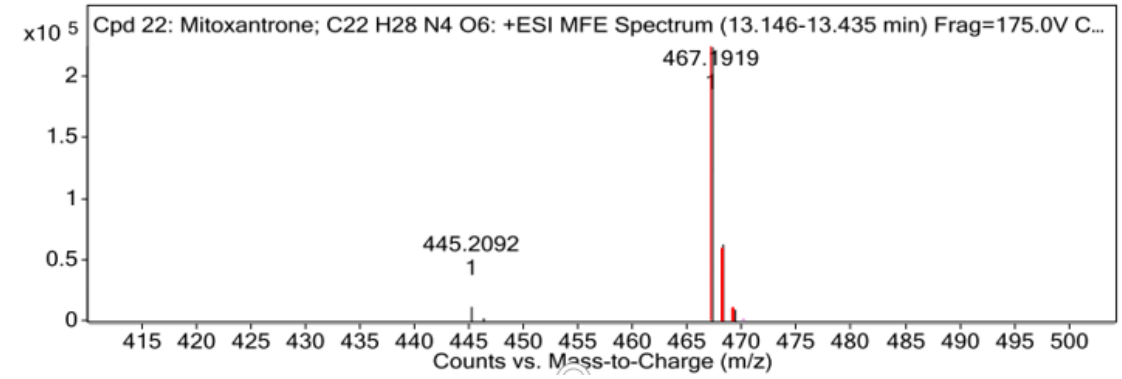
Mitoxantrone is an antineoplastic drug that inhibits DNA synthesis by intercalating DNA, inducing DNA strand breaks, and causing DNA aggregation and compaction, and delays cell cycle progression, particularly in late S phase. It is used for the treatment of hematological malignancies, in breast cancer and in advanced hepatic or ovarian carcinoma. (Faulds, Balfour, Chrisp, & Langtry, 1991). It is a cytotoxic immunosuppressive drug for multiple Sclerosis (MS) (Edan, 2016).

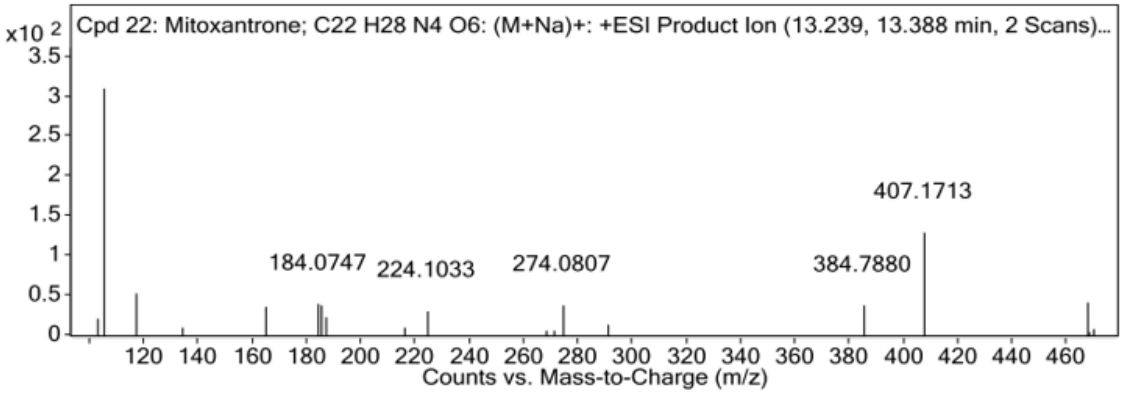
Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 22: Mitoxantrone; C22 H28 N4 O6	**Mitoxantrone**	467.1919	13.356	Find by Molecular Feature	444.2027

MFE MS Spectrum



MFE MS Zoomed Spectrum

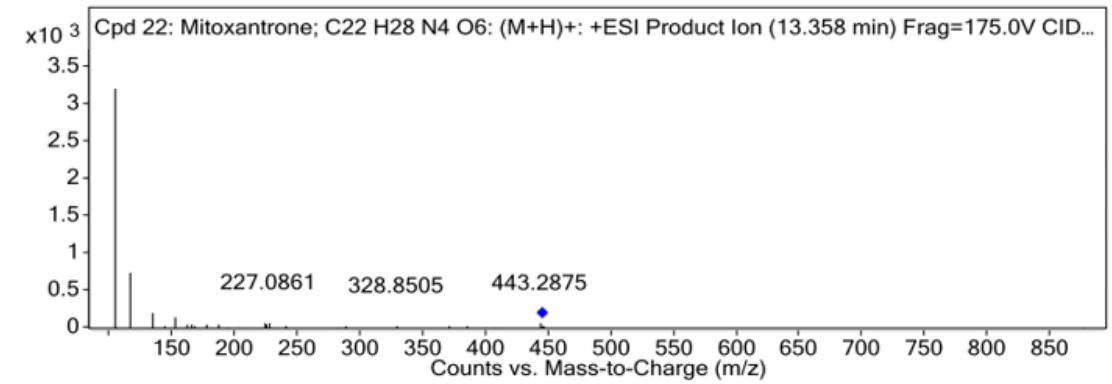




MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
445.2092	1	12175.64		(M+H)+
446.2134	1	3457.21		(M+H)+
467.1919	1	224578.8	C22 H28 N4 O6	(M+Na)+
468.1947	1	63784.74	C22 H28 N4 O6	(M+Na)+
469.1977	1	9921.32	C22 H28 N4 O6	(M+Na)+

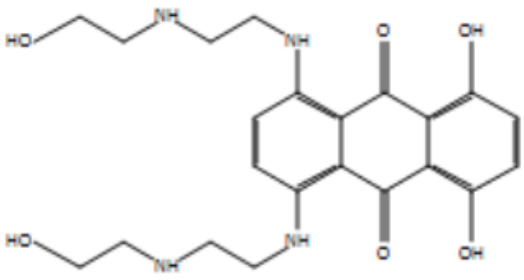
MSMS Spectrum



MS/MS Spectrum Peak List

m/z	z	Abund
105.0315	1	310.98
117.0671	1	53.17
164.9238	1	37.35
184.0747	1	40.8
185.1297	1	38.83
224.1033	1	32.02
274.0807	1	39.48
384.788	1	38.87
407.1713	1	130.59
467.1881	1	42.13
105.0322	1	3223.13
117.0679	1	754.59
134.0945	1	205.65
135.0987	1	65.14
152.1076	1	151.29
166.0945	1	59.27
224.1063	1	83.78
225.1054	1	66.83
227.0861	1	85.41
443.2875	1	78.82

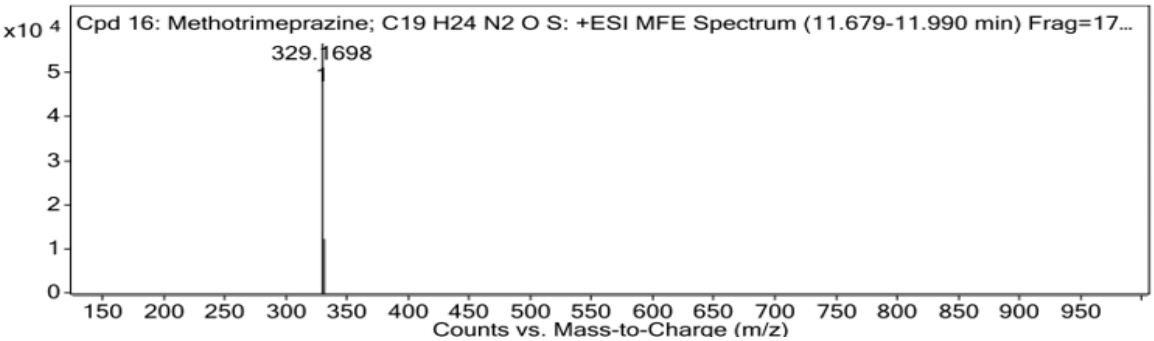
Compound structure



b. Methotrimeprazine:

Methotrimeprazine is a group 1 aliphatic phenothiazine. It is a neuroleptic drug used as sedative and analgesic drug to treat antipsychotic and used in palliative care. However, it has moderate side effects like anti-muscarinic and extrapyramidal (Davis, 2007).

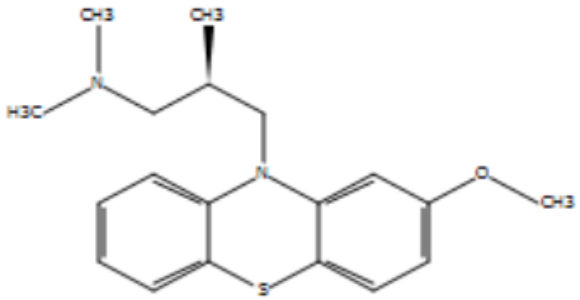
Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 16: Methotrimeprazine; C19	**Methotrimeprazine**	329.1698	11.763	Find by Molecular Feature	328.1624



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
329.1698	1	56801.45	C19 H24 N2 O S	(M+H)+
330.1724	1	12687.08	C19 H24 N2 O S	(M+H)+

Compound Structure

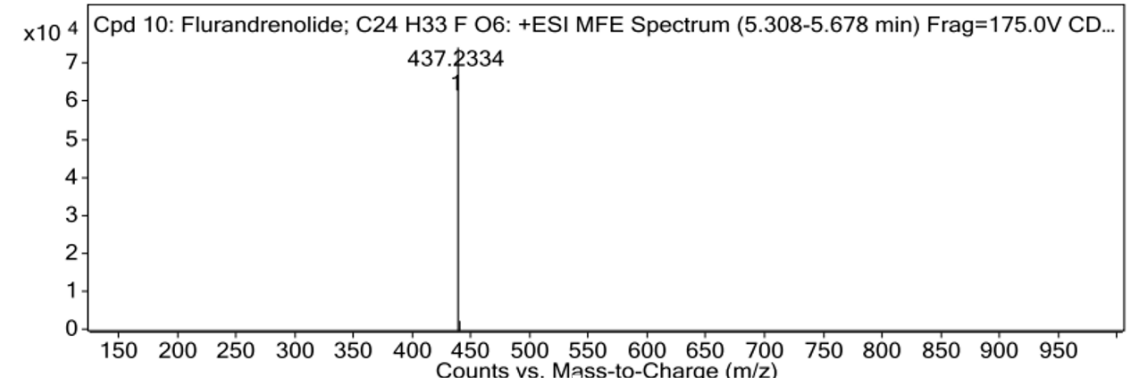


c. Flurandrenolide

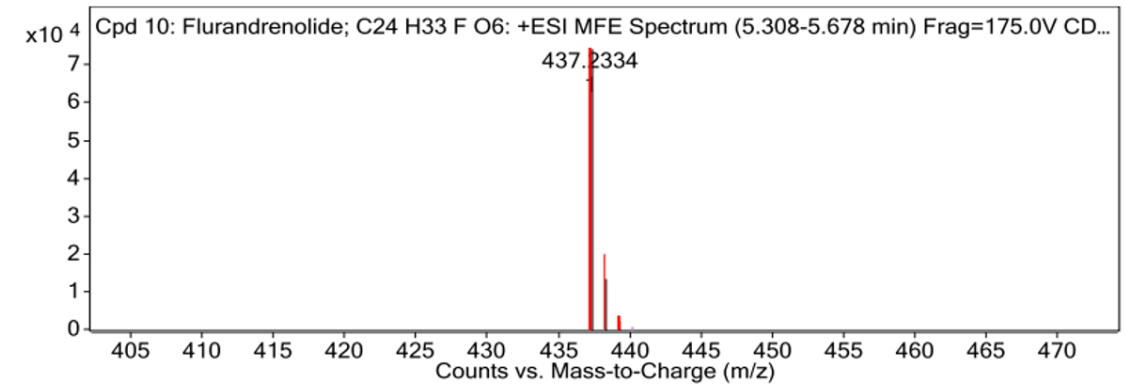
Flurandrenolide is an antipruritic, anti-inflammatory and vasoconstrictive in nature. It shows anti-inflammatory action by stabilizing cell and lysome membranes. It reduces the inflammation by preventing the release of proteolytic enzymes (Daily Med, n.d.).

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 10: Flurandrenolide; C24 H33 F O6	**Flurandrenolide**	437.2334	5.501	Find by Molecular Feature	436.226

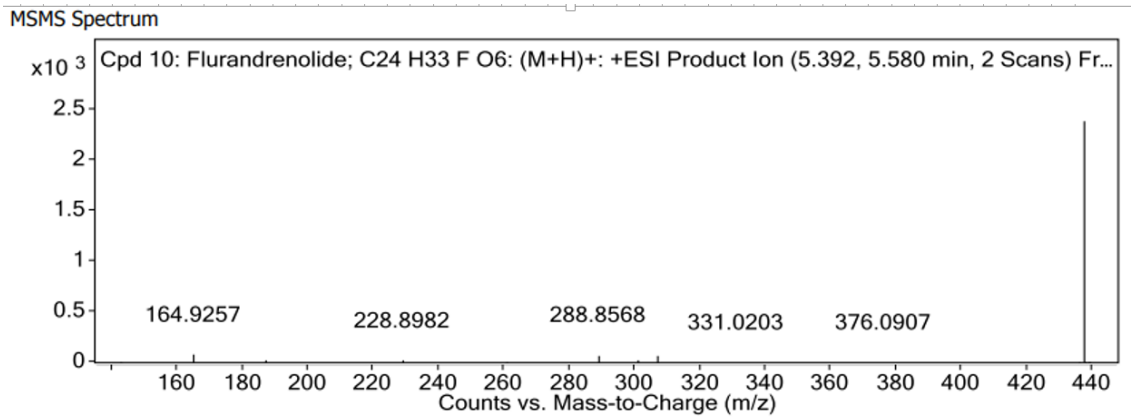
MFE MS Spectrum



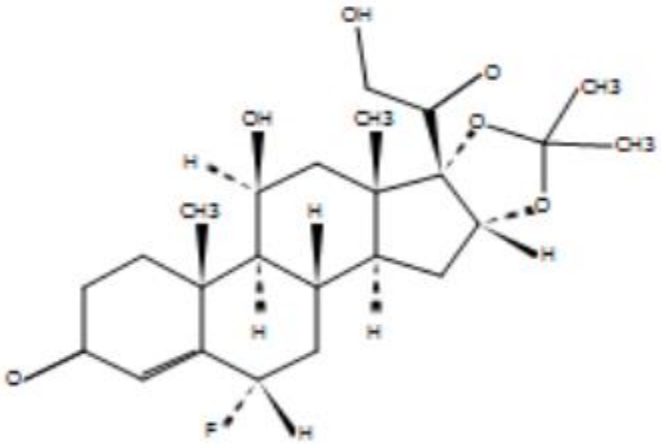
MFE MS Zoomed Spectrum



m/z	z	Abund	Formula	Ion
437.2334	1	74349.76	C24 H33 F O6	(M+H)+
438.2364	1	13904.39	C24 H33 F O6	(M+H)+
439.2381	1	2808.58	C24 H33 F O6	(M+H)+



Compound Structure

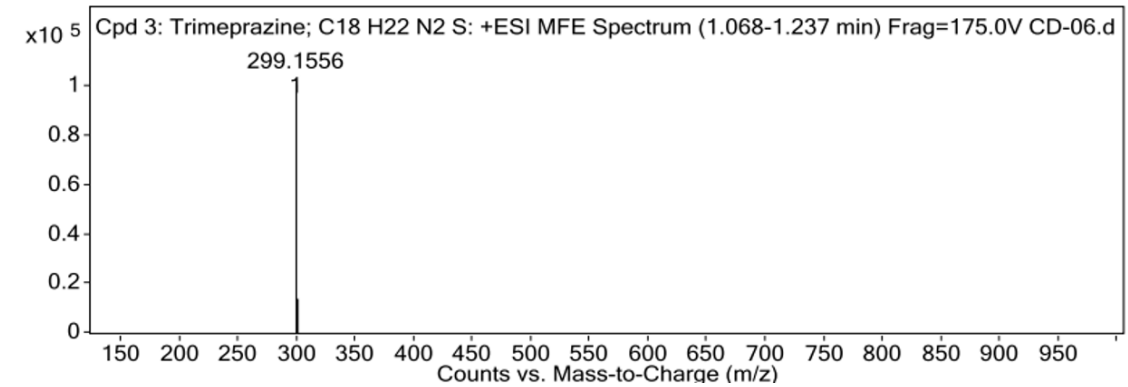


d. Trimeprazine

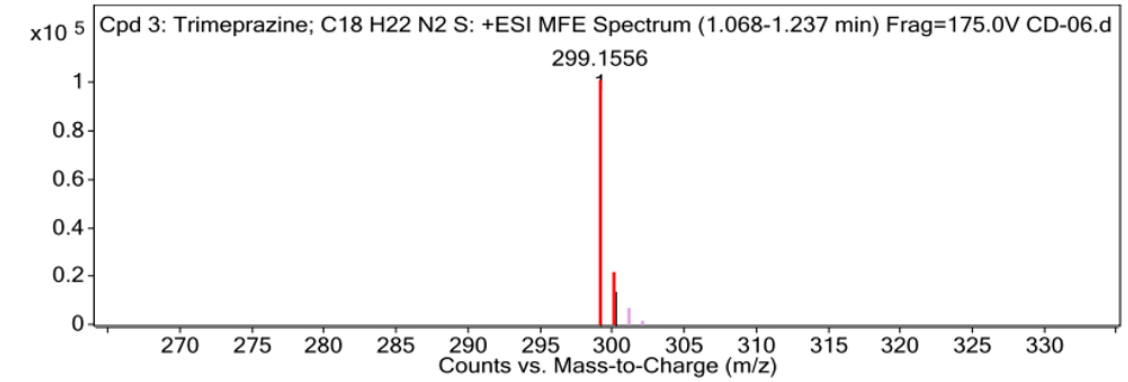
Trimeprazine is an antihistamine & antipruritic agent used in the treatment of insomnia, and cough. It also shows a potent analgesic effect (Kintz, Villain, & Cirimele, 2006).

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 3: Trimeprazine; C18 H22 N2 S	**Trimeprazine**	299.1556	1.105	Find by Molecular Feature	298.1484

MFE MS Spectrum



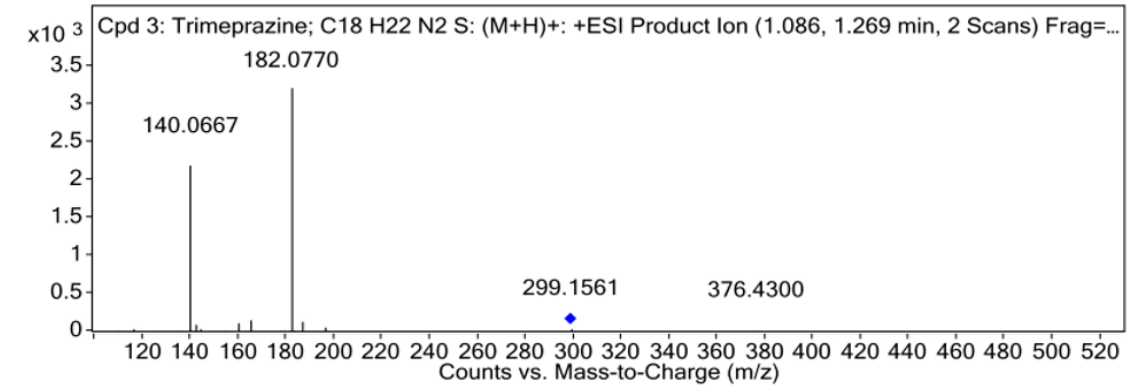
MFE MS Zoomed Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
299.1556	1	100845.78	C18 H22 N2 S	(M+H)+
300.159	1	13982.15	C18 H22 N2 S	(M+H)+

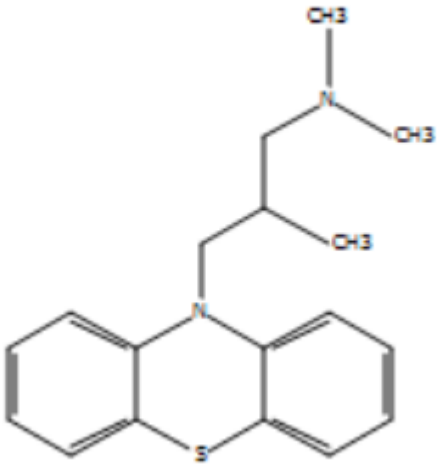
MSMS Spectrum



MS/MS Spectrum Peak List

m/z	z	Abund
116.0667	1	34.62
140.0667	1	2189.93
142.1183	1	94.24
144.0632	1	40.5
160.096	1	115.28
164.9279	1	162.52
182.077	1	3210
186.9071	1	135.41
196.0916	1	63.11
299.1561	1	47

Compound Structure

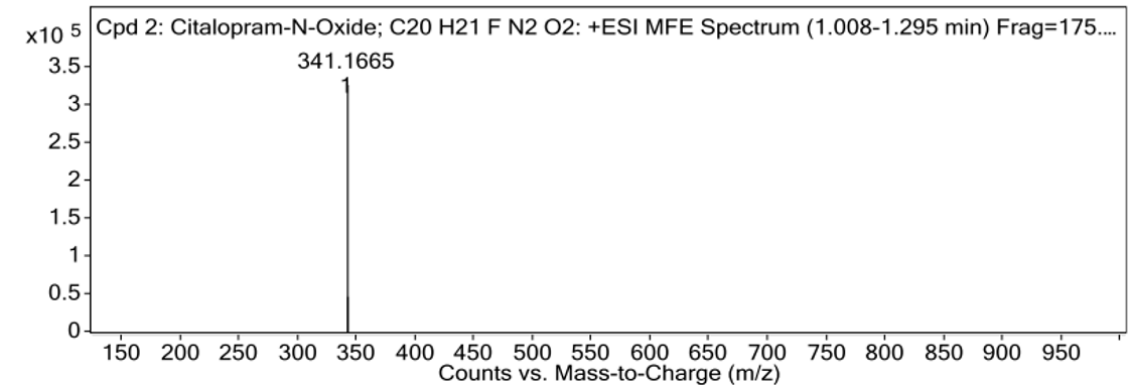


e. Citalopram N-Oxide:

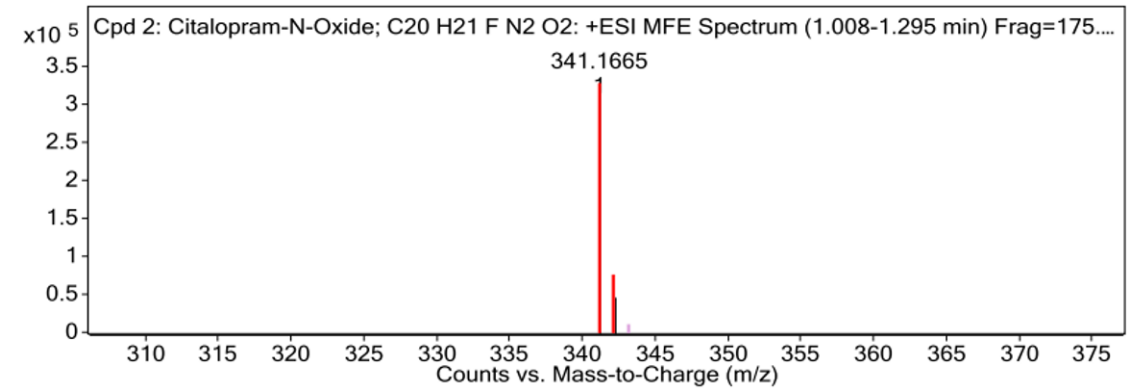
Citalopram n-oxide is metabolite of Citalopram. Citalopram selectively inhibits 5-hydroxytryptamine transporter (serotonin). Citalopram is useful for studying gene expression patterns in circulating lymphocytes (Torres, Gainetdinov, & Caron, 2003) (Gabriel, et al., 2019).

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 2: Citalopram-N-Oxide; C20 H21 F N2 O2	**Citalopram-N-Oxide**	341.1665	1.092	Find by Molecular Feature	340.1592

MFE MS Spectrum



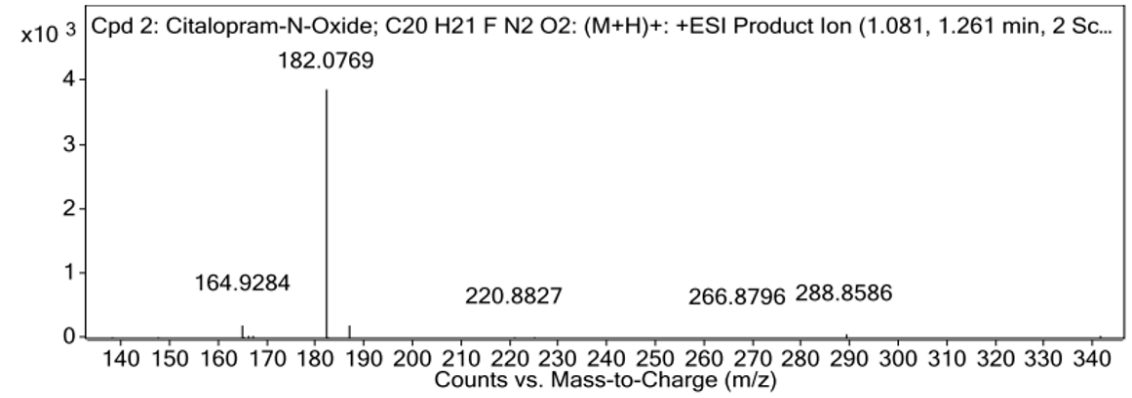
MFE MS Zoomed Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
341.1665	1	326549.53	C20 H21 F N2 O2	(M+H)+
342.1692	1	47040.06	C20 H21 F N2 O2	(M+H)+

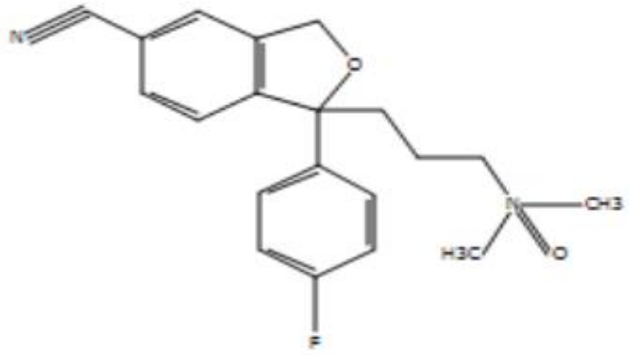
MSMS Spectrum



MS/MS Spectrum Peak List

m/z	z	Abund
138.0883	1	24.81
147.431	1	21.36
164.9284	1	219.84
165.0054	1	18.5
166.0809	1	55.54
167.0836	1	43.47
182.0769	1	3874.1
186.9115	1	212.52
288.8586	1	62.96
341.1673	1	37.4

Compound Structure

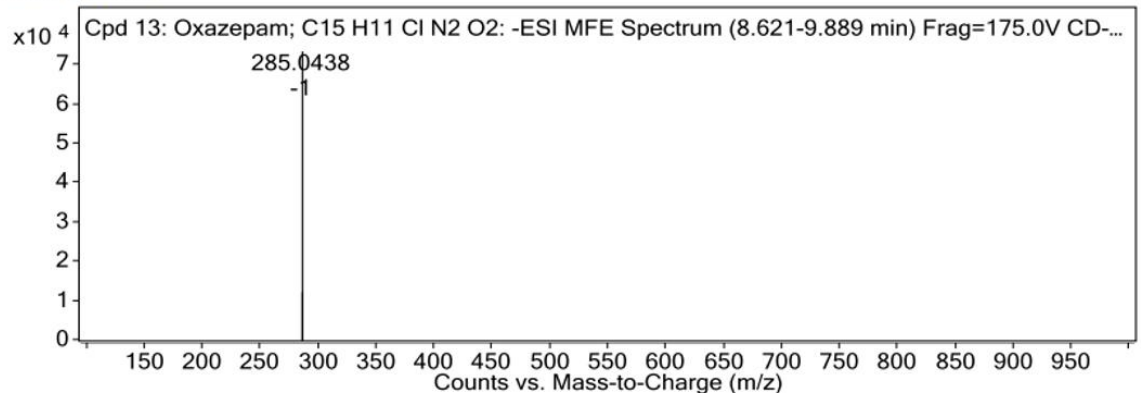


f. Oxazepan:

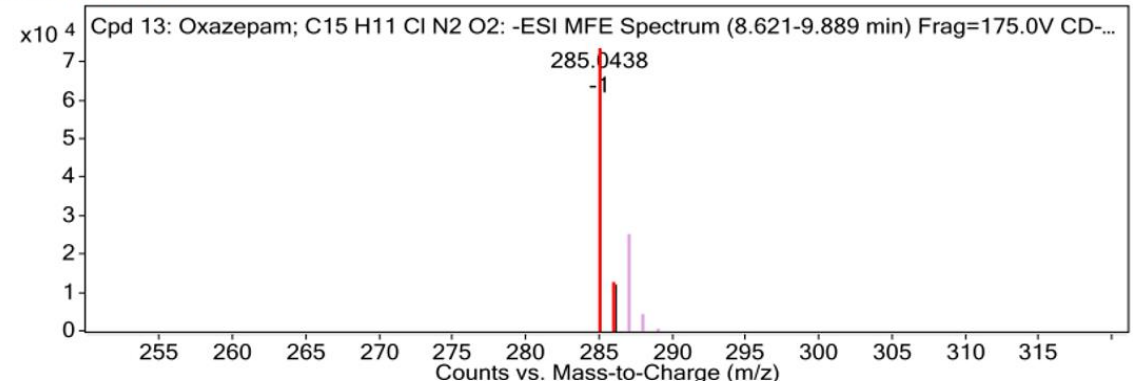
Oxazepan is a type of benzodiazepine and is us as a sedative, management of behavior, anxiety, tension and seizure disorder. It acts as CNS depressant through amplifying the neurological impacts of endogenous GABA, which is major regulatory neurotransmitter of Central nervous system. It is also used to treat alcohol withdrawal (Singh & Abdijadid, 2023) (Griffin, Kaye, Bueno, & Kaye, 2013).

Compound Label	Name	*m/z*	RT	Algorithm	Mass
Cpd 13: Oxazepam; C15 H11 Cl N2 O2	**Oxazepam**	285.0438	9.006	Find by Molecular Feature	286.0511

MFE MS Spectrum



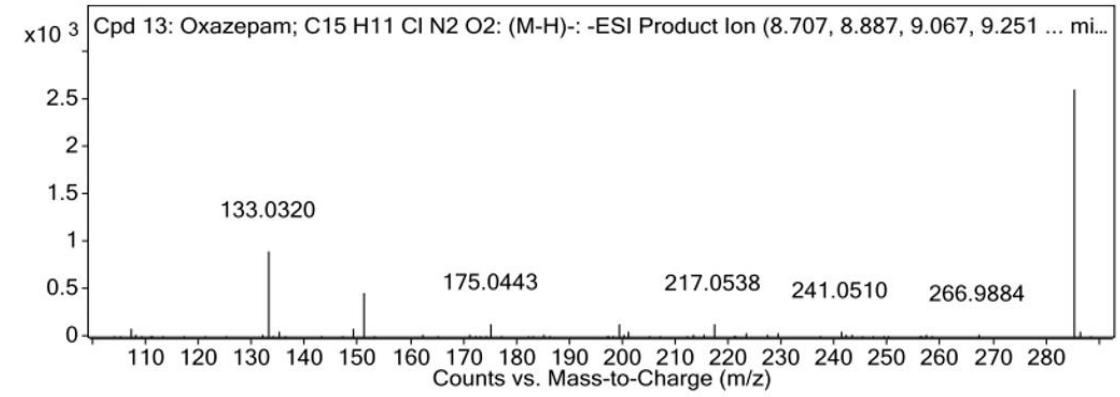
MFE MS Zoomed Spectrum



MS Spectrum Peak List

m/z	z	Abund	Formula	Ion
285.0438	-1	73476.34	C15 H11 Cl N2 O2	(M-H)-
286.0468	-1	12447.28	C15 H11 Cl N2 O2	(M-H)-

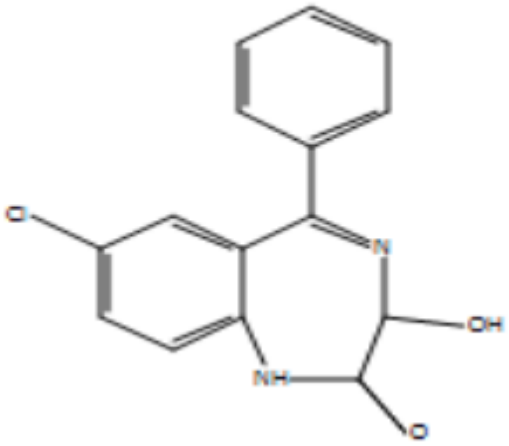
MSMS Spectrum



MS/MS Spectrum Peak List

m/z	z	Abund
107.0142	1	100.79
133.032	1	909.88
135.0481	1	60.36
149.0264	1	101.38
151.0061	1	472.24
175.0443	1	146.85
199.0432	1	135.82
217.0538	1	137.39
285.0432	1	2616.33
286.0244	1	67.16

Compound Structure



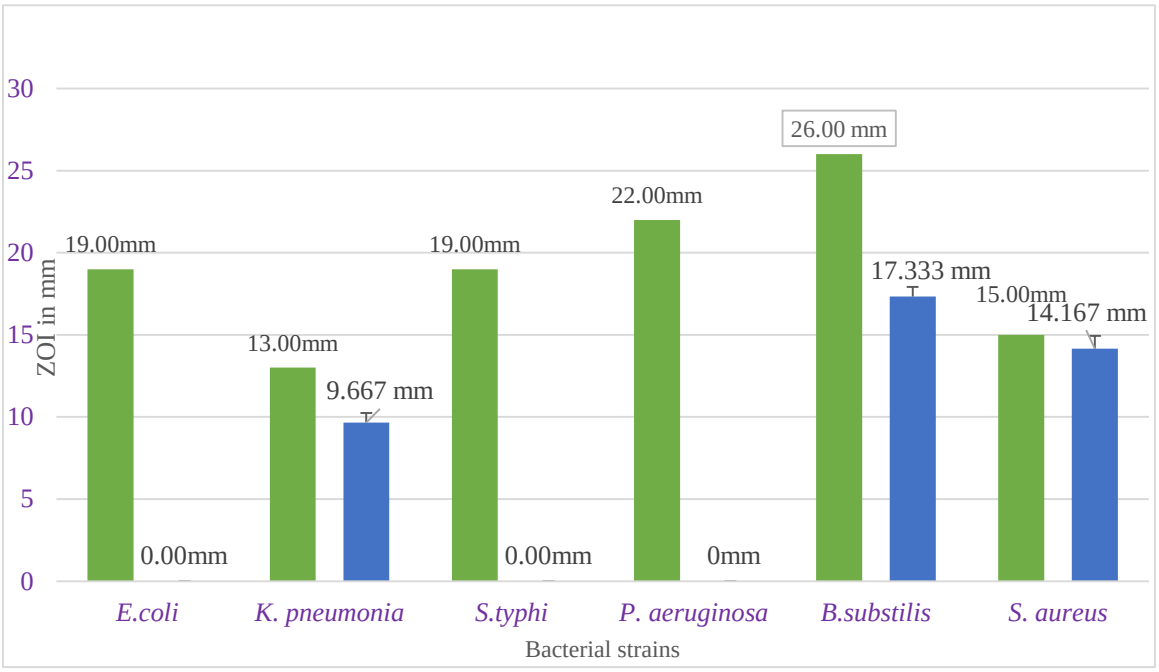
4.2.3. Antimicrobial activity:

The plant has useful phytochemicals which fight against the various micro-organism and inhibits their growth.

Table 4: ZOI shown by the methanol extract of C.macrophylla leaves.

S.no	Name of the micro organism	Name of the standard	Zone inhibition of standard (mm)	Zone of inhibition of 100 mg/ml of *C.macrophylla*			Average ZOI (mm)
				ZOI_1 (mm)	**ZOI_2 (mm)**	**ZOI_3 (mm)**	
1.	*Escherichia coli*	GEN 10 mcg	19	No activity	No activity	No activity	No activity
2.	*Klebsiella pneumonia*	E 15 mcg	13	10	9	10	9.667
3.	*Salmonella typhi*	GEN 10 mcg	19	No activity	No activity	No activity	No activity
4.	*Pseudomonas aeruginosa*	GEN 10 mcg	22	No activity	No activity	No activity	No activity
5.	*Bacillus subtilis*	E 15 mcg	26	17	18	18	17.333
6.	*Staphylococcus aureus*	VA 30 mcg	15	14	14	15	14.167

- GEN- Gentamicin, E- Erythromycin, VA- Vancomycin



Graph 1: Antimicrobial activity of standards versus extract

From this study, the crude methanoic extract of the leaves of *C.macrophylla* showed activity against both the gram positive bacteria tested, *Bacillus subtilis* & *Staphylococcus aureus* while it showed activity against a gram negative bacteria, *Klebsiella pneumonia* (ZOI= 9.667mm) only but it was inactive against the other three gram negative bacteria tested, *Escherichia coli*, *Staphylococcus typhi* & *Pseudomonas aeruginosa*. Highest zone of inhibition of 17.333 mm was observed against *Bacillus subtilis*, a gram positive bacteria, in comparison to other extract sensitive bacteria.

4.3. Quantitative Analysis of the Selected Phytochemicals

4.3.1. Total Phenol Content Determination

Different concentrations of Gallic acid was prepared for plotting the calibration curve. The sample extract of 250µg/mL was also prepared. Absorbance of the standard along with the

sample was observed at the wavelength of 760 nm in a UV-Visible spectrophotometer (Pg + double beam) and concentration v/s absorbance calibration curve was plotted for Gallic Acid. The total phenolic content was estimated as milligram of Gallic Acid equivalent by applying the standard calibration curve of Gallic acid. Absorbance of different Gallic acid concentrations is given in the table 5 below.

Table 5: Absorbance of Gallic acid at 760 nm

Concentration (µg/mL)	Absorbance			Average absorbance	SD Deviation
	A1	**A2**	**A3**		
0	0	0	0	0	0
2	0.173	0.17	0.175	0.173	0.003
4	0.324	0.337	0.34	0.334	0.009
6	0.481	0.474	0.486	0.48	0.006
8	0.662	0.674	0.667	0.668	0.006
10	0.876	0.863	0.865	0.868	0.007
12	1.01	1.033	1.017	1.02	0.012

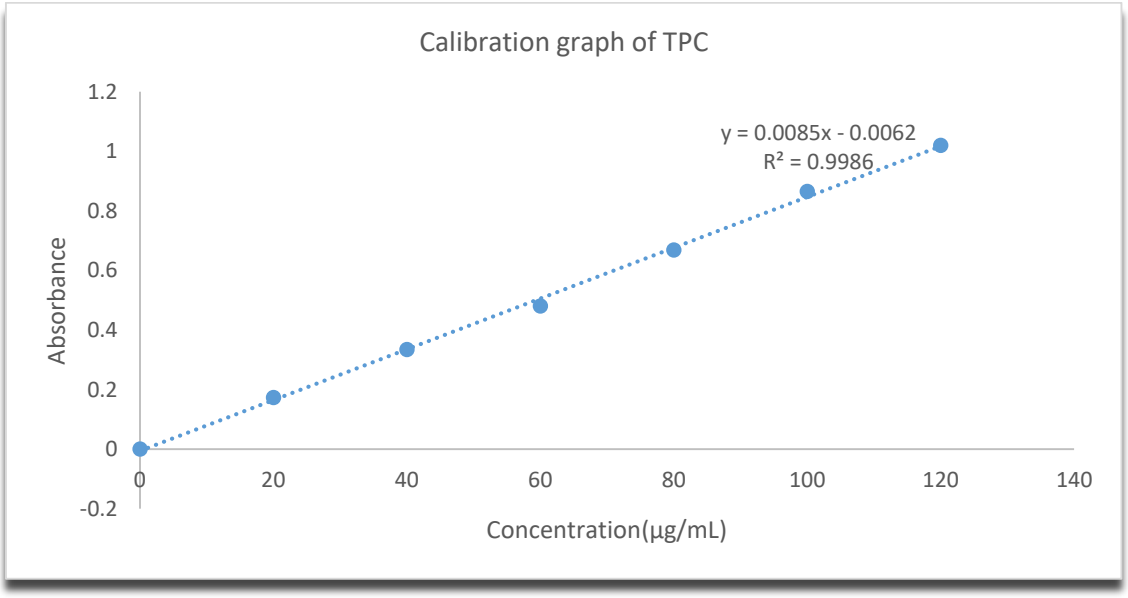


Figure 3: Standard correlation graph for TPC

The total phenol concentration of the leaf methanol extract of *C.macrophylla* was calculated by employing a linear regression equation derived from the Gallic acid curve obtained from graph.

The equation is as follows

$$y = 0.0085x - 0.0062$$

$$R^2 = 0.9986$$

Where,

y = absorbance;

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

m = slope = 0.0085

c = y–intercept = - 0.0062

$$(x = \frac{(y + 0.0062)}{0.0085})$$

The total phenol content along with the essential statistics is shown below:

Table 6: Total phenolic content in the methanol extract of C.macrophylla leaves

Conc. Of extract (mg/mL)	Conc. Of extract (g/mL)	Absorbance	GAC C (µg/mL)	GAC C (mg/mL)	TPC as GAE = $\boldsymbol{c} * \frac{\boldsymbol{v}}{\boldsymbol{m}}$ (mg/g)	TPC ± SD
2.5	0.0025	0.919	108.8471	0.108847	43.539	43.489 ± 0.175
2.5	0.0025	0.921	109.0824	0.109082	43.633	
2.5	0.0025	0.920	108.2353	0.108235	43.294	

From the above data and calibration graph, the total phenol content of methanol extract of *C.macrophylla* leaves was found to be 43.489±0.175 mg Gallic acid equivalent/g of dry extract.

4.3.2. Total Flavonoid Content Determination:

Flavonoids has various biochemical actions as well as therapeutic characteristics which includes antimicrobial, antithrombotic and carcinogenic action. Total flavonoid content was determined as mg RE/g of dried material.

Table 7: Absorbance of Rutin observed at 510 nm

Concentration (µg/mL)	Absorbance			Average absorbance	Standard deviation
	A_1	**A_2**	**A_3**		
0	0	0	0	0	0
25	0.199	0.206	0.196	0.2	0.005
50	0.431	0.429	0.429	0.43	0.001
75	0.606	0.601	0.607	0.605	0.003
100	0.758	0.748	0.751	0.752	0.005
150	1.061	1.058	1.062	1.06	0.002

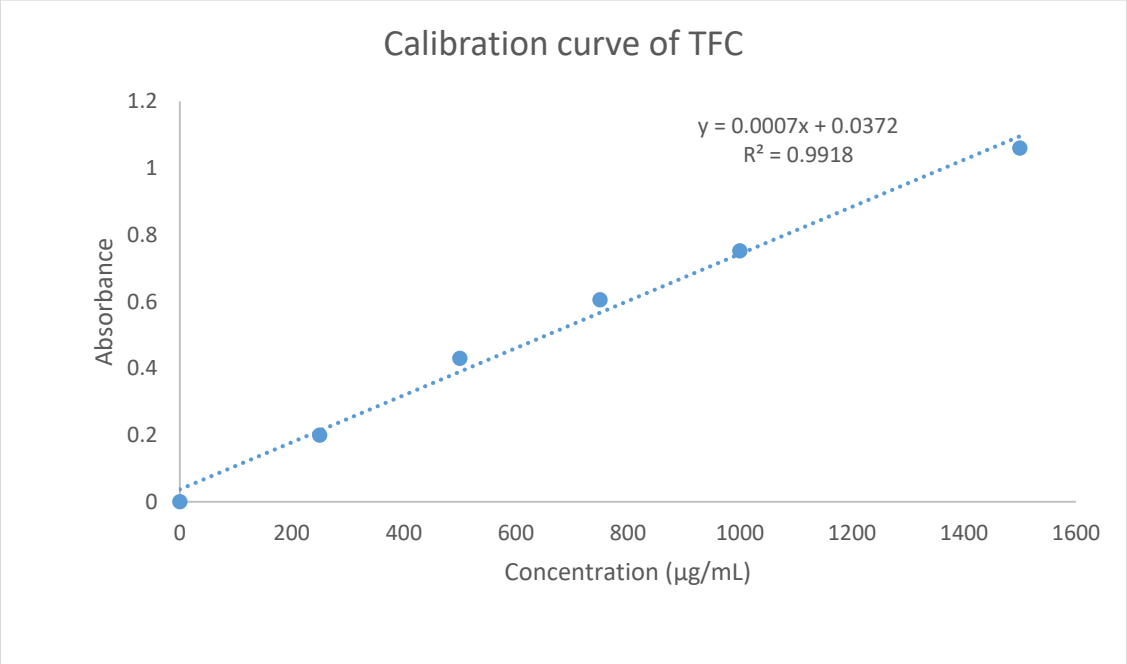


Figure 4: Standard correlation graph for TFC

The concentration of total flavonoids of the methanolic extract of *C.macrophylla* leaves was estimated by employing a linear regression equation derived from the standard Rutin curve obtained from graph.

The equation is as follows

$$y = 0.0007x + 0.0372$$

$$R^2 = 0.9918$$

Where,

y = absorbance;

x = Rutin concentration (RC) (µg/mL)

m = slope = 0.0007

c = y–intercept = 0.0372

$$x = \frac{(y - 0.0372)}{0.0007}$$

The total flavonoid content along with the essential statistics is shown below:

Table 8: Total flavonoid content in methanol extract of C.macrophylla leaves

Conc. of extract (mg/mL)	Extract weight (g/mL)	Absorbance	RC C (µg/mL)	RC C (mg/mL)	TFC as RE = $\boldsymbol{c} * \frac{\boldsymbol{v}}{\boldsymbol{m}}$ (mg/g)	TFC ± SD
10	0.01	1.363	1894.000	1.894000	189.400	189.829 ± 0.429
10	0.01	1.369	1902.571	1.902571	190.257	
10	0.01	1.366	1898.286	1.898286	189.829	

From the above table and graph, the total flavonoid content of methanol extract of *C.macrophylla* leaves was calculated to be 189.829±0.429 mg RE/g.

4.3.3. Total Antioxidant Capacity (Phosphomolybdenum Assay)

The total antioxidant capacity of the methanol extract of *C.macrophylla* leaves was calculated by using Phosphomolybdenum assay. The different concentration of ascorbic acid was used as reference for obtaining the calibration curve. The absorbance was observed at 695 nm.

Table 9: Absorbance of Ascorbic acid observed at 695 nm

Concentration (µg/mL)	Absorbance			Average absorbance ± SD
	A_1	**A_2**	**A_3**	
0	0	0	0	0.00 ± 0.00
40	0.481	0.486	0.476	0.481 ± 0.004
60	0.685	0.681	0.686	0.684 ± 0.002
80	0.869	0.854	0.864	0.862 ± 0.006
100	1.235	1.249	1.239	1.241 ± 0.006
120	1.354	1.356	1.362	1.357 ±0.003
140	1.677	1.672	1.677	1.675 ± 0.002

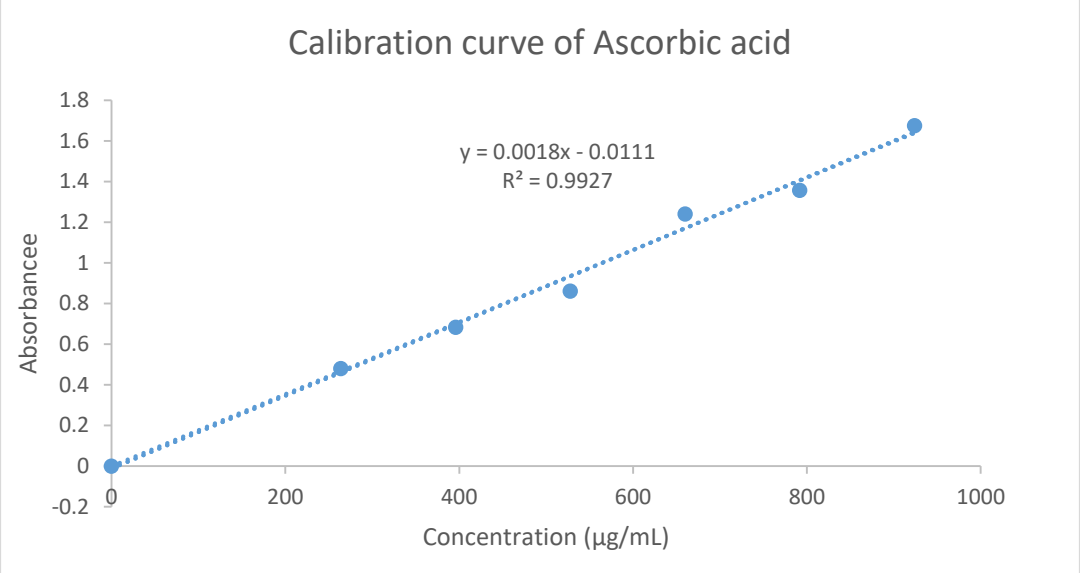


Figure 5: Standard correlation graph for TAC

The total antioxidant capacity of the methanolic extract of *C.macrophylla* leaves was calculated by employing the linear regression equation derived from the Ascorbic acid curve obtained from the graph.

The equation is as follows

$$y = 0.0018x - 0.0111$$

$$R^2 = 0.9927$$

Where,

y = absorbance;

x = Ascorbic acid concentration (AAC) (µg/mL)

m = slope = 0.0018

c = y–intercept = - 0.0111

$$x = \frac{(y + 0.0111)}{0.0018}$$

The total antioxidant content along with the essential statistics is shown below:

Table 10: Total antioxidant capacity of the methanol extract of C.macrophylla leaves

Extract concentration (mg/mL)	extract weight (g/mL)	Absorbance	AAC C (µg/mL)	AAC C (mg/mL)	TFC as AAE = $c \times v / m$ (mg/g)	TPC ± SD
6.6	0.0066	0.745	420.0556	0.4200556	63.645	63.953 ± 0.271
6.6	0.0066	0.750	422.8333	0.4228333	64.066	
6.6	0.0066	0.751	423.3889	0.4233889	64.150	

From the above table and figure, the total antioxidant capacity the methanol extract of *C. macrophylla* leaves was obtained to be 63.953±0.271 mg ascorbic acid equivalent/g of dry extract indicating the plant extract contains antioxidant properties

CHAPTER-5
CONCLUSION AND RECOMMENDATION

The biological analysis of the methanolic extract of *Callicarpa macrophylla* leaves from Ramjakot, Tanahun resulted in the presence of alkaloids, flavonoids, phenols, saponnins, amino acids, coumarins, gums and resins, fixed oils and fats.

The anti-bacterial test of the extract revealed the zone of inhibition of 9.667mm, 17.333mm, and 14.167mm against *Klebsiella pneumonia, Bacillus subtilis* and *Staphylococcus aureus*, respectively, while it was inactive against rest of the tested bacteria, i.e., *Escherichia coli, Salmonella typhi* and *Pseudomonas aeruginosa*. This infers that *C.macrophylla* extract is active against gram positive bacterial strains only.

The methanolic extract of *C.macrophylla* showed the total phenolic content of about 43.489±0.175 mg Gallic acid equivalent/g of the dry material while the flavonoid content was found to be 189.8287±0.429 mg/g Rutin equivalent/g of dry material. The total antioxidant capacity was estimated to be 63.953±0.271 mg Ascorbic acid/g of the dry material.

The HRLCMS spectra of the extract showed a total of 75 compounds out of which 70 compounds were known and the remaining 5 compounds were unknown compounds. There were 16 compounds belonging to terpenoids, 6 compounds belonging to amino acids, 5 compounds belonging to steroids, and 4 compounds belonging to esters. 12 compounds out of the 70 known compounds have high medicinal value and are in present use and 3 compounds are used by the pharmaceutical industries. The compounds like Sakacin A & 6,10,14-trimethyl-5,9,13-pentavecatrene-2-one are used as bio preservatives and flavoring agents in food industry. The compound Mitoxantrone is a throcenedione derived drug and is being used as chemotherapeutic drug for Nonlymphocytic leukemia, peripheral T-cell Lymphoma (Orphan) and secondary progressive multiple sclerosis. Methotrimeprazine, a phenothiazine derived drug is used for schizophrenia and bipolar disorder due to its histamine-antagonist effects on the CNS. Flurandrenolide is a

corticosteroid derived drug used as cream or ointment of skin diseases due to its anti-inflammatory, antipruritic and vaso-constrictive actions. Oxazepan, a sedative drug and used in the treatment of seizure. On the other hand, presence of anti-pathogenic compounds such as Sakacin, Antimycin A and Pleuromutilins in the extract showed positive results to anti-bacterial test against selected bacterial strains. Probol, 4-amino-2-6-dimitrotouluene and diplodiatoxin observed in the HR-LCMS spectra are harmful compounds.

Traditionally, *Callicarpa macrophylla* leaves have been used to treat cough, inflammation, mouth ulcer, etc. HR-LCMS spectra of the leaf extract of the plant revealed that it contains compounds that exhibit anti-inflammatory, analgesic effect and compounds that treat insomnia and cough. The traditional use of the plant could thus be correlated to those compounds present in the extract

Recommendations:

The bioactive compounds in this study were extracted from single solvent i.e. methanol. Since not all the compounds can be dissolved in the single solvent hence comparative research on phytochemical activities of different solvents is essential. Further, isolation, purification and characterization of the bioactive compounds from leaves of *C.macrophylla* is also promising. The study for cytotoxic effect by brine shrimp lethality assay (BSLA) for preliminary screening and anticancer property should be examined in human cancer cell lines. The study of isolation of vital bioactive compounds could lead to finding new drugs in the time ahead.

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APPENDIX- I: PHYTOCHEMICAL SCRENNING METHODS

Phytochemical Analysis

Test for alkaloids

Mayer's test

2 mL of filtrate was treated with 1 mL of Mayer's reagent (Potassium mercuric iodide solution). Appearance of cream colored precipitate indicates the positive results of alkaloids.

Hager's test/ Picric Acid test

To a 2mL of filtrate, few drops of freshly prepared Hager's reagent (saturated picric acid) was added. Formation of yellow precipitate indicates the presence of alkaloids.

Wagner's test

To a 2 mL of filtrate, few drops of Wagner's reagent was added carefully along the side of the tube. Appearance of brown color precipitate confirms the presence of alkaloids.

Test for flavonoids

Alkaline reagent test

To a small amount of filtrate, few drops of 2%NaOH was added. Appearance of intense yellow color which becomes colorless on the addition of dil. acid indicates the presence of flavonoids.

Lead acetate test

To 1 mL of filtrate, add few drops of 10% lead acetate solution. Formation of yellow color precipitation indicates the presence of flavonoids.

Pew's test

To a 2 mL of filtrate, 0.1 g of zinc was added and 8 mL conc. HCl was added drop wise slowly. Appearance of purple red or cherry red indicates the positive results of flavonoids.

Test for phenols

Ferric chloride test

To 2 mL of filtrate, 10% ferric chloride (freshly prepared) was added. Formation of blue or green color indicates the presence of phenols.

Liebermann's test

About 2 mL of filtrate and sodium nitrite (acidic) was heated and allowed to cool. Then dil.H_2S0_4 and excess of dil. NaOH was added.

Lead acetate test

2mL of filtrate was treated with small amount of 10% Lead acetate solution was added. Appearance of bulky white precipitate indicates the positive results of phenols.

Test for saponins.

Froth test

0.5 g of extract was dissolved in 10 mL of distilled water and shook well. Formation of forth which persists even after warming in water bath for 5 minutes indicates the presence of saponins.

Foam test

5 mL of extract was dissolved in 5 mL of distilled water, heated to boil and filtered. The filtrate was again mixed with 3mL of distilled water and shake well for 5 minutes. Appearance of foam after shaking designates the presence of saponins.

Test for tannins

Ferric chloride test

To a small amount of aqueous extract, add few drops of 5% $FeCl_3$ solution. Formation of black or blue-green coloration or precipitation indicates the presence of tannins.

Potassium dichromate test

To a 5 mL of aqueous extract, add 1ml of 10% aqueous potassium dichromate solution. Formation of yellowish brown precipitate indicates the tannins.

Gelatin test

To a 5 mL of aqueous extract, add 1% gelatin solution and 10% NaCl solution. Appearance of a white precipitate indicates the presence of tannins.

Test for cardiac glycosides.

Modified borntrager's test

To a 2mL of extract, a few drops of $FeCl_3$ solution was added and boiled for 5 minutes and cooled down. Add equal volume of benzene, separate layer of benzene was formed and

treated with a few drops of ammonium solution. Appearance of a rose –pink to blood red colored solution indicates the presence the glycosides.

Keller killani test

1mL of filtrate was treated with 2mL of glacial acetic acid and a few drops of freshly prepared $FeCl_3$ was added then Conc. H_2SO_4 was added along the side of the test tubes. Appearance of brown ring at the interference indicates the positive results of cardiac glycosides.

Legal's test

50 mg of extract was dissolved in pyridine and then treated with sodium nitropruside solution (freshly prepared). Then, 10% Na0H solution was added to make the solution alkaline. Appearance of pink color indicates the presence of glycosides.

Test for Carbohydrates

100 mg of extract was dissolved in the distilled water and filtered. The filtrate was subjected to various test of carbohydrates.

Molish's test

2 mL of filtrate was treated with 2 drops of alcoholic α-naphthanol and shook well. Then conc. H_2SO_4 was added alongside of the test tube slowly. Formation of violet ring at the junction indicates the presence of carbohydrates.

Benedict's test

Benedict's reagent was added to a small amount of filtrate and heat gently for 2 minutes. Formation of orange-red precipitate indicates the presence of reducing sugars.

Fehling's test

Equal amount of filtrate was heated with Fehling's A and B solution. Formation of red precipitation indicates the presence of carbohydrates.

Test for terpenoids

Chloroform/ Salkowski test

To 100 mg of extract, 2 mL of chloroform was added and then 2 mL of conc. H_2SO_4 was added along the test tube. Presence of terpenoids was confirmed by reddish brown color appearance at the interface.

Test for amino acids and proteins

Millon's test

2 mL of filtrate was treated with few drops of Millon's reagent. Appearance of flesh to red precipitate indicates the positive to amino test.

Ninhydrin test

To 2 mL of filtrate, 2 drops of ninhydrin solution is added. Appearance of purple color indicates the presence of amino acids.

Xanthoproteic test

To a 2 mL of filtrate, few drops of conc. Nitric acid. Appearance of yellow precipitate indicates the presence of proteins.

Test for Coumarins

To a 1mL of the sample solution, 10% of NaOH was added. Appearance of yellow color indicates the presence of coumarins.

Test for phlobatannins

To a 2 mL of aqueous sample, 1% of 1 mL hydrochloric acid was added and boiled for few minutes. Phlobatannins was confirmed by the appearance of red precipitation.

Test for Quinones

To a 2mL of extract, few drops of conc.H_2SO_4 was added. Color formation indicates the presence of quinones

Test for gums and resins

To 20 mL of distilled water, 15 mL of (ethanol extract) filtrate was added. Presence of resins is confirmed by the precipitation.

Test for fixed oils and fats

Spot test

A small quantity of extract is pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oils.

APPENDIX- II: HR-LCMS INSTRUMENT AND ACQUISITION METHOD

Acquisition Method Info

Method Name	metabolite_ESI_+VE_MSMS.m
Method Path	D:\MassHunter\Methods\2022\metabolite_ESI_+VE_MSMS.m
Method Description	Default Method

Device List

HiP Sampler
Binary Pump
Column Comp.
Q-TOF

TOF/Q-TOF Mass Spectrometer

Component Name	MS Q-TOF	**Component Model**	G6550A
Ion Source	Dual AJS ESI	**Stop Time (min)**	30.00
Can wait for temp.	Enable	**Fast Polarity**	N/A
MS Abs. threshold	200	**MS Rel. threshold(%)**	0.010
MS/MS Abs. threshold	5	**MS/MS Rel. threshold(%)**	0.010
Tune File	AutoTune (3).tun		

Time Segments

Time Segment #	Start Time (min)	Diverter Valve State	Storage Mode	Ion Mode
1	0	MS	Both	Dual AJS ESI

Time Segment 1

Acquisition Mode AutoMS2

MS Min Range (m/z)	126
MS Max Range (m/z)	1200
MS Scan Rate (spectra/sec)	1.00
MS/MS Scan Rate (spectra/sec)	1.00
Isolation Width MS/MS	Medium (~4 amu)

Ramped Collision Energy

Charge	Slope	Offset
1	8	-2.6
2	6	-2.6
3	4	-2.6

Auto MS/MS Preferred/Exclude Table

Mass	Delta Mass (ppm)	Charge	Type	Retention Time (min)	Delta Ret. Time (min)	Isolation Width	Collision Energy
197.8075	500	1	Exclude	0		Medium (~4 amu)	

Precursor Selection

Max Precursors Per Cycle	10
Threshold (Abs)	10000
Threshold (Rel)(%)	0.010
Precursor abundance based scan speed	Yes
Target (counts/spectrum)	25000.000
Use MS/MS accumulation time limit	Yes
Use dynamic precursor rejection	No
Purity Stringency (%)	100.000
Purity Cutoff (%)	30.000
Isotope Model	Common
Active exclusion enabled	Yes
Active exclusion excluded after (spectra)	1
Active exclusion released after (min)	0.20
Sort precursors	By abundance only

Charge State Preference

Selected Charges
1
2
Unk

Source Parameters

Parameter	Value
Gas Temp (°C)	250
Gas Flow (l/min)	13
Nebulizer (psig)	35
SheathGasTemp	300
SheathGasFlow	11

Scan Segments

Scan Seg #	Ion Polarity	Collision Energy
1	Positive	0

Scan Segment 1

Scan Source Parameters

Parameter	Value
VCap	3500
Nozzle Voltage (V)	1000
Fragmentor	175
Skimmer1	65
OctopoleRFPeak	750

ReferenceMasses

Ref Mass Enabled	Disabled
Ref Nebulizer (psig)	

Chromatograms

Chrom Type	Label	Offset	Y-Range
TIC	TIC	15	10000000

Acquisition Method Report

Agilent Technologies

Name: HiP Sampler **Model:** G4226A

Auxiliary	
Draw Speed	100.0 µL/min
Eject Speed	100.0 µL/min
Draw Position Offset	0.0 mm
Wait Time After Drawing	2.0 s
Sample Flush Out Factor	5.0
Vial/Well bottom sensing	Yes
Injection	
Injection Mode	Injection with needle wash
Injection Volume	5.00 µL
Needle Wash	
Needle Wash Location	Flush Port
Wash Time	3.0 s
High throughput	
Automatic Delay Volume Reduction	No
Overlapped Injection	
Enable Overlapped Injection	No
Valve Switching	
Valve Movements	0
Valve Switch Time 1	
Switch Time 1 Enabled	Yes
Switch Time 1	0.01 min
Valve Switch Time 2	
Switch Time 2 Enabled	No
Valve Switch Time 3	
Switch Time 3 Enabled	No
Valve Switch Time 4	
Switch Time 4 Enabled	No
Stop Time	
Stoptime Mode	As pump/No limit
Post Time	
Posttime Mode	Off

Acquisition Method Report

Agilent Technologies

Name: Binary Pump Model: G4220B

Flow	0.300 mL/min
Use Solvent Types	Yes
Stroke Mode	Synchronized
Low Pressure Limit	0.00 bar
High Pressure Limit	1200.00 bar
Max. Flow Ramp Up	100.000 mL/min²
Max. Flow Ramp Down	100.000 mL/min²
Expected Mixer	No check
Stroke A	
Automatic Stroke Calculation A	Yes
Stop Time	
Stoptime Mode	Time set
Stoptime	35.00 min
Post Time	
Posttime Mode	Off

Solvent Composition

	Channel	Ch. 1 Solv.	Name 1	Ch2 Solv.	Name 2	Selected	Used	Percent
1	A	100.0 % Water V.02	0.1% FA in water	100.0 % Water V.02	0.1% FA in water	Ch. 2	Yes	95.00 %
2	B	100.0 % Methanol V.03		100.0 % Acetonitrile V.02		Ch. 2	Yes	5.00 %

Timetable

	Time	A	B	Flow	Pressure
1	1.00 min	95.00 %	5.00 %	0.300 mL/min	1200.00 bar
2	25.00 min	0.00 %	100.00 %	0.300 mL/min	1200.00 bar
3	30.00 min	0.00 %	100.00 %	0.300 mL/min	1200.00 bar
4	31.00 min	95.00 %	5.00 %	0.300 mL/min	1200.00 bar
5	35.00 min	95.00 %	5.00 %	0.300 mL/min	1200.00 bar

Name: Column Comp. Model: G1316C

Ready when front door open	Yes
Left Temperature Control	
Temperature Control Mode	Temperature Set
Temperature	40.00 °C
Enable Analysis Left Temperature	
Enable Analysis Left Temperature On	Yes
Enable Analysis Left Temperature Value	0.80 °C
Right Temperature Control	
Right temperature Control Mode	Temperature Set
Right temperature	40.00 °C
Enable Analysis Right Temperature	
Enable Analysis Right Temperature On	Yes
Enable Analysis Right Temperature Value	0.80 °C
Stop Time	
Stoptime Mode	As pump/injector
Post Time	
Posttime Mode	Off

APPENDIX- III: HR-LCMS PROFILE OF COMPOUNDS OBSERVED IN POSITIVE ESI MODE

Qualitative Compound Report

Data File	CD-06.d	**Sample Name**	CD-06
Sample Type	Sample	**Position**	P1-F1
Instrument Name	QTOF	**User Name**	
Acq Method	metabolite_ESI_+VE_MSMS.m	**Acquired Time**	8/7/2023 9:21:56 AM
IRM Calibration Status	Success	**DA Method**	Default.m
Comment			

Sample Group **Info.**
Acquisition SW 6200 series TOF/6500 series
Version Q-TOF B.05.01 (B5125.3)

Compound Table

Compound Label	RT	Mass	Name	Formula	MFG Formula	DB Formula	DB Diff (ppm)	Hits (DB)
Cpd 1: 4-Amino-2,6-dinitrotoluene; C7 H7 N3 O4	1.027	197.0435	4-Amino-2,6-dinitrotoluene	C7 H7 N3 O4	C7 H7 N3 O4	C7 H7 N3 O4	0.9	10
Cpd 2: Citalopram-N-Oxide; C20 H21 F N2 O2	1.092	340.1592	Citalopram-N-Oxide	C20 H21 F N2 O2	C20 H21 F N2 O2	C20 H21 F N2 O2	-1.42	3
Cpd 3: Trimeprazine; C18 H22 N2 S	1.106	298.1494	Trimeprazine	C18 H22 N2 S	C18 H22 N2 S	C18 H22 N2 S	6.61	1
Cpd 4: N-Isovalerylglycine methyl ester; C8 H15 N O3	1.139	173.1054	N-Isovalerylglycine methyl ester	C8 H15 N O3	C8 H15 N O3	C8 H15 N O3	6.68	8
Cpd 5: Medicanine; C7 H13 N O3	1.167	159.0883	Medicanine	C7 H13 N O3	C7 H13 N O3	C7 H13 N O3	7.65	10
Cpd 6: Isoamyl nitrite; C5 H11 N O2	1.167	117.0776	Isoamyl nitrite	C5 H11 N O2	C5 H11 N O2	C5 H11 N O2	11.73	9
Cpd 7: 3beta,6beta-Dihydroxynortropane; C7 H13 N O2	1.249	143.0935	3beta,6beta-Dihydroxynortropane	C7 H13 N O2	C7 H13 N O2	C7 H13 N O2	7.61	3
Cpd 8: Dibenzothiophene; C12 H8 S	2.894	184.033	Dibenzothiophene	C12 H8 S	C12 H8 S	C12 H8 S	8.93	1
Cpd 9: Paramethasone; C22 H29 F O5	5.103	392.2	Paramethasone	C22 H29 F O5	C22 H29 F O5	C22 H29 F O5	-0.32	10
Cpd 10: Flurandrenolide; C24 H33 F O6	5.502	436.226	Flurandrenolide	C24 H33 F O6	C24 H33 F O6	C24 H33 F O6	0.23	5
Cpd 11: Lucidenic acid A; C27 H38 O6	5.833	458.2702	Lucidenic acid A	C27 H38 O6	C27 H38 O6	C27 H38 O6	-7.39	3
Cpd 12: Tuckolin F15; C20 H26 O4	11.169	330.1789	Tuckolin F15	C20 H26 O4	C20 H26 O4	C20 H26 O4	12.82	7
Cpd 13: Ethyl 2E,4Z-hexadecadienoate; C18 H32 O2	11.18	280.2402	Ethyl 2E,4Z-hexadecadienoate	C18 H32 O2	C18 H32 O2	C18 H32 O2	0.28	10
Cpd 14: 6,10,14-Trimethyl-5,9,13-pentadecatrien-2-one; C18 H30 O	11.186	262.2296	6,10,14-Trimethyl-5,9,13-pentadecatrien-2-one	C18 H30 O	C18 H30 O	C18 H30 O	0.12	10
Cpd 15: 4,4-Difluoro-17beta-hydroxy-17alpha-methyl-androst-5-en-3-one; C20 H28 F2 O2	11.233	338.2071	4,4-Difluoro-17beta-hydroxy-17alpha-methyl-androst-5-en-3-one	C20 H28 F2 O2	C20 H28 F2 O2	C20 H28 F2 O2	-3.95	10
Cpd 16: Methotrimeprazine; C19 H24 N2 O S	11.763	328.1624	Methotrimeprazine	C19 H24 N2 O S	C19 H24 N2 O S	C19 H24 N2 O S	-4.46	5
Cpd 17: Dimethyl 3-methoxy-4-oxo-5-(8,11,14-pentadecatrienyl)-2-hexenedioate; C24 H36 O6	12.034	420.2493	Dimethyl 3-methoxy-4-oxo-5-(8,11,14-pentadecatrienyl)-2-hexenedioate	C24 H36 O6	C24 H36 O6	C24 H36 O6	4.54	4
Cpd 18: Dimethyl 3-methoxy-4-oxo-5-(8,11,14-pentadecatrienyl)-2-hexenedioate; C24 H36 O6	12.252	420.2491	Dimethyl 3-methoxy-4-oxo-5-(8,11,14-pentadecatrienyl)-2-hexenedioate	C24 H36 O6	C24 H36 O6	C24 H36 O6	4.93	4
Cpd 19: Valdiate; C17 H26 O5	12.958	310.1763	Valdiate	C17 H26 O5	C17 H26 O5	C17 H26 O5	5.67	3
Cpd 20: Santin; C18 H16 O7	13.076	344.0874	Santin	C18 H16 O7	C18 H16 O7	C18 H16 O7	6.33	10
Cpd 21: 6,10,14-Trimethyl-5,9,13-pentadecatrien-2-one; C18 H30 O	13.147	262.2299	6,10,14-Trimethyl-5,9,13-pentadecatrien-2-one	C18 H30 O	C18 H30 O	C18 H30 O	-0.86	10
Cpd 22: Mitoxantrone; C22 H28 N4 O6	13.396	444.2003	Mitoxantrone	C22 H28 N4 O6	C22 H28 N4 O6	C22 H28 N4 O6	-4.63	2
Compound 23	13.433	227.2597						
Cpd 24: Mitoxantrone; C22 H28 N4 O6	13.56	444.2007	Mitoxantrone	C22 H28 N4 O6	C22 H28 N4 O6	C22 H28 N4 O6	-4.08	2
Cpd 25: [7]-Paradol; C18 H28 O3	14.777	292.2032	[7]-Paradol	C18 H28 O3	C18 H28 O3	C18 H28 O3	5.67	10
Cpd 26: Isolimonic acid; C26 H34 O10	15.476	506.2181	Isolimonic acid	C26 H34 O10	C26 H34 O10	C26 H34 O10	-5.77	2
Cpd 27: Phthalic acid Mono-2-ethylhexyl Ester; C16 H22 O4	16.958	278.1502	Phthalic acid Mono-2-ethylhexyl Ester	C16 H22 O4	C16 H22 O4	C16 H22 O4	6.16	9
Cpd 28: Lucidenic acid M; C27 H42 O6	17.493	462.2988	Lucidenic acid M	C27 H42 O6	C27 H42 O6	C27 H42 O6	-1.47	4
Cpd 29: Cavipetin D; C25 H38 O5	17.548	418.2728	Cavipetin D	C25 H38 O5	C25 H38 O5	C25 H38 O5	-1.99	3
Compound 30	17.62	736.49						
Compound 31	17.62	550.344						

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Qualitative Compound Report

Cpd 32: (3S,3'S,5R,5'R,6R)-6,7-Didehydro-5,6-dihydro-3,3',5,8'-tetrahydroxy-beta,kappa-caroten-6'-one; C40 H56 O5	17.668	616.4157	(3S,3'S,5R,5'R,6R)-6,7-Didehydro-5,6-dihydro-3,3',5,8'-tetrahydroxy-beta,kappa-caroten-6'-one	C40 H56 O5	C40 H56 O5	C40 H56 O5	-4.78	3
Cpd 33: N-tert-Butyloxycarbonyl-deacetyl-leupeptin; C23 H44 N6 O5	17.82	484.3361	N-tert-Butyloxycarbonyl-deacetyl-leupeptin	C23 H44 N6 O5	C23 H44 N6 O5	C23 H44 N6 O5	2.44	3
Cpd 34: Acetylvalerenolic acid; C17 H24 O4	17.828	292.165	Acetylvalerenolic acid	C17 H24 O4	C17 H24 O4	C17 H24 O4	8.45	8
Cpd 35: Ganosporelactone A; C30 H40 O7	21.177	512.2787	Ganosporelactone A	C30 H40 O7	C30 H40 O7	C30 H40 O7	-2.57	10
Cpd 36: Ganosporelactone A; C30 H40 O7	21.53	512.2788	Ganosporelactone A	C30 H40 O7	C30 H40 O7	C30 H40 O7	-2.73	10
Cpd 37: Antimycin A1; C28 H40 N2 O9	22.932	548.2752	Antimycin A1	C28 H40 N2 O9	C28 H40 N2 O9	C28 H40 N2 O9	-3.37	1
Cpd 38: D8'-Merulinic acid A; C24 H38 O4	23.11	390.274	D8'-Merulinic acid A	C24 H38 O4	C24 H38 O4	C24 H38 O4	7.65	10
Cpd 39: Antimycin A1; C28 H40 N2 O9	23.301	548.2752	Antimycin A1	C28 H40 N2 O9	C28 H40 N2 O9	C28 H40 N2 O9	-3.39	1
Cpd 40: D8'-Merulinic acid A; C24 H38 O4	23.39	390.2739	D8'-Merulinic acid A	C24 H38 O4	C24 H38 O4	C24 H38 O4	7.84	10
Compound 41	24.072	337.3319						

APPENDIX- IV: HR-LCMS PROFILE OF COMPOUNDS OBSERVED IN NEGATIVE ESI MODE.

Qualitative Compound Report

Data File	CD-06_-ve.d	**Sample Name**	CD-06
Sample Type	Sample	**Position**	P1-F1
Instrument Name	QTOF	**User Name**	
Acq Method	metabolite_ESI_-VE_MSMS.m	**Acquired Time**	8/10/2023 1:56:46 AM
IRM Calibration Status	Success	**DA Method**	Default.m
Comment			

Sample Group **Info.**
Acquisition SW 6200 series TOF/6500 series
Version Q-TOF B.05.01 (B5125.3)

Compound Table

Compound Label	RT	Mass	Name	Formula	MFG Formula	DB Formula	DB Diff (ppm)	Hits (DB)
Cpd 1: Dictyoquinazol C; C18 H18 N2 O5	1.119	342.1201	Dictyoquinazol C	C18 H18 N2 O5	C18 H18 N2 O5	C18 H18 N2 O5	4.34	10
Cpd 2: O-1,4-α-L-Dihydrostreptosyl-streptidine 6-phosphate; C14 H29 N6 O11 P	3.97	488.1577	O-1,4-α-L-Dihydrostreptosyl-streptidine 6-phosphate	C14 H29 N6 O11 P	C14 H29 N6 O11 P	C14 H29 N6 O11 P	11.2	2
Cpd 3: Quercetin 3,7-dirhamnoside; C27 H30 O15	5.27	594.1611	Quercetin 3,7-dirhamnoside	C27 H30 O15	C27 H30 O15	C27 H30 O15	-7.84	10
Cpd 4: Citbismine A; C35 H32 N2 O10	5.667	640.2056	Citbismine A	C35 H32 N2 O10	C35 H32 N2 O10	C35 H32 N2 O10	0.19	4
Cpd 5: Lavandulifolioside; C34 H44 O19	6.221	756.2526	Lavandulifolioside	C34 H44 O19	C34 H44 O19	C34 H44 O19	-6.54	2
Cpd 6: Citbismine F; C36 H34 N2 O10	6.376	654.2206	Citbismine F	C36 H34 N2 O10	C36 H34 N2 O10	C36 H34 N2 O10	1.09	2
Cpd 7: Isoacteoside; C29 H36 O15	6.562	624.2115	Isoacteoside	C29 H36 O15	C29 H36 O15	C29 H36 O15	-9.71	5
Cpd 8: Leonoside A; C35 H46 O19	6.596	770.2682	Leonoside A	C35 H46 O19	C35 H46 O19	C35 H46 O19	-6.27	2
Cpd 9: Isoacteoside; C29 H36 O15	6.974	624.2106	Isoacteoside	C29 H36 O15	C29 H36 O15	C29 H36 O15	-8.35	5
Cpd 10: Cinncassiol D2; C20 H32 O6	7.355	368.2242	Cinncassiol D2	C20 H32 O6	C20 H32 O6	C20 H32 O6	-11.75	10
Cpd 11: Steviobin G; C20 H34 O5	7.405	354.2445	Steviobin G	C20 H34 O5	C20 H34 O5	C20 H34 O5	-11.81	10
Cpd 12: Nigakihemiacetal A; C22 H34 O7	8.964	410.2294	Nigakihemiacetal A	C22 H34 O7	C22 H34 O7	C22 H34 O7	-12.17	10
Cpd 13: Oxazepam; C15 H11 Cl N2 O2	9.006	286.0511	Oxazepam	C15 H11 Cl N2 O2	C15 H11 Cl N2 O2	C15 H11 Cl N2 O2	-0.68	10
Compound 14	9.084	396.2555						
Cpd 15: Auxin a; C18 H32 O5	10.054	328.2289	Auxin a	C18 H32 O5	C18 H32 O5	C18 H32 O5	-11.85	4
Cpd 16: Corchorifatty acid F; C18 H32 O5	10.884	328.2286	Corchorifatty acid F	C18 H32 O5	C18 H32 O5	C18 H32 O5	-11.18	5
Cpd 17: Zoapatanol; C20 H34 O4	11.083	338.2455	Zoapatanol	C20 H34 O4	C20 H34 O4	C20 H34 O4	-10.48	8
Cpd 18: Phorbol; C20 H28 O6	11.1	364.1933	Phorbol	C20 H28 O6	C20 H28 O6	C20 H28 O6	-13.87	10
Cpd 19: Diplodiatoxin; C18 H28 O4	11.847	308.2021	Diplodiatoxin	C18 H28 O4	C18 H28 O4	C18 H28 O4	-10.88	10
Cpd 20: PGB2; C20 H30 O4	12.048	334.2181	PGB2	C20 H30 O4	C20 H30 O4	C20 H30 O4	-11.16	10
Cpd 21: Fortimicin AP; C14 H30 N4 O5	12.221	334.2184	Fortimicin AP	C14 H30 N4 O5	C14 H30 N4 O5	C14 H30 N4 O5	9.65	10
Cpd 22: Nakacin A; C12 H24 N4 O4	12.447	288.1769	Nakacin A	C12 H24 N4 O4	C12 H24 N4 O4	C12 H24 N4 O4	11.4	10
Cpd 23: Sulfaphenazole; C15 H14 N4 O2 S	12.909	314.0811	Sulfaphenazole	C15 H14 N4 O2 S	C15 H14 N4 O2 S	C15 H14 N4 O2 S	1.92	10
Cpd 24: Austalide J; C25 H32 O7	13.28	444.2102	Austalide J	C25 H32 O7	C25 H32 O7	C25 H32 O7	10.44	2
Cpd 25: Austalide J; C25 H32 O7	13.47	444.21	Austalide J	C25 H32 O7	C25 H32 O7	C25 H32 O7	10.85	2
Cpd 26: Pleuromutilin; C22 H34 O5	13.753	378.2447	Pleuromutilin	C22 H34 O5	C22 H34 O5	C22 H34 O5	-10.87	5
Cpd 27: Pleuromutilin; C22 H34 O5	13.934	378.2449	Pleuromutilin	C22 H34 O5	C22 H34 O5	C22 H34 O5	-11.29	5
Cpd 28: [7]-Paradol; C18 H28 O3	14.499	292.2073	[7]-Paradol	C18 H28 O3	C18 H28 O3	C18 H28 O3	-11.94	10
Cpd 29: [7]-Paradol; C18 H28 O3	14.697	292.2075	[7]-Paradol	C18 H28 O3	C18 H28 O3	C18 H28 O3	-12.43	10
Cpd 30: 10-Oxo-11-octadecen-13-olide; C18 H30 O3	15.076	294.2226	10-Oxo-11-octadecen-13-olide	C18 H30 O3	C18 H30 O3	C18 H30 O3	-10.69	10
Cpd 31: 8-Epideoxyloganin tetraacetate; C25 H34 O13	15.389	542.2007	8-Epideoxyloganin tetraacetate	C25 H34 O13	C25 H34 O13	C25 H34 O13	-1.37	2
Cpd 32: Hordatine A; C28 H38 N8 O4	15.945	550.2943	Hordatine A	C28 H38 N8 O4	C28 H38 N8 O4	C28 H38 N8 O4	11.28	2
Cpd 33: 16-fluoro-hexadecanoic acid; C16 H31 F O2	16.504	274.2314	16-fluoro-hexadecanoic acid	C16 H31 F O2	C16 H31 F O2	C16 H31 F O2	-9.28	10
Cpd 34: Fortimicin AP; C14 H30 N4 O5	16.758	334.2184	Fortimicin AP	C14 H30 N4 O5	C14 H30 N4 O5	C14 H30 N4 O5	9.65	10
Cpd 35: Fortimicin AP; C14 H30 N4 O5	17.041	334.2188	Fortimicin AP	C14 H30 N4 O5	C14 H30 N4 O5	C14 H30 N4 O5	8.44	10
Cpd 36: Tsugaroside B; C37 H60 O7	17.553	616.4291	Tsugaroside B	C37 H60 O7	C37 H60 O7	C37 H60 O7	7.74	2

Cpd 37: Yucalexin B20; C20 H30 O3	17.972	318.2211	Yucalexin B20	C20 H30 O3	C20 H30 O3	C20 H30 O3	-11.4	10
Cpd 38: Istamycin X0; C14 H30 N4 O4	18.218	318.2235	Istamycin X0	C14 H30 N4 O4	C14 H30 N4 O4	C14 H30 N4 O4	9.97	10
Cpd 39: APGPR Enterostatin; C21 H36 N8 O6	18.707	496.2759	APGPR Enterostatin	C21 H36 N8 O6	C21 H36 N8 O6	C21 H36 N8 O6	-0.25	6
Cpd 40: 16-Hydroxy hexadecanoic acid; C16 H32	19.452	272.2386	16-Hydroxy hexadecanoic acid	C16 H32 O3	C16 H32 O3	C16 H32 O3	-11.72	1
Cpd 41: 3-Hydroxy-beta-ionol 3-[glucosyl-(1->6)-glucoside]; C25 H42 O12	21.333	534.2677	3-Hydroxy-beta-ionol 3-[glucosyl-(1->6)-glucoside]	C25 H42 O12	C25 H42 O12	C25 H42 O12	-0.19	5
Cpd 42: 3-Hydroxy-beta-ionol 3-[glucosyl-(1->6)-glucoside]; C25 H42 O12	21.641	534.2679	3-Hydroxy-beta-ionol 3-[glucosyl-(1->6)-glucoside]	C25 H42 O12	C25 H42 O12	C25 H42 O12	-0.42	5
Cpd 43: PE(22:1(11Z)/15:0); C42 H82 N O8 P	23.726	759.5802	PE(22:1(11Z)/15:0)	C42 H82 N O8 P	C42 H82 N O8 P	C42 H82 N O8 P	-3.15	10
Cpd 44: Alloxanthin; C40 H52 O2	23.736	564.3975	Alloxanthin	C40 H52 O2	C40 H52 O2	C40 H52 O2	-1.39	5
Cpd 45: Ritterazine A; C54 H76 N2 O10	23.943	912.5395	Ritterazine A	C54 H76 N2 O10	C54 H76 N2 O10	C54 H76 N2 O10	11.49	1
Cpd 46: 3'-N-Acetyl-4'-O-(10,12-octadecadienoyl)fusarochromanone; C35 H52 N2 O6	24.082	596.3797	3'-N-Acetyl-4'-O-(10,12-octadecadienoyl)fusarochromanone	C35 H52 N2 O6	C35 H52 N2 O6	C35 H52 N2 O6	4.83	4
Cpd 47: Alloxanthin; C40 H52 O2	24.122	564.3997	Alloxanthin	C40 H52 O2	C40 H52 O2	C40 H52 O2	-5.25	5
Cpd 48: 3-Hydroxy-b,e-caroten-3'-one; C40 H54 O2	24.208	566.4139	3-Hydroxy-b,e-caroten-3'-one	C40 H54 O2	C40 H54 O2	C40 H54 O2	-0.96	10
Cpd 49: Ritterazine A; C54 H76 N2 O10	24.289	912.5392	Ritterazine A	C54 H76 N2 O10	C54 H76 N2 O10	C54 H76 N2 O10	11.79	1
Cpd 50: 3-Hydroxy-b,e-caroten-3'-one; C40 H54 O2	24.621	566.4139	3-Hydroxy-b,e-caroten-3'-one	C40 H54 O2	C40 H54 O2	C40 H54 O2	-2.74	10

APPENDIX- V: REAGENT PREPARATION METHOD

Aluminium chloride (1:10)

Dissolve 10 g of $AlCl_3$ in 100mL of water.

FCR (1:10)

Dissolve 10 ml of FCR in 100mL of distilled water.

75g/L sodium carbonate:

Dissolve 7.5 g of sodium carbonate in100mL of water.

Wagner's Reagent

1.27g of iodine and 2g of potassium iodide was mixed in the 100mL of water.

Alcohol α-Naphthanol

3.75g of α-naphthanol in 25 mL of 99% ethanol.

2% NaOH

1.031 g of NaOH dissolved in 50 mL of distilled water.

10% Lead acetate solution:

5.078 g of lead acetate was dissolved in 50 mL of distilled water.

10% Ferric chloride

5.101 g of ferric chloride in 50 mL of distilled water.

10% NaOH

10.309 g of NaOH was dissolved in 100 mL distilled water.

10% $KMNO_4$

10.1 g of $KMNO_4$ was dissolved in 100 mL distilled water.

1% Gelatin

1.00 g of gelatin was dissolved in 100mL distilled water.

10% NaCl

10.010 g of NaCl was dissolved in 100 mL of distilled water.

APPENDIX- VI: PHOTOS OF THE SELECTED PLANT, DRIED LEAVES AND POWDERED LEAF SAMPLE

Figure 6: Callicarpa macrophylla Vahl.plant, its dried leaves and powdered leaf sample

APPENDIX- VII: PLANT IDENTIFICATION REPORT

नेपाल सरकार
वन तथा वातावरण मन्त्रालय
राष्ट्रिय हर्बेरियम तथा वनस्पति प्रयोगशाला
गोदावरी, ललितपुर

प्राविधिक विशेषज्ञको प्रतिवेदन

१. नमूना परिक्षण गर्न पठाउने व्यक्ति/निकायः- श्री वीरेन्द्र बहुमुखी क्याम्पस, भरतपुर, चितवन।

१(क) विद्यार्थीको नाम- Sachin Silwal, Swikar Giri and Sabi Thapa

२. प्राप्त नमूनाको विवरणः- हर्बेरियमको नमूना थान - ८

३. यस कार्यालयमा प्राप्त मितिः- २०७९/१०/१८

४. परिक्षणका आधारहरुः- (क) हर्बेरियममा भएको नमूनाहरु संगको तुलनात्मक अध्ययन ।
(ख) सन्दर्भ सामग्रीहरुको अध्ययन ।

५. पहिचान प्रतिवेदनः- प्राप्त नमूनाहरुको Morphological अध्ययन र यस राष्ट्रिय हर्बेरियम तथा वनस्पति प्रयोगशालाको हर्बेरियम संग्रहालयमा राखिएका नमूनाहरुसंगको तुलनात्मक अध्ययन गर्दा उक्त नमूना निम्नानुसार भएको पहिचान हुन गएको ।

S. N	Locality	Scientific Name	Family	Collector	Remarks
1	Ramjhakots, Tanahun	*Callicarpa macrophylla* Vahl	Lamiaceae	Sabi Thapa	
2	Bharatpur-15Mangalpur	*Cuscuta reflexa* Roxb.	Convolvulaceae	Sabi Thapa	
3	Shivaahats Chitwan	*Justicia adhatoda* L.	Acanthaceae	Sabi Thapa	
4	Dhading, Sakhu	*Scutellaria discolor* Wall.ex Benth.	Lamiaceae	Sachin Silwal	
5	Khaireni-12, Chitwan	*Euphorbia hirta* L.	*Euphorbiaceae*	Sachin Silwal	
6	Khaireni, Chitwan	*Carica papaya* L.	Caricaceae	Sachin Silwal	
7	Butwal	*Aegle marmelos* (L.) Corrêa	Rutaceae	Swikar Giri	
8	Butwal	*Parthenium hysterophorus* L.	Asteraceae	Swikar Giri	

६. परिक्षण गर्ने अधिकारीः-

धनराज कँडेल
अनुसन्धान अधिकृत
(१९८१९८)

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Bharatpur, Chitwan

पत्रसंख्या :
च. नं.(Ref.) :

मिति : २०८०।०८।२४
Date :

जो जस सँग सम्वन्धित छ ।

तपाई SABI THAPA ले यस पुस्तकालयमा प्लेजारिजम परीक्षण गर्नका लागि हार्डकपी र सफ्टकपीको विषय वस्तुमा कुनै फरक छैन भनी स्वघोषणा गरी पेनड्राइभ/ईमेल मार्फत विभागमा पेश गर्नुभएकोले विभागबाट इमेल मार्फत प्राप्त सफ्टकपी BIOLOGICAL ACTIVITIES OF CALLICARPA MACROPHYLLA VAHL. LEAVES AND HRLCMS BASED PROFILING शिर्षकको .. रसायन शास्त्र ...एम् एस्सी तहको उपाधिका लागि तयार गरिएको Dissertation / Thesis मा प्लेजारिजम परीक्षण पछिको समानता सूची (Similarity Index) १४ (चौध) प्रतिशत रहेको व्यहोरा प्रमाणित गरिन्छ ।

महेन्द्रप्रसाद अधिकारी
पुस्तकालय प्रमुख

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