PHYSICOCHEMICAL AND HR-LCMS ANALYSIS OF SECONDARY METABOLITES PRESENT IN HONEY COLLECTED FROM DIFFERENT FARMS OF NEPAL

A THESIS SUBMITTED TO THE
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FOR THE PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
MASTER OF SCIENCE DEGREE
IN CHEMISTRY

BY
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DECLARATION

Thesis entitled **"PHYSICOCHEMICAL AND HR-LCMS ANALYSIS OF SECONDARY METABOLITES PRESENT IN HONEY COLLECTED FROM DIFFERENT FARMS OF NEPAL"** is my research work 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, under the supervision of Assoc. Prof. Dr. Ganga Raj Pokhrel, Department of Chemistry, Birendra Multiple Campus, Tribhuvan University and co-supervised by Assoc. Prof. Dr. Bodh Babu Bhattarai, 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.

Ranjana Khanal

RECOMMENDATION

This is to recommend that **Ranjana Khanal** has carried out research entitled **PHYSICOCHEMICAL AND HR-LCMS ANALYSIS OF SECONDARY METABOLITES PRESENT IN HONEY COLLECTED FROM DIFFERENT FARMS OF NEPAL** 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 to 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 Master of Science Degree in Chemistry.

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FOREWORD

The thesis work "**PHYSICOCHEMICAL AND HR-LCMS ANALYSIS OF SECONDARY METABOLITES PRESENT IN HONEY COLLECTED FROM DIFFERENT FARMS OF NEPAL**" submitted by Ranjana Khanal as a part of M.Sc. Coursework in Chemistry at Birendra Multiple Campus is carried out under my supervision. Any part of this thesis work has not been submitted for any other degree award.

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Date: Feb , 2025

On the recommendation of Ganga Raj Pokhrel, Ph.D. (Assoc. Prof.) and Bodh Babu Bhattarai, Ph.D. (Asst. Prof.), this M.Sc. thesis submitted by Ranjana Khanal entitled **"PHYSICOCHEMICAL AND HR-LCMS ANALYSIS OF SECONDARY METABOLITES PRESENT IN HONEY COLLECTED FROM DIFFERENT FARMS OF NEPAL"** is forwarded by Department of Chemistry, Birendra Multiple Campus (BMC) to the office of Dean, IOST, T.U.

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Date: February 06, 2025

This dissertation entitled **"Physicochemical and HR-LCMS Analysis of Secondary Metabolites Present in Honey Collected From Different Farms of Nepal,"** by **Ranjana Khanal**, under the supervision of ***Ganga Raj Pokhrel, Ph.D. (Assoc. Prof.)*** and ***Bodh Babu Bhattarai, Ph.D. (Asst. Prof.),*** Department of Chemistry, Tribhuvan University is submitted for the partial fulfillment of the Master of Science (M.Sc.) degree in Chemistry. It is importantly note that this work has not been previously presented to any other institution for pursue any degree.

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LIST OF ABBREVIAATION

AAC	Ascorbic acid Concentration
AAE	Ascorbic acid Equivalent
C	Concentration
Conc.	Concentration
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
GC-MS	Gas 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
µg/mL	Microgram per milliliter
Nm	Nanometer
RE	Rutin concentration
RE	Rutin equivalent
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species

Rpm	Revolution per minute
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

ABSTRACT

This study examined the quality and composition of Nepalese honeys by analyzing their physicochemical properties, antioxidant potential, and secondary metabolite profiles. Thirteen honey samples were collected from various farms across Nepal during November and December 2023, representing diverse floral sources such as Rudilo (*Pogostemon benghalensis*), Chiuri (*Diploknema butyracea*), Mustard (*Brassica napus*), Rhododendron (*Rhododendron arboreum*), Litchi (*Litchi chinensis*), and Fapar (*Fagopyrum esculentum*). The analysis focused on key physicochemical parameters, including moisture content and electrical conductivity. Antioxidant capacity was evaluated using DPPH, while secondary metabolites were profiled using High-Resolution Liquid Chromatography-Mass Spectrometry (HR-LCMS). Among the key constituents identified were naringenin-7-O-glucuronide, chlorogenic acid, 6-demethoxycapillarisin, and ophthalmic acid. The moisture content of the honey samples ranged from 13.86±0.02% to 19.89±0.03%, with the highest value observed in sample H2 (19.89±0.03%). Electrical conductivity ranged from 0.20±0.02 to 1.72±0.02 mS/cm, with sample H12 exhibiting the highest value (1.72±0.02 mS/cm). All honey samples exhibited acidity, with pH values ranging from 3.86 to 4.95, aligning with the typical pH range found in honey. Sample H6 displayed a notably higher pH (4.95±0.02) compared to the other samples. Citric acid content ranged from 14.04±0.02 to 68.64 ±0.03mg/100g, demonstrating an inverse correlation with pH, as evidenced by the higher citric acid level in sample H5 (68.64 ±0.02mg/100g) which corresponded to a pH of 3.86±0.02. Lactic acid content varied between 12.05±0.02and 19.04±0.02mg/100g across the different floral origins, with sample H5 exhibiting the highest concentration (19.04 mg/100g). The riboflavin content varied between 0.01 ± 0.001 mg/100g and 0.09 ± 0.004 mg/100g, with sample H7 (0.0187 mg/100g) representing a typical level for *Apis cerana* honey. Antioxidant activity, as measured by DPPH, ranged from 33.24% to 53.10%, with sample H5 displaying the highest activity (53.10%). The IC50 values ranged from 1158.08 µg/mL to 1662.63 µg/mL. Total phenolic content ranged from 21.71±3.42 to 72.02±2.103 mg GAE/100mg/ml, with H13 honey showing significantly higher levels compared to other samples. Similarly, total flavonoid content ranged from 9.82±0.96 to 38.025±1.350 mg GAE/100mg/ml, with H13 honey again exhibiting significantly higher levels. These findings emphasize the significant influence of mention

parameter above on honey quality in Nepal, providing a scientific basis for applications such as targeted marketing, quality control, and exploration of potential health benefits. This research provides important insights into Nepalese honeys and their potential uses.

शोधसार

नेपालमा पाइने महको गुणस्तर र संरचनाको अध्ययन गर्न यस अनुसन्धानले तिनीहरूको भौतिक-रासायनिक गुण, एन्टिअक्सिडेन्ट क्षमता, र द्वितीय पदार्थहरूको पहिचान गरेको छ। कात्तिक र मंसिर २०८० मा नेपालका विभिन्न कृषि फार्महरूबाट जम्मा १३ वटा महका नमूना सङ्कलन गरियो । यी नमूनाहरू विभिन्न वनस्पतिजन्य स्रोतहरूबाट प्राप्त गरिएका थिए, जस्तै रुदिलो, चिउरी, तोरी, लालीगुराँस, लिची, र फापर ।

यस अध्ययनमा महका प्रमुख भौतिक-रासायनिक तत्वहरू जस्तै नमी मात्रा र विद्युत चालकता जाँच गरियो । एन्टिअक्सिडेन्ट क्षमताको मूल्याङ्कनका लागि निश्चित परीक्षणहरू गरियो । साथै, उच्च गुणस्तरको भिल्ली प्रविधि प्रयोग गरी द्वितीय पदार्थहरूको पहिचान गरियो । अनुसन्धानमा प्रमुख जैविक पदार्थहरू न्यारिन्जेनिन-७-ओ-ग्लुक्युरोनाइड, क्लोरोजेनीक अम्ल, ६-डेमेथोक्सीक्यापिलारिसिन, र ओफ्थाल्मिक अम्ल पहिचान गरियो ।

महको नमी मात्रा १३.८६ ±०.०२% देखि १९.८९ ±०.०३% को दायरामा पाइयो, जसमा २ नमूनामा सबैभन्दा बढी (१९.८९±०.०३%) देखियो। विद्युत चालकता ०.२० ±०.०२ देखि १.७२ ±०.०२ सम्म पाइयो, जसमा १२ नमूनामा सबैभन्दा उच्च (१.७२±०.०२) देखियो । सबै मह अम्लीय प्रकृतिका थिए, जसको अम्ल-मात्रा ३.८६ देखि ४.९५ को दायरामा रह्यो । ६ नमूनाको अम्ल-मात्रा सबैभन्दा उच्च (४.९५±०.०२) थियो ।

साइट्रिक अम्लको मात्रा १४.०४ ±०.०२ देखि ६८.६४±०.०३ सम्म देखियो, जसले अम्ल-मात्रासँग उल्टो सम्बन्ध देखायो । ५ नमूनामा सबैभन्दा धेरै साइट्रिक अम्ल (६८.६४±०.०२) पाइयो, जसको अम्ल-मात्रा ३.८६+०.०२ थियो। ल्याक्टिक अम्लको मात्रा १२.०५ ±०.०२ देखि १९.०४ ±०.०२ सम्म पाइयो, जसमा ५ नमूनामा सबैभन्दा उच्च (१९.०४) थियो ।

राइबोफ्लाभिनको मात्रा ०.०१ ±०.००१ देखि ०.०९ ±०.००४ सम्म पाइयो । ७ नमूनाको राइबोफ्लाभिन सामग्री (०.०१८७) सामान्यस्तरको महको लागि उपयुक्त पाइयो । एक विशेष परीक्षणद्वारा मापन गरिएको एन्टिअक्सिडेन्ट गतिविधि ३३.२४% देखि ५३.१०% सम्म थियो, जसमा ५ नमूनाको सबैभन्दा उच्च (५३.१०%) गतिविधि देखियो ।कुल फिनोलिक पदार्थ २१.७१±३.४२ देखि ७२.०२±२.१०३ सम्म पाइयो, जसमा १३ नमूनामा सबैभन्दा उच्च मात्रा देखियो। कुल फ्लाभोनोइड पदार्थ ९.८२±०.९६ देखि ३८.०२५±१.३५० सम्म थियो, जसमा १३ नमूनामा सबैभन्दा उच्च मात्रा देखियो ।

यस अनुसन्धानले नेपालमा पाइने महको गुणस्तरमा विभिन्न तत्त्वहरूको महत्वपूर्ण प्रभाव रहेको देखाएको छ । यसले लक्षित बजार प्रवर्द्धन, गुणस्तर नियन्त्रण, र स्वास्थ्य सम्बन्धी सम्भावित फाइदाहरूको अनुसन्धानका लागि वैज्ञानिक आधार प्रदान गर्दछ ।

CHAPTER 1

INTRODUCTION

1.1 Background

Honey is a naturally occurring, viscous liquid produced by honeybees from the nectar of flowers. The bees collect the nectar and transform it by combining it with specific substances from their own bodies. They then deposit, dehydrate, and store the honey in the honeycomb, allowing it to ripen and mature (Poli, n.d.).

Historically, honey has been used for various medical and dietary applications, dating back to ancient times. Studies suggest that its composition and antioxidant properties are influenced by the type of floral nectar, along with seasonal and environmental conditions. Additionally, processing techniques can also affect honey's composition and antioxidant potential.(Al-Mamary et al., 2002; Frankel et al., 1998; Gheldof & Engeseth, 2002; Yao et al., 2004) .

Recent studies have underscored the therapeutic potential of honey, particularly its antioxidant properties against reactive oxygen species (Ferreira et al., 2009). As a result, research in recent years has concentrated on the composition of honey and its various biological properties, including antioxidants (Al-Mamary et al., 2002), anti-inflammatory (Tonks et al., 2003), and antimicrobial activities (Brudzynski & Sjaarda, 2021). These properties are particularly relevant in the contexts of wound healing (Nasir et al., 2010), wound treatment (Molan, 2006), and gastrointestinal disorders (Ladas et al., 1995). While the antibacterial activity of honey was first recognized in 1892, its use in modern medicine has been somewhat restricted due to a lack of robust scientific support (Mohapatra et al., 2011a). Nonetheless, honey's antimicrobial activity is of considerable therapeutic significance, particularly in situations where the body's immune response is insufficient to eliminate infections Recent studies have underscored the therapeutic potential of honey, particularly its antioxidant properties against reactive oxygen species (Ferreira et al., 2009) The therapeutic potential of honey has long been celebrated, particularly due to its remarkable antioxidant properties, which are instrumental in neutralizing reactive oxygen species (Ferreira et al., 2009). In recent years, an increasing number of studies have homed in on the intricate composition of various honeys and their multifaceted biological properties.

These include their potent antioxidant capabilities (Al-Mamary et al., 2002), anti-inflammatory effects (Tonks et al., 2003), and significant antimicrobial activities (Brudzynski & Sjaarda, 2021). Additionally, honey has gained recognition for its beneficial applications in wound healing (Nasir et al., 2010) and its effectiveness as a dressing for cuts and abrasions (Molan, 2006), as well as for its potential to alleviate gastrointestinal disorders (Ladas et al., 1995). While the antibacterial properties of honey were first documented in 1892, its use in modern medical practices has been somewhat restricted due to a lack of robust scientific evidence supporting its efficacy (Mohapatra et al., 2011a). Nonetheless, the importance of its antimicrobial activity cannot be overstated, particularly in scenarios where the body's immune response is inadequate to combat infections. Honey has demonstrated powerful antimicrobial effects against a wide array of microorganisms, including both pathogenic and non-pathogenic species, such as yeasts and fungi. Remarkably, it has shown effectiveness against strains that have developed resistance to various antibiotics, highlighting its potential as a natural therapeutic agent (Zaghloul et al., 2001). In the realm of analytical chemistry, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has emerged as a highly sensitive and selective technique that is invaluable for the detection and quantification of trace compounds. This method is particularly crucial in the analysis of complex matrices such as honey, where it is used for the accurate identification of antibiotic residues and other bioactive components.

1.1 Hypothesis: The physicochemical properties, organic acids, vitamins, phenolic content, and antioxidant activity in honey are correlated with its antimicrobial and therapeutic effects, which can be further explored through HR-LCMS-based profiling.

1.2 Objectives

1.2.1 General Objective

The general objective of this research is to measure physiochemical properties as well as antioxidant and HR-LCMS analysis of honey.

1.2.2 Specific Objectives

a. To measure physicochemical properties such as moisture content and electrical conductivity.

b. To quantify organic acids (citric acid, lactic acid) and vitamins (riboflavin).
c. To estimate Total Phenolic Content (TPC) and Total Flavonoid Content (TFC).
d. To determine antioxidant activity through DPPH and phosphomolybdenum assays.
e. To analyze antimicrobial activity.
f. To evaluate secondary metabolites using HR-LCMS.

1.3. Research Problem

Honey has been used in traditional medicine due to its perceived health benefits. However, the scientific understanding of its therapeutic potential remains underexplored, especially in terms of its chemical composition and bioactive properties. Despite numerous studies on honey's properties, a comprehensive analysis connecting its physicochemical properties, bioactive compounds, and therapeutic effects is still lacking. Understanding the connection between these components could open the door to new applications for honey in health and medicine.

1.4. Rationale of the Study

Honey has been used for its promising health benefits, but the comprehensive understanding of its therapeutic properties is still lacking. While traditional methods for studying honey's medicinal effects can be time-consuming, the use of HR-LCMS enables precise identification of bioactive compounds. Although. By combining HR-LCMS with antioxidant assays and microbial testing, this research aims to uncover the therapeutic properties of honey and its bioactive compounds, providing new insights into its potential for medical use.

CHAPTER 2

LITERATURE REVIEW

Honey is a captivating and complex natural substance, cherished for its delightful sweetness and remarkable therapeutic, antioxidant, and antimicrobial properties. This multifaceted food and medicinal product are revered across cultures and widely used in various culinary and health applications. The chemical composition of honey can significantly differ based on several factors, including its geographic origin, the specific floral sources from which the nectar is collected, and the methods employed during processing. Produced by honeybees through a fascinating process, honey begins its journey when bees gather nectar from vibrant blossoms or extract honeydew secretions from certain insects. Through a combination of regurgitation, enzymatic breakdown, and water evaporation, these industrious insects transform the delicate nectar into a luscious, viscous liquid that not only satisfies the palate but also offers an array of health benefits (Seraglio et al., 2019) .This natural sweetener is recognized as a nutritious alternative, boasting an impressive array of chemical components that contribute to its esteemed medicinal and preventive qualities. These include antibacterial, healing, and antioxidant properties, making honey a highly sought-after natural food product (Brudzynski, 2006). The global production of honey reached an impressive estimate of 1.8 million tons in 2022 (FAOSTAT 2022). The leading honey-producing countries include China, which accounts for 25% of the total production, followed by Turkey at 6%, India at 4%, and the USA at 3%. In Nepal, according to the Ministry of Agriculture and Livestock Development's report for the fiscal year 2021/2022, a total of 5,168 tons of honey was produced, highlighting the growing significance of honey in the region. The physical characteristics of honey are influenced by a range of factors, including temperature, moisture content, the type of flowers visited by bees, and the concentration of sugars present. Fresh honey is often described as a supersaturated solution, containing a higher concentration of sugar than water can typically dissolve at ambient temperatures, resulting in its unique texture and viscosity (Tomasik et al., 2004). At normal temperatures, honey can be considered a supercooled liquid, with glucose occasionally precipitating into solid granules, a phenomenon that showcases the intricate chemistry underlying this natural marvel. This produces a semisolid solution of precipitated glucose crystals in a solution

containing fructose and other components. At 20°C, honey's density normally ranges between 1.38 and 1.45 kg/L

In general, honey is comprised of sugars, vitamins, minerals, amino acids, and bioactive substances like phenolic acids and flavonoids. These substances enhance their antioxidant and antimicrobial qualities. Research indicates that darker types of honey exhibit greater antioxidant activity in comparison to lighter varieties, likely due to their elevated phenolic content and capacity to neutralize free radicals (Estevinho et al., 2008).

2.1 Chemical Composition and Therapeutic Activity

In general, honey is comprised of sugars, vitamins, minerals, amino acids, and bioactive substances like phenolic acids and flavonoids. These substances enhance its antioxidant and antimicrobial qualities. Research indicates that darker types of honey exhibit greater antioxidant activity in comparison to lighter varieties, likely due to their elevated phenolic content and capacity to neutralize free radicals (Estevinho et al., 2008) . Honey is created by honeybees and is a natural supersaturated sugar solution consisting of trace ingredients such as proteins, enzymes, and volatile compounds.(Meo et al., 2017) (Meo et al., 2017). Predominantly, honey is made up of sugars, chiefly monosaccharides like fructose and glucose, which account for approximately 80% to 95% of its dry weight (Lawal et al., 2009). Generally, honey contains 80-85% carbohydrates, 15-17% water, 0.3% protein, and 0.2% ash, in addition to small quantities of amino acids, phenolic compounds, pigments, and vitamins (Bogdanov, et al., 2004). The various physical characteristics of honey, including color, pH, enzyme activity, ash content, electrical conductivity, and even flavor, can vary based on the species of honeybee, geographical origin, and the presence of impurities)(Terrab et al., 2003). The moisture content in honey is vital for its stability, helping to prevent fermentation and crystallization. A lower moisture level plays a key role in inhibiting microbiological activity, enabling honey to be stored for long periods without going bad(Hassan et al., 2014). The tendency to form granules is a key characteristic that sets honey apart from other sweeteners ((Bogdanov,et al., 2004). Like other physical properties, the pH of honey serves as an indicator of its purity and quality, though it can vary based on geographic region. For

example, Pakistani honey typically has a pH ranging from 2.4 to 4.7 (Hussain et al., 2013).

In a study, honey samples were randomly collected from farmers in various locations in the Western States of Nigeria. These samples were analyzed for their physicochemical properties and mineral content. The average results revealed a pH of 3.34, moisture content of 16.6%, ash content of 0.46%, ascorbic acid at 2.61 mg/100g, citric acid at 10.67%, lactic acid at 121.71%, riboflavin at 0.002 mg/100g, sugar content of 65.78%, a refractive index of 1.49, and a relative density of 1.41 g/cm³ from proximate analysis (Coolborn & Adetoun, 2016). In contrast, samples of Algerian honey showed a mean pH of 4.41 ± 0.58, moisture content of 15.3 ± 1.5%, electrical conductivity of 0.324 ± 0.016 ms/cm, and water-soluble solids at 0.14 ± 0.065% (Rebiai & Lanez, et al., 2014)

Dark honey samples tend to have a high level of phenolic compounds, which enhance their antimicrobial properties. The phenolic profiles can vary with the honey's geographic origin, making honey a unique product with significant health-promoting potential (Moreira et al., 2008). Different types of honey exhibit distinct electrical conductivities; for instance, honey derived from honeydew has a higher conductivity compared to nectar honey, which is attributed to its greater mineral content. This property is useful for differentiating between honeydew and nectar honey (El Sohaimy et al., 2015).

Honey from the Amhara region has demonstrated greater antioxidant activity and higher total phenolic content compared to honey from the Tigray region, indicating a strong correlation (Lewoyehu & Amare, 2019). The chemical composition of honey is influenced by various abiotic and biotic factors surrounding the bee colony, including floral sources, climate conditions, soil type, and beekeeping practices (A. Sharma et al., 2021).

Honey contains numerous free radical scavengers, which can help balance the production of free radicals and antioxidant levels (Kishore et al., 2011). Research has demonstrated that honey's antioxidant potential correlates with the concentration of total phenolics and honey color, with darker honey containing more phenolic compounds and, consequently, a higher antioxidant capacity (Alvarez-Suarez et al.,

2010; Moreira et al., 2008). Regular consumption of honey with high antioxidant activity could contribute to cardiovascular health by decreasing oxidative stress and enhancing overall heart function (Mandal & Mandal et al., 2011).

2.2 HR-LCMS Analysis

Recent studies using LC-MS have identified abscisic acid, a plant hormone, as a significant compound in honey samples. Abscisic acid regulates plant development, growth, and stress

responses and is commonly found in honey. It has been proposed as a quality marker and adulteration detector, particularly in Acacia and Erica honey, even in the absence of corresponding pollen types. These findings highlight the role of LC-MS in distinguishing authentic honey and correlating its chemical composition with its bioactive properties.

2.3 Antimicrobial Activity

Numerous studies have demonstrated honey's antibacterial activity against various pathogens, including *E. coli* and *P.aeruginosa*. However, detailed statistical analyses are necessary before honey can be fully integrated into clinical settings for treating bacterial infections (Wilkinson & Cavanagh, 2005). Honey demonstrates both bacteriostatic and bactericidal activity against a variety of pathogens, with methanol extracts showing the most potent effects. Study has shown significant antibacterial activity against both Gram-positive (S. *aureus*, *B. subtilis, B. cereus, E. faecalis, M. luteus*) and Gram-negative bacteria (*E. coli, P. aeruginosa, S. typhi*), with zones of inhibition ranging from 6.94 to 37.94 mm. These findings support the potential of honey as an alternative therapy for bacterial infections (Mohapatra et al., 2011).The antibacterial effectiveness of honey has been compared to conventional antibiotics.The efficacy of undiluted honey is recorded at 96.4% against Pseudomonas aeruginosa and Escherichia coli when diluted in the range of 1:2 to 1:6, showcasing superior effectiveness compared to gentamicin at concentrations of 8.0 and 4.0 μg/ml. Furthermore, honey samples derived from Oman have demonstrated considerable antibacterial activity, supporting the potential of honey as a viable alternative to conventional antibiotics (Albaridi, 2019).

2.4. Quality and Stability of Honey

The quality of honey is inherently linked to the bees responsible for its production. The foraging behavior of honeybees and the various floral sources they exploit significantly influence the chemical composition of honey. However, there is a limited body of research concerning the stability of these chemical compounds during storage. Given the importance of maintaining honey's therapeutic properties, it is essential to conduct further studies that establish a comprehensive control system to ensure the preservation of these beneficial attributes over time (Da Silva et al., 2016).

Despite extensive investigative efforts, notable gaps remain in the understanding of honey's bioactive properties. Current research frequently isolates parameters such as total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity without adequately correlating them with microbial activity or chemical composition through advanced methodologies such as liquid chromatography-mass spectrometry (LC-MS). Additionally, there is often insufficient emphasis on geographical and botanical variances, the synergistic effects of bioactive compounds, and the application of LC-MS in elucidating chemical constituents. This study aims to address these identified gaps by integrating analyses of TPC, TFC, antioxidant activity, and microbial efficacy with LC-MS-based profiling. The research will explore the influences of floral and geographical sources while uncovering potential synergistic mechanisms that contribute to the therapeutic efficacy of honey.

CHAPTER 3

MATERIALS AND METHODS

3.1. Collection of Honey Samples

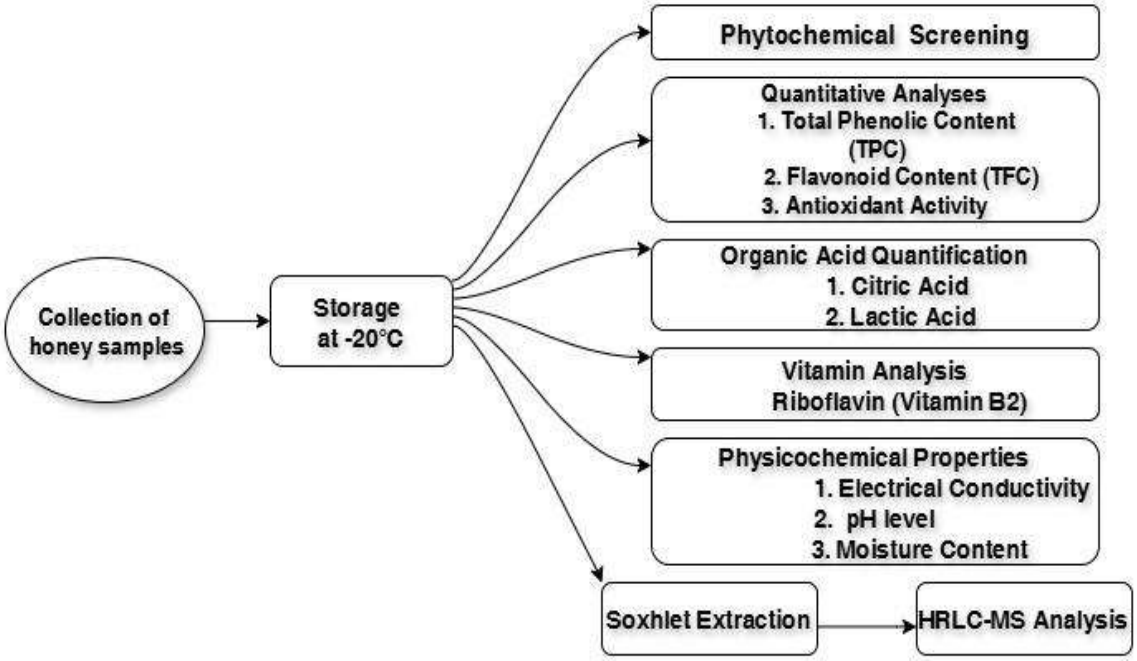
In total thirteen different samples were collected from different farms of various vegetative origin representing honey of 6 different floral sources'Rudilo' (Pogostemon benghalensis), 'Chiuri' (Diploknema butyracea), 'Mustard' (Brassica napus), 'Rhododendron' (Rhododendron arboreum), 'Litchi' (Litchi chinensis), 'Fapar' (Fagopyrum esculentum)etc. Samples were collected in Nov/Dec 2023.Honey samples were collected in clean plastic containers sealed tightly and transported to the laboratory at Birendra Multiple Campus and stored at -20ºc before analysis. Chemicals used for the analysis of honey samples were all of analytical grade.

Table 1: Collection of honey from different farm of Nepal

Honey Sample Code	Collecting Bee Species	Apiary Location	Botanical Origin
H_1	Apis melifera	Kalika-7,Chitwan	Chiuri
H_2	Apis melifera	Tulsipur-7,Dang	Tori+Rudilo
H_3	Apis melifera	Kalika -6,Chitwan	Tori
H_4	Apis melifera	Rolpa	Chiuri
H_5	Apis cerena	rolpa	(wild honey)
H_6	Apis melifera	Kadakhute-1,Dang	Chiuri
H_7	Apis cerena	Parasnagar-Chitwan	Tori
H_8	Apis melifera	Tarechodi-Mahottari	Rudilo
H_9	Apis melifera	Khairahani municipality,Chitwan	Tori
H_{10}	Apis melifera	Shaktikhor,chitwan	Chiuri
H_{11}	Apis melifera	Gulmi	Rhododendron
H_{12}	Apis melifera	Chyangli-Gorkha	Litchi
H_{13}	Apis melifera	Bharatpur-26, Chitwan	Fapar+Tori

3.2. Scheme 1:

Flow chart for extraction, screening, and analysis



3.3. Phytochemical Screening

The phytochemical screening of different honey samples were carried using the protocol as mentioned (Jaradat et al., 2015)

3.4. Determination of Moisture Content:

Clean crucibles were thoroughly dried in an oven set to a moderate temperature for 15 minutes, after which they were carefully transferred to desiccators to cool and maintain low humidity. Next, precisely two grams of samples were measured and added to each crucible. These crucibles were then placed back in the oven, where the samples underwent a drying process for two hours at a consistent temperature of 100°C. This process continued until a constant weight was obtained, indicating that all moisture had been removed. Finally, the percentage of moisture content in the samples was calculated using the protocol outlined by), ensuring accurate and reliable results.(Olugbemi *et al.,*2013)**.**

$$\text{Moisture Content} = \frac{W1-W2}{W1-Wo}$$

Where:

W_0 = Wt. of the crucible

W_1 = Wt. of fresh sample +crucible

W_2= Wt. of heated sample + crucible

3.5. Measurement of electrical conductivity:

A 20.0 g sample of dry honey was meticulously dissolved in 100 mL of distilled water to make a uniform solution. To ensure accurate measurements, the electrical conductivity of this solution was then assessed at a temperature of 25°C. This measurement was conducted using a Crison conductometer, specifically the Basic 30 model, which is designed to provide precise readings of conductivity. This procedure aligns with internationally recognized standards for measuring the electrical properties of solutions Honey Commission Methods (2009).

3.6. Determination of pH

The pH of the honey sample was measured using the method suggested by the International Honey Commission. A digital pH meter was used to check the pH of a 10% (w/v) honey solution made with distilled water.(Sharma et al., 2024a)

3.7. Estimation of citric acid

0.5 ml of the honey sample was mixed with 50 mL of distilled water and then slowly titrated with 1M NaOH using 0.5 mL of dilute phenolphthalein as an indicator.(Sharma et al., 2024a)

$$\text{Factor} = \frac{Titrevalue \times equivalentlength \times 100}{\text{Weight taken}}$$

3.8. Estimation of Lactic acid

1 mL of the honey sample was mixed with 10 mL of distilled water and 20 mL of 1M NaOH in a volumetric flask. The flask was sealed with a stopper and allowed to react for 30 minutes. Following this, 0.5 mL of dilute phenolphthalein solution was added and the mixture was titrated with 1M HCl.(Sharma et al., 2024a)

$$\text{Factor} = \frac{\text{Titre value} \times \text{equivalent factor} \times \text{factor} \times 100}{\text{Weight taken}}$$

3.9. Determination of Riboflavin (Vitamin B2)

The assay solution was diluted in buffer solution of pH 4.0 (i.e., acetate buffer- a mixture of acetic acid and sodium acetate) to achieve a concentration between 10 and

20 µg/mLof sample. The samples were then analyzed using a spectrophotometer set at 445 nm with an absorbance A^1 % 1cm = 30.(Sharma et al., 2024a)

$$\text{Riboflavin concentration (mg/mL)} = \frac{(\text{Absorbance} \times 100)}{(\text{A1\% 1cm})}$$

3.10. Total Phenolic Content (TPC):

The total phenolic content of the plant extract was analyzed using the Folin-Ciocalteu colorimetric method based on an oxidation-reduction reaction, following the procedure outlined by (Javanmardi et al., 2003)Gallic acid was used as a standard reference.

3.10.1 Preparation of the Standard Gallic Acid Solution.

A 1000 µg/mL stock solution was prepared by dissolving 10 mg of gallic acid in 10 mL of methanol. Serial dilutions of this stock solution were made to obtain concentrations of 5, 10, 20, 120, and 160 µg/mL.

3.10.2 Construction of the Calibration Curve

Each test tube was filled with 1 mL of gallic acid solution (at different concentrations), followed by the addition of 4 mL of sodium carbonate (Na_2CO_3) solution and 5 mL of 10% Folin-Ciocalteu reagent (FCR), bringing the total volume to 10 mL. The mixture was thoroughly shaken and incubated at 40°C for 30 minutes in a water bath, allowing a blue-colored complex to develop. Absorbance was measured at 760 nm using a spectrophotometer, with a blank solution (containing all reagents except gallic acid) as the reference. Each experiment was conducted in triplicate, and the calibration curve was generated by plotting the average absorbance against gallic acid concentration.

3.10.3 Preparation of the Sample Solution.:

A stock solution of the extract was prepared by dissolving 10 g of the extract in 50 mL of methanol. Serial dilutions were performed to achieve a 100 µg/mL concentration, and the absorbance was measured following the same method used for gallic acid.

The total phenolic content was calculated using the following relation,

$$C = \frac{cV}{m} \quad \text{……..... (3)}$$

Where, C = Total phenolic content (mg/g) in gallic acid equivalent (GAE)

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

m = Mass of extract (g)

3.10.4 Statistical Analysis:

Data were recorded as the mean absorbance of three replicates for each concentration, from which the linear correlation coefficient (R^2) was determined. The regression equation used was:

$y = mx + c$ …… (4)

Where y = Absorbance of the extract

m = Slope of the calibration curve

x = Concentration of the extract

c = Intercept

Using this regression equation, the extract concentration was calculated and used to determine TPC using equation (3).

3.11 Estimation of Total flavanoid content (TFC)

3.11.1 Preparation of the Standard Quercetin Stock Solution:

Total flavonoid content was quantified using the aluminium chloride colorimetric method as described by Hasan et al. (2015). A 1000 µg/mL (ppm) quercetin stock solution was prepared by dissolving 10 mg of quercetin in 10 mL of methanol. Serial dilutions were carried out to obtain concentrations of 4, 16, 40, 80, 120, and 150 µg/mL.

For the experimental setup, separate test tubes were labeled for each concentration . To each test tube, the corresponding concentrations of quercetin were then added. Following this step, 3 mL of methanol was introduced to each tube to provide a suitable solvent environment. The next critical component, 200 µL of a 10% AlCl3

solution, was added to facilitate the formation of a complex with flavonoids, while 200 µL of 1 M potassium acetate solution was included to assist in stabilizing this complex. Finally, each test tube was topped off with 5.6 mL of distilled water, ensuring a total volume conducive to accurate measurements.

The reaction was allowed to take through incubation at room temperature for a duration of 30 minutes, during which time the flavonoid-aluminium complex developed, yielding a colour change that corresponded to flavonoid concentration. Following the incubation, the absorbance of each solution was meticulously measured at a wavelength of 415 nm using a UV-Vis spectrophotometer. This measurement was compared against a blank control, which contained no flavonoids, ensuring the accuracy of the data.

A calibration curve was generated by plotting the average absorbance values corresponding to different quercetin concentrations, enabling an accurate correlation between absorbance and flavonoid concentration in the honey extract.

3.11.2 Preparation of the Sample Solutions

To prepare the stock solution of sample, 5g of the extract were dissolved in 25 mL of methanol. Then, using the same method as previously described for quercetin, triplicate concentrations of the extract at 100 µg/mL were prepared by serial dilution, and their absorbance values were measured.

3.11.3 Calculation of the Total Flavonoid Content

The following formula was used to calculate the total flavonoid content of the extract

$$C = \frac{cV}{m} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (5)$$

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

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

V = Volume of the extract (in mL) m = Weight of the plant extract (in g)

3.11.4 Statistical Analysis

Data were recorded as a mean of three determinations of absorbance for each concentration, from which the Linear Correlation Coefficient (R2) value was calculated. The regression equation is given as,

$y = mx + c$ … …… … … … (6)

Where y = Absorbance of the extract

m = Slope from the calibration curve

x = Concentration of the extract,

c = Y-Intercept

Using this regression equation, the concentration of the extract was calculated. Thus, with the calculated value of the concentration of extract, the flavonoid content was calculated by equation (5).

3.12. Quantitative analysis Antimicrobial Activity

The agar well diffusion procedure was applied for antibacterial activity.

3.12.1 Preparation of honey solutions

Honey solutions were prepared prior to the experiment by diluting the pure extracts to a 90% (w/v) concentration in distilled water. Specifically, 9 g of honey was dissolved in the appropriate volume of sterile distilled water to yield a 10 mL solution. The vials were subsequently stored at -4°C for future use.(Mama et al., 2019)

3.12.2 Preparation of Media:

The media used in the study were prepared following the manufacturer's instructions. The detailed procedure is provided below.

3.12.3 Nutrient Agar

It was prepared by mixing 28 g of nutrient agar with distilled water in a conical flask to produce a final volume of 1000 ml (28 g/l). The mixture was boiled while being continuously shaken, then autoclaved for 15 minutes at 121°C. After sterilization, the medium was cooled to approximately at 50°C, and 25 mL of the solution was

aseptically transferred into sterilized 90 mm Petri dishes, which were properly labeled.. Plates were then left for solidification.

3.12.4. Mueller Hinton Agar (MHA)

The Mueller Hinton Agar medium was prepared following the manufacturer's instructions. To make it, 9.5 g of the medium was suspended in 250 mL of distilled water, boiled to dissolve, and autoclaved for 15 minutes at 121°C and 15 pounds of pressure for sterilization. After cooling to around 50°C, 20 mL of the medium was poured into Petri dishes. The plates were left undisturbed to solidify.

3.12.5. Preparations of the bacterial inoculums and Susceptibility testing of honey samples

Standard pathogenic bacteria were collected from Bharatpur Hospital in Chitwan, Nepal. The tested bacteria included both Gram-positive (*Staphylococcus aureus* and *Bacillus sp.*) and Gram-negative (*Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*). Positive controls (e.g., Ampicillin) and a negative control (distilled water) were employed for comparison.

3.12.6. Susceptibility testing was performed by Kirby–Bauer disk diffusion technique.

Inoculums were prepared by isolating test organisms using a sterile wire loop and suspending them in sterile normal saline. The density of the suspension was adjusted by comparing it to the McFarland 0.5 barium sulfate standard. A sterile swab was dipped into the suspension, excess fluid was removed by pressing it against the tube’s inner wall, and the swab was then used to evenly spread the suspension across the surface of Mueller-Hinton agar (Oxoid). The plates were left at room temperature to allow the excess fluid to be absorbed. Sterile tips (6 mm in diameter) were used to create wells in the agar, and 100 µL of 90% honey was added to each well. The plates were incubated at 37°C for 24 hours. After incubation, the diameters of the inhibition zones were measured in millimeters and recorded. A positive control was included for each bacterial strain, while sterile distilled water served as the negative control. The experiment was conducted in triplicate for each bacterial strain. 4o : Following the (Kirby-Bauer Disk Diffusion Susceptibility Test Protocol, 2009)

3.13.HR-LC-MS Analysis

The extract with the highest antioxidant and antimicrobial potential was selected for LC-MS profiling, following the method described by(Nursanty et al., 2023)

CHAPTER 4

RESULT AND DISCUSSIONS

4.1. Phytochemical testing Result

Thirteen different honey samples were analyzed for phytochemical screening, and the results obtained from the screening tests are as follows. (Table 2).

The presence of various bioactive compounds in the honey sample are assessed using preliminary phytochemical screening as summarized in Table 2. Positive test results are indicated by a (+) sign, while negative results are shown with as (−) sign. The phytochemical screening of honey reveals that the honey samples contain Carbohydrates, flavonoids, phenolic compounds, polyphenols, Protein saponins, tannins, and steroids which are responsible for its antioxidant, antimicrobial, and anti-inflammatory properties. These phytochemicals contribute to honey's medicinal value, including immune support, wound healing, and protection against oxidative stress. The variation in these compounds across different honey types is influenced by the floral source and bee species, affecting its therapeutic potential (Silva *et al.,* 2013)

Table 2 : Results on the qualitative analysis of phytochemical constituents of different honey samples

S.N.	Parameter	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13
1.	Alkaloid:	-	-	-	-	-	-	-	-	-	-	-	-	-
	a) Mayer Test	-	-	-	-	-	-	-	-	-	-	-	-	-
	b) Wagner Test													
2.	Flavonoid:	+	+	+	+	+	+	+	+	+	+	+	+	+
	a) Alkali Test	+	+	+	+	+	+	+	+	+	+	+	+	+
	b) Lead acetate Test													
3.	Carbohydrate:	+	+	+	+	+	+	+	+	+	+	+	+	+
	a) Molisch Test	+	+	+	+	+	+	+	+	+	+	+	+	+
	b) Fehling Test													
4.	Tannins:	+	+	+	+	+	+	+	+	+	+	+	+	+
	a) $Fecl_3$ Test													
5.	Phenolic Compounds	+	+	+	+	+	+	+	+	+	+	+	+	+
	a) Ferric Chloride Test	+	+	+	+	+	+	+	+	+	+	+	+	+
	b) Lead Acetate Test													
6.	Polyphenols	+	+	+	+	+	+	+	+	+	+	+	+	+
	a) Ferric Chloride Test													
7.	Protein	+	+	+	+	+	+	+	+	+	+	+	+	+
	a) Ninhydrin test													
8.	Saponins	+	+	+	+	+	+	+	+	+	+	+	+	+
9.	Starch	-	-	-	-	-	-	-	-	-	-	-	-	-
10.	Steroids	+	+	+	+	+	+	+	+	+	+	+	+	+

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4.2. Determination of Moisture Content:

The moisture content of honey is influenced by environmental factors and the harvesting practices of beekeepers, varying across different seasons and years (Ogidi & Otenep et al., 2019). In this study, the moisture content of the analyzed honey samples ranged from 13.86% to 19.89% (Table no.3). Statistical analysis revealed significant differences in moisture content among honey derived from different floral sources. However, all values remained within the permissible limit of ≤20%, as per international quality regulations (“Council Directive 2001/110/EU” 2002). The highest moisture content was recorded in the H2 honey sample (19.89%), while the lowest was observed in the H11 sample (13.86%). These findings are consistent with previously reported moisture content values for various honey types, which ranged between 14.4% and 17.8%. The moisture content of honey is influenced by environmental factors and the harvesting practices of beekeepers, varying across different seasons and years (Ogidi & Otenep et al., 2019). In this study, the moisture content of the analyzed honey samples ranged from 13.86% to 19.89% (Table no.3). Statistical analysis revealed significant differences in moisture content among honey derived from different floral sources. However, all values remained within the permissible limit of ≤20%, as per international quality regulations (“Council Directive 2001/110/EU” 2002). The highest moisture content was recorded in the H2 honey sample (19.89%), while the lowest was observed in the H11 sample (13.86%). These findings are consistent with previously reported moisture content values for various honey types, which ranged between 14.4% and 17.8%.(Coolborn & Adetoun*et al.,* 2016).The moisture content of honey is crucial for determining its quality, freshness,

and shelf life. Honey with lower moisture levels (≤ 20%) is less likely to ferment, ensuring better preservation and microbial stability ((Bogdanov et al., 2004). However, higher moisture content can lead to fermentation, impacting the honey's quality and reducing its shelf life. Moisture content also affects the texture and viscosity of honey (Terrab et al., 2003).

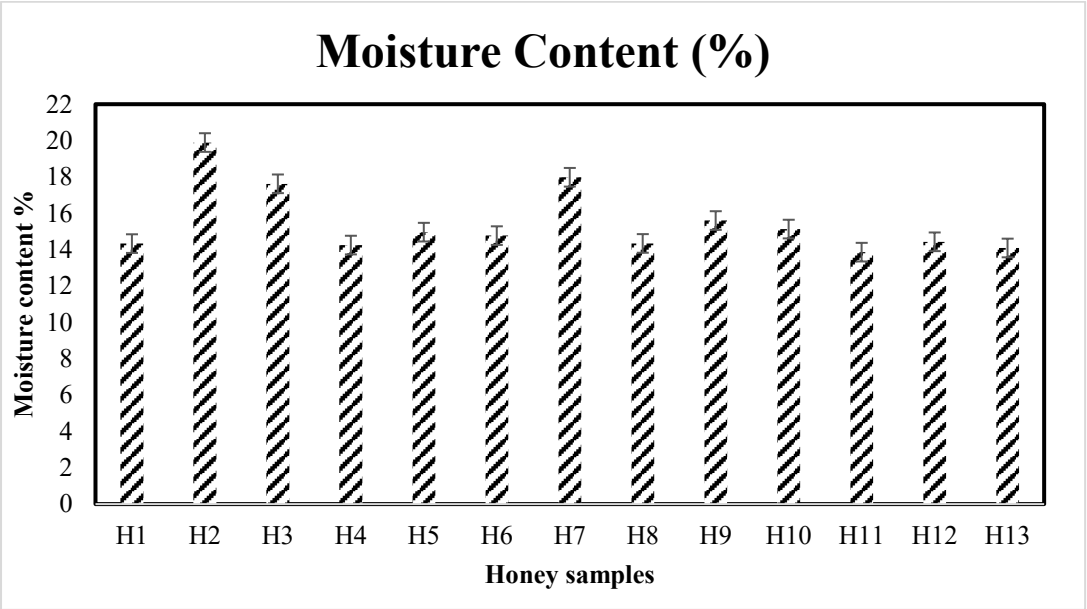


Figure 1 : Moisture content in honey

4.3. Measurement of Electrical Conductivity

Electrical conductivity has a close link with the concentration of minerals and organic acids. The samples exhibited significant variability in electrical conductivity between different groups of honey.The highest Electrical Conductivity value is found in the H12 honey sample (1.72±0.2 ms/cm), whereas the lowest Electrical conductivity value is found in the H2 (0.20±0.2 ms/cm). Higher EC values generally correlate with higher levels of mineral content and dissolved salts. High conductivity can sometimes suggest adulteration or contamination with substances like sugar syrups(Sharma et al., 2024b). Honey with high EC is rich in minerals (potassium, calcium, magnesium) and organic acids, which enhance honey's antimicrobial and antioxidant properties (Khalil et al., 2012).

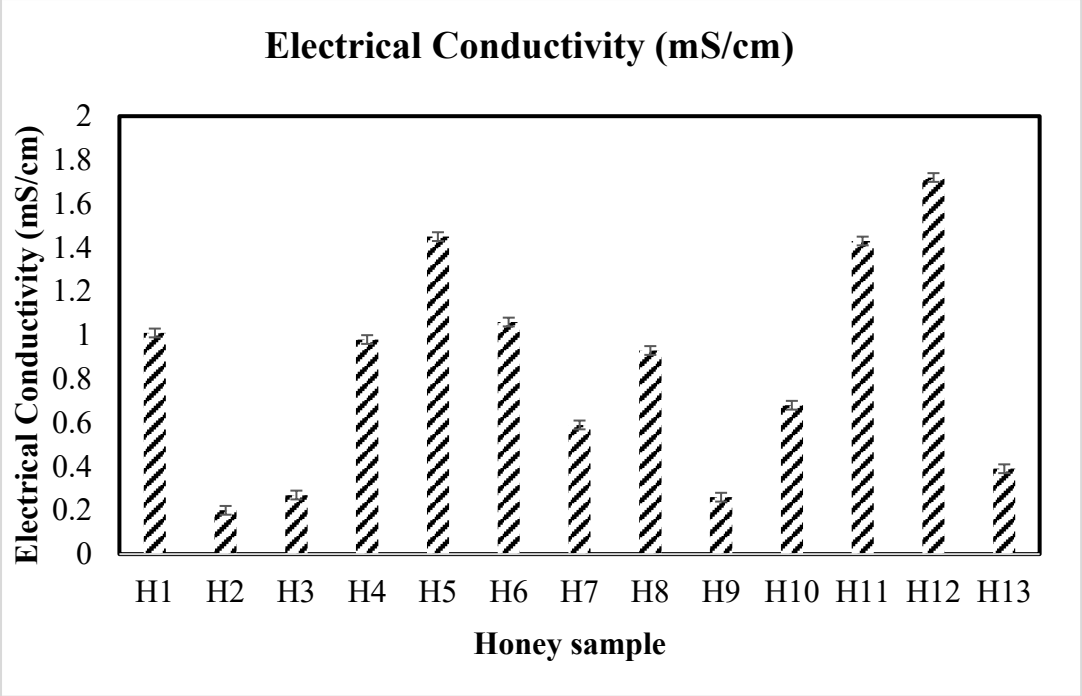


Figure 2: Electrical Conductivity (in mS/cm) of honey samples

4.4. Determination of pH

The pH of honey is an important factor influencing its storage stability and resistance to microbial growth, which in turn affects its texture and overall quality(El & Abdalla, 2015). In this study, the pH values of thirteen honey samples were measured, revealing that all samples exhibited acidic properties, with pH levels ranging from 3.86 ±0.2to 4.95±0.2. These values fall within the acceptable pH range of 3.40–6.10, as specified by the Codex Alimentarius (2001), indicating the freshness of the honey samples. Among them, the H6 sample recorded the highest pH (4.95±0.2), while the H5 sample had the lowest (3.86±0.2). The pH values obtained in this study are consistent with previously reported findings for different honey varieties, where values ranged from 3.35 to 4.95 (Bhattarai et al., n.d.) The natural acidity of honey is a key factor in its antimicrobial effectiveness, as the low pH creates an unfavorable environment for bacterial and microbial growth Honey's low pH creates an environment that inhibits the growth of bacteria and other microorganisms. Apis cerana honey might have different acidic profiles compared to Apis mellifera due to differences in nectar sources and processing. The lower pH value of H7(4.1) compared to some Apis mellifera samples (which have pH values up to 4.95) suggests that H7 may have a slightly more acidic composition, which could be influenced by

the specific floral sources or environmental conditions affecting Apis cerana.Measured pH values of different honey samples are shown in figure no.4.

Table 3 : pH level in Honey samples

S.N.	Sample	pH value± SD
1.	H1	4.83±0.1
2.	H2	4.75±0.2
3.	H3	4.92±0.2
4.	H4	4.8±0.4
5.	H5	3.86±0.2
6.	H6	4.95±0.2
7.	H7	4.10±0.1
8.	H8	3.94±0.3
9.	H9	4.57±0.4
10.	H10	4.75±0.5
11.	H11	4.81±0.2
12.	H12	4.85±0.3
13.	H13	4.17±0.2

4.5. Estimation of Citric Acid

The citric acid content in the honey samples analyzed ranged from 14.04±0.02 to 68.64 ±0.03 mg per 100 g (Table 5). The variation in citric acid levels among different honey samples is influenced by the floral source from which the nectar is derived. This variation may result from the activity of resident microorganisms that ferment the sugar components in honey, thereby influencing its taste and enhancing its resistance to microbial spoilage. The acidity of honey primarily stems from the presence of organic acids, particularly gluconic acid, which exists in equilibrium with its lactones or esters, along with inorganic ions such as phosphate and chloride (Alqarni et al., 2016). The citric acid values observed in this study align with previous reports on honey samples from Latvia, where concentrations ranged from 1 mg to 92 mg per 100 g.(Keke & Cinkmanis *et al.,*2019).All the examined samples meet the required standards, confirming their freshness and showing no signs of fermentation. The acidity in honey plays a role in its flavor, helps prevent microbial growth,

promotes chemical reactions, and enhances both its antibacterial and antioxidant properties(Gheldof & Engeseth, 2002) .Citric acid content in honey inversely correlates with pH value; higher citric acid levels, such as those found in sample H5 (68.64 ±0.03 mg/100g) with a pH of 3.86, result in a lower pH and increased acidity (Bogdanov *et al.,* 2009) whereas lower citric acid content as in sample H6 (14.04±0.03 mg/100g) with a pH of 4.95, leads to a higher pH and less acidic honey (White *et al.,* 1980).Honey produced by Apis melifera typically shows a wide range of citric acid content due to diverse nectar sources, such as high levels in sample H5 (68.64±0.04 mg/100g) and lower levels in sample H6 (14.04 ±0.03 mg/100g) ((Bogdanov, n.d.) *et al.,* 2009). In contrast, Apis cerana honey generally has more consistent citric acid levels, with sample H7 exhibiting moderate citric acid content (21.84±0.03 mg/100g) (White *al.,*1980).

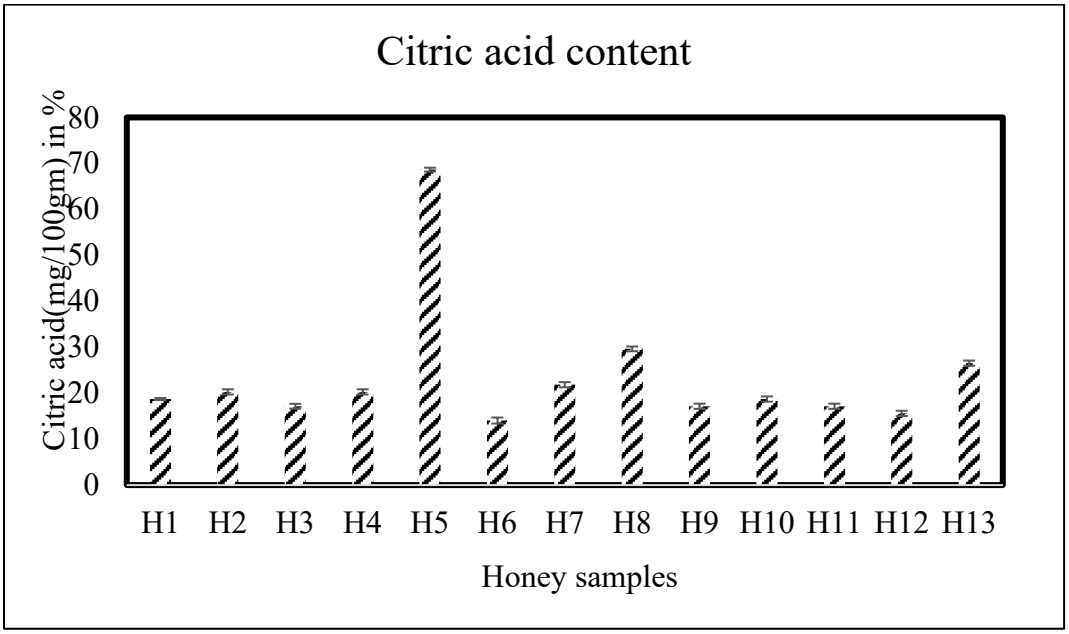


Figure 3 : Citric acid content in Honey samples

4.6. Estimation of Lactic Acid

The content of Lactic acid in the sample of different floral origin ranges from 12.05 to 19.04 mg 100 g^{-1}(Table no.6).These variation of lactic acid content are similar to those previously reported for Lactic acid content of honey samples from Morocco i.e. ranges from10.35 mg 100 g^{-1} to 17.96 mg 100 g^{-1}(Bouhlali *et al.,* 2019).The sample H5 honey has considerably highest lactic acid content (19.04±0.02mg /per 100gm) than the other honey samples & the lowest pH content is recorded in the sample H6

(12.47±0.03 mg/ 100gm).Lactic acid content in honey inversely correlates with pH value; higher lactic acid levels result in lower pH and increased acidity (Molan *et al.,* 1992). The examined sample H5, with the highest lactic acid content (19.04±0.02mg/100g), has a lower pH, indicating greater acidity, while sample H6, with the lowest lactic acid content (12.47±0.03 mg/100g), shows a higher pH, reflecting milder acidity.

Table 4: Lactic acid content in Honey samples

S.N.	Samples	Lactic Acid content (mg/100g± SD)
1.	H1	13.26±0.02
2.	H2	15.94±0.01
3.	H3	13.03±0.03
4.	H4	14.46±0.02
5.	H5	19.04±0.02
6.	H6	12.47±0.03
7.	H7	13.40±0.04
8.	H8	16.85±0.04
9.	H9	13.15±0.02
10.	H10	13.45±0.01
11.	H11	12.97±0.01
12.	H12	13.05±0.02
13.	H13	16.78±0.03

4.7. Determination of Riboflavin content

Riboflavin content in the sample varies from sample to sample of honey that ranges from 0.01±0.001to 0.09±0.004mg/100g (Table no. 7)**.** The concentration of riboflavin in honey can range from very low levels of 0.01-0.02 mg/kg up to 6.1 mg/kg, with the higher end of the range typically found in honeydew honey(Asaduzzaman *et al.,* 2015 ; Coolborn & Adetoun, 2016; Popkova et al., 2021)**.**The riboflavin content varies significantly based on the floral source and geographical origin of the honey(Popkova *et al.,* 2021)**.**Honey from Apis melifera shows considerable variability in riboflavin content, ranging from 0.0118 mg/100g in sample H2 to 0.0938 mg/100g in sample H13, reflecting the diversity in floral sources and environmental conditions (Molan,

2006). In contrast, honey from Apis cerana typically exhibits more consistent riboflavin levels, such as in sample H7 (0.0187 mg/100g), suggesting a more uniform profile due to its nectar sources.

Table 5: Riboflavin Content (mg/100g ± SD)

S.N.	Sample	Riboflavin Content (mg/100g ± SD)
1.	H1	0.05±0.006
2.	H2	0.01±0.001
3.	H3	0.02±0.002
4.	H4	0.05±0.001
5.	H5	0.04±0.018
6.	H6	0.02±0.023
7.	H7	0.01±0.004
8.	H8	0.02±0.002
9.	H9	0.04±0.014
10.	H10	0.05±0.001
11.	H11	0.02±0.002
12.	H12	0.03±0.015
13.	H13	0.09±0.004

Values mean standard deviation values of three replicate samples (n=3).

4.8. Total phenolic content

A broad range of biological and pharmacological activities of phenolic is widely recognised. gallic provides a standard for quantifying their content. Total phenolic content (TPC) value were from 21.71±3.42 to 72.02±2.103 mg GAE/100mg/ml, with sample H13 showing significantly higher levels.

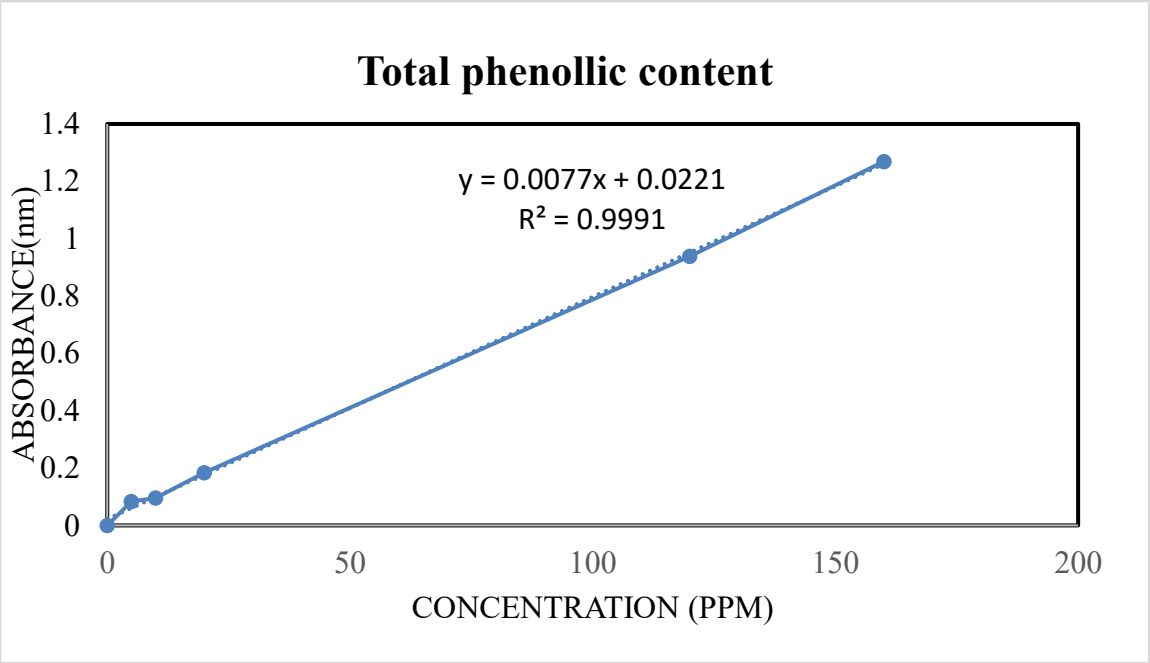


Figure 4 : Calibration of gallic acid

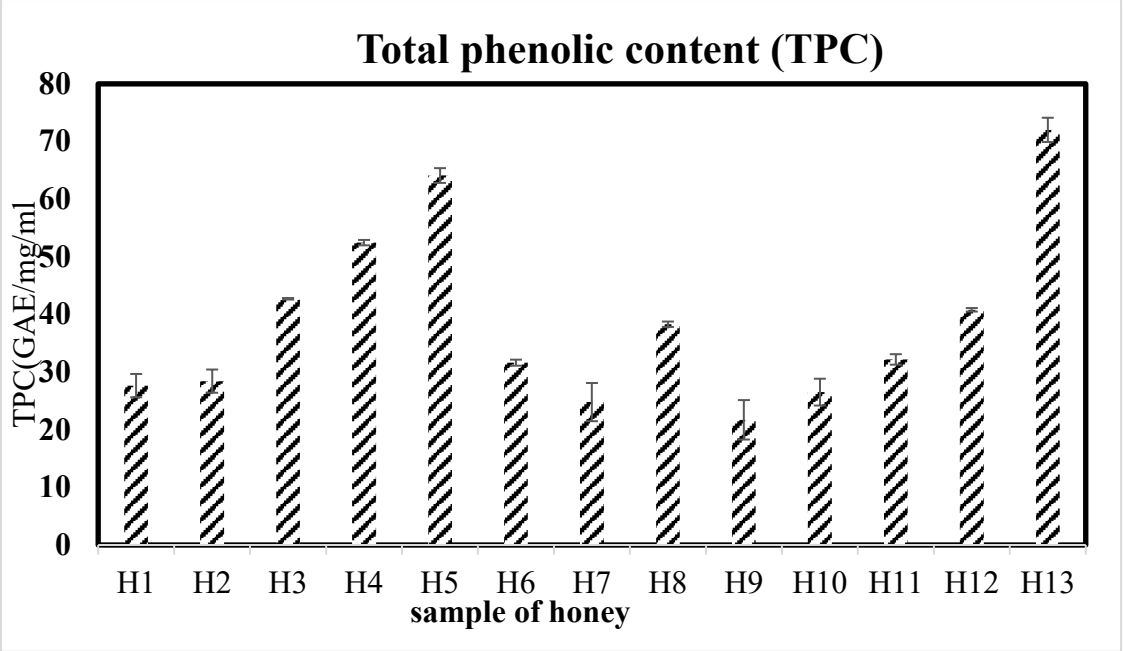


Figure 5 : Total phenolic content present in honey

4.9. Estimation of total flavonoid content

A broad range of biological and pharmacological activities of flavonoids is widely recognised. Quercetin provides a standard for quantifying their content. Total flavonoid content (TFC) varies from 9.43±1.23 to 39.69±1.67 mg GAE/100mg/ml. Sample H5(wild honey) exhibit the highest levels with value 39.69±1.67 mg GAE/100mg/ml.

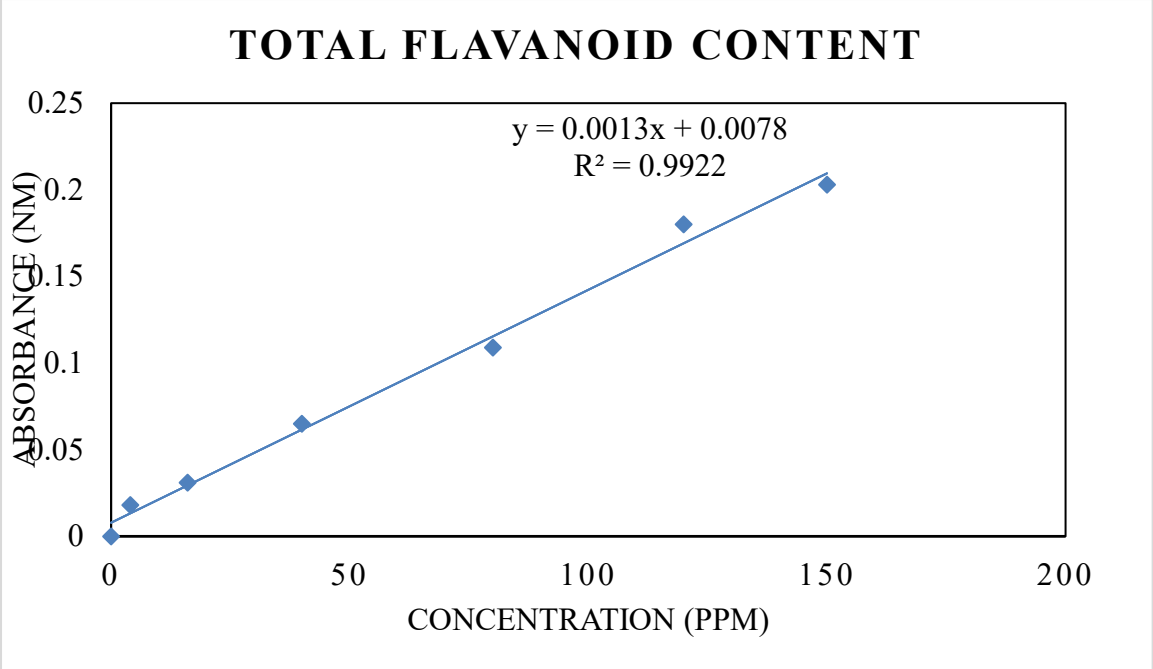


Figure 6 : Calibration of quercetin

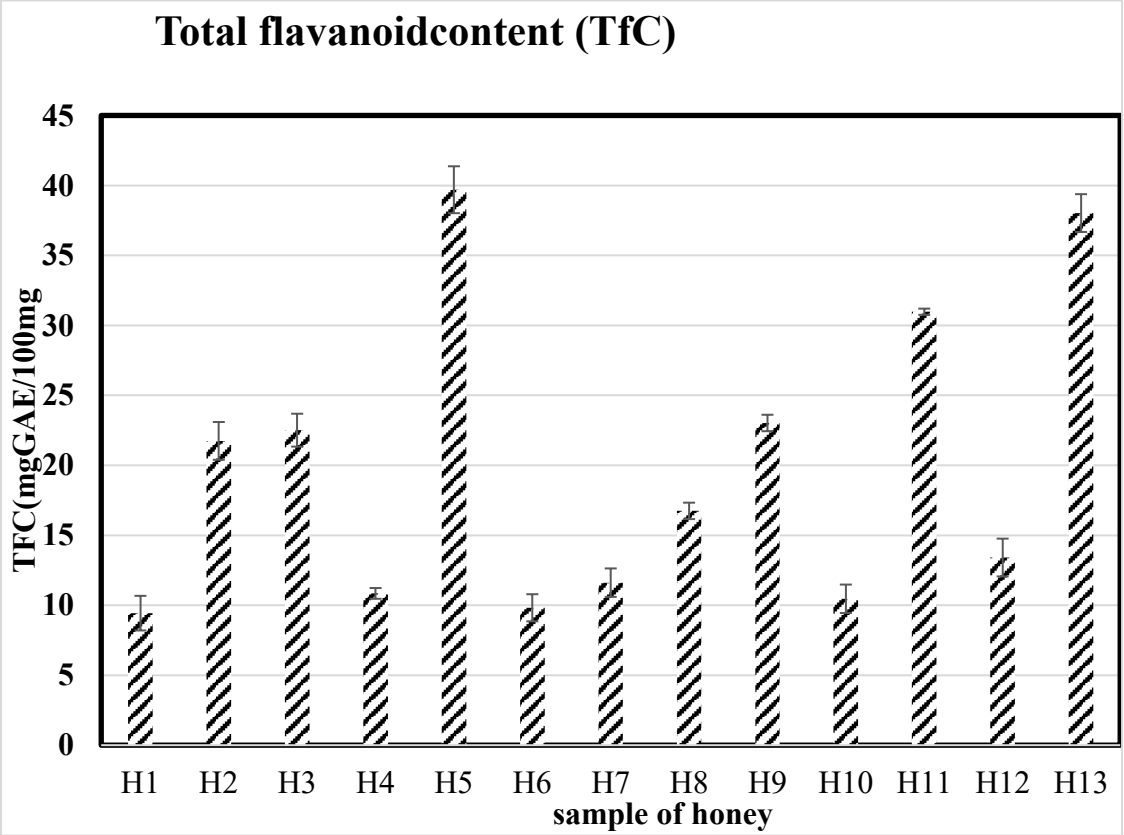


Figure 7 : Total flavonoid content in honey

4.10 Determination of Antioxidant activity

The antioxidant activity of the honey samples of different origin are found in the range between 33.24% and 53.10% in DPPH radical scavenging table no.8.(Neupane *et al.,* 2015, reported similar antioxidant activity (i.e. 25.69% to 76.66% DPPH radical scavenging)in the honey from various altitudes of Nepal. Samples 5, 7 and 10 exhibited the highest percentage of RSA in the DPPH experiment which is the strongest antioxidant activity among all the honey samples.

Table 6 : Evaluation of antioxidant activity by DPPH test on collected honey samples

Sample no.	**Scavenging activity(% inhibition)**
H1	46.38
H2	44.01
H3	44.63
H4	42.44
H5	53.10
H6	40.35
H7	50.69
H8	43.85
H9	41.45
H10	47.74
H11	42.45
H12	33.24
H13	39.97

The inverse correlation exists between the IC50 value and antioxidant potential. This value can be calculated by plotting the percentage of RSA correlated with varying extract concentrations. This is applicable in determining the antioxidant properties and the extract concentration needed to achieve 50% radical scavenging (IC50). The IC50 value of different honey samples ranges from 1158.08µg/mL to 1662.63 µg/mL

Table 7 : IC50 value (µg/mL) in Honey samples

S.N.	Honey samples	IC50 value (µg/mL)
1.	H1	1316.28
2.	H2	1413.17
3.	H3	1394.47
4.	H4	1447.56
5.	H5	1158.08
6.	H6	1456.22
7.	H7	1204.29
8.	H8	1354.79
9.	H9	1432.67
10.	H10	1274.42
11.	H11	1486.93
12.	H12	1662.63
13.	H13	1485.79

4.11. Antimicrobial Activity

The antibacterial potential of various honey types was assessed following the procedure outlined in the relevant section, using a fixed concentration of 90% m/v. The results are presented in the table below, expressed as the diameter of the inhibition zone, including the well's diameter (6 mm).

Table 8 : Standard antibacterial discs (Positive control) create an inhibition zone.

S.N	Antibacterial standard disc	Conc. (µg)	Bacterial strains	Zone of inhibition (mm) (Mean ±S.D)
1	Ampicillin	10 ppm	Staphylococcus aureus	17.667±0.57
2	Amikacin	5 ppm	klebsiella pneumonia	28±1.0
3	Amikacin	5 ppm	Pseudomonas aeruginosa	22±1.0
4	Ciprofloxacin(CIP)	10ppm	Escherichia Coli	24.5± 0.57
5	Ciprofloxacin(CIP)	10ppm	Salmonella typhirium	24.67±0.57
6.	Amikacin	5 pmm	A. baumannii	-
7.	Ampicillin	10 ppm	Bacillus	-

Note: "+" indicating presence of antibacterial activity and "-" indicating absence of antibacterial activity.

Table 9 : Antimicrobial activity of honey

HONEY SAMPLE	S.aureus(food) Z.O.I (mm) (Mean ±S.D)	E. Coli Z.O.I (mm) (Mean ±S.D)	Salmonella typhirium Z.O.I (mm) (Mean ±S.D)	K.pneumonia Z.O.I (mm) (Mean ±S.D)	P.aeruginosa Z.O.I (mm) (Mean ±S.D)	Bacillus Z.O.I (mm) (Mean ±S.D)	A. baumannii Z.O.I (mm) (Mean ±S.D)
H1	13.6±1.53	10±0	12±0	12.3±0.57	-	11±0	14±1
H2	12.3±0.57	12±0	13.6±0.5	14.3±0.57	-	0	14±0
H3	12.6±0.57	12.3±0.57	11.6±0.57	13.6±0.57	-	10.03±0.57	14.3±1.15
H4	13.6±0.57	12±1	12±1	12.6±1.52	-	-	16±1
H5	-	-	0	0	-	-	0
H6	14.3±0.57	13±0	10±0.57	12.66±0.54	-	10±0	15±1
H7	10.6±0.57	12±1.73	11.6±0.57	9.3±0.57	-	11±0	12.6±0.57
H8	11±0	11.6±0.57	13.6±0.52	12±1	-	11±0	14.3±0.57
H9	12±0	11±0	12.6±0.57	10.6±0.57	-	11.66±1.52	13±0
H10	14±0.57	15±1	15±1	12.4±0.60	-	0	15±0.1
H11	13.6±0.55	13±1	14±1	16±1	-	8±1	14.1±0.2
H12	7.6±0.57	8±0.57	8±0.57	15±1	-	10±2	15±1
H13	12±0.12	8.66±1.15	11±1	13.6±1.52	-	7.06±0.057	15±1.15

Note: "+" indicating presence of antibacterial activity and "-" indicating absence of antibacterial activity.

The antimicrobial activity of honey samples (H1–H13) varied across different bacterial strains, as measured by the zone of inhibition (Z.O.I) in millimeters. Most samples exhibited antibacterial effects against *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Bacillus*, and *Acinetobacter baumannii*, while *Pseudomonas aeruginosa* was resistant to all tested honey samples. Notably, H5 demonstrated no inhibitory effect against any bacterial strain, and several samples (H2, H4, H5, and H10) showed no activity against *Bacillus*. The variability in antibacterial potency suggests differences in the chemical composition of honey, potentially influenced by factors such as flavonoid and phenolic content, which warrant further investigation.

4.12.HR LCMS analysis

Honey (H3,H7,H12) were analysed by HR-LC-ESI-QTOF-MS to better interpret the diversity of available phytochemical. Summarise all the compounds that were characterised in Honey (H3,H7,H12)including retention time (RT), molecular formula, experimental m/z, and properties. H3 honey contained a total of 67 compounds along with 30 unknown compounds. The identified compounds included 6 flavonoids, 7 phenolic compounds, 8 alkaloids, 4 amide compounds, 6 peptides, 2 amino acid compounds, and 3 carbohydrates. Additionally, 21 compounds were classified under the "other" category, which included pesticides, fatty acids, pharmaceuticals, and antibiotics. H7 honey contained a total of 120 compounds along with 38 unknown compounds. The identified compounds included 22 flavonoids, 18 phenolic compounds, 10 alkaloids, 10 amide compounds, 14 peptides, 9 amino acid compounds, and 8 carbohydrates. Additionally, 21 compounds were classified under the "other" category, which included pesticides, fatty acids, pharmaceuticals, and antibiotics. H12 honey contained a total of 150 compounds along with 23 unknown compounds. The identified compounds included 37 flavonoids, 15 phenolic compounds, 18 alkaloids, 12 amide compounds, 8 peptides, 7 amino acid compounds, and 6 carbohydrates. Additionally, 47compounds were classified under the "other" category, which included pesticides, fatty acids, pharmaceuticals, and antibiotics.

Thus, honey contains a wide variety of molecules having medicinal importance for human health. The traditional use of this plant as a medicine paid attention to the scientific investigation. The molecules that are reported from HR- LCMS are

separated according to phenolic compound and its derivative, flavonoid compound and its derivative, alkaloid compound and its derivative and other compound in both ESI mode.

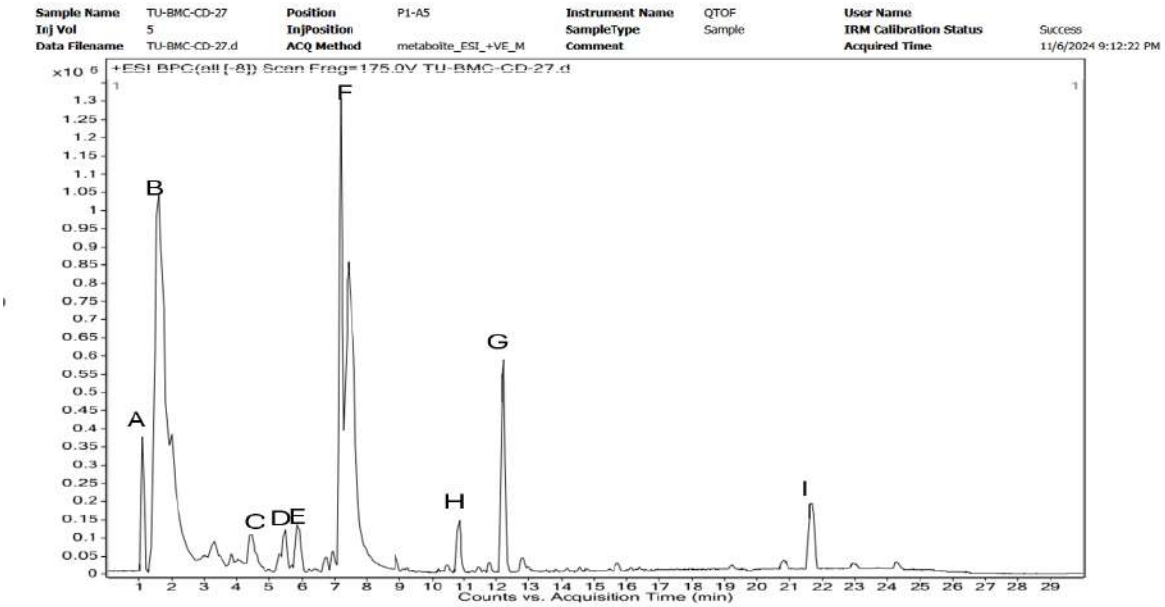


Figure 8: Base peak chromatogram of H3 in positive mode

A=Kinobeon

B=Moracin L

C= Talampanel

D=Cyclacilin

E=Opthalmic acid

F=Lagerstroemine

H=Na-L-Glutamyl-L-aspartic acid

G=L-alpha-Aspartyl-L-hydroxyproline

I= Unknown

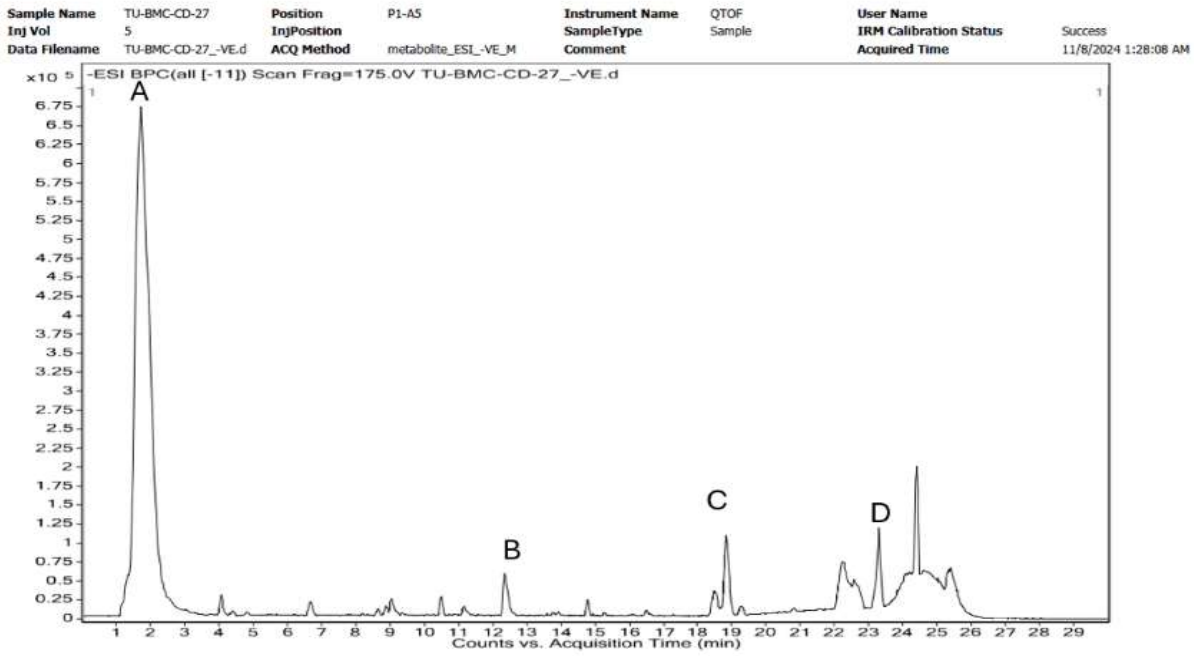


Figure 9 : Base peak chromatogram of H3 in negative mode mode

A=Palmidin C

B=6-Demethoxycapillarisin

C=Valdiate

D=Canrenone

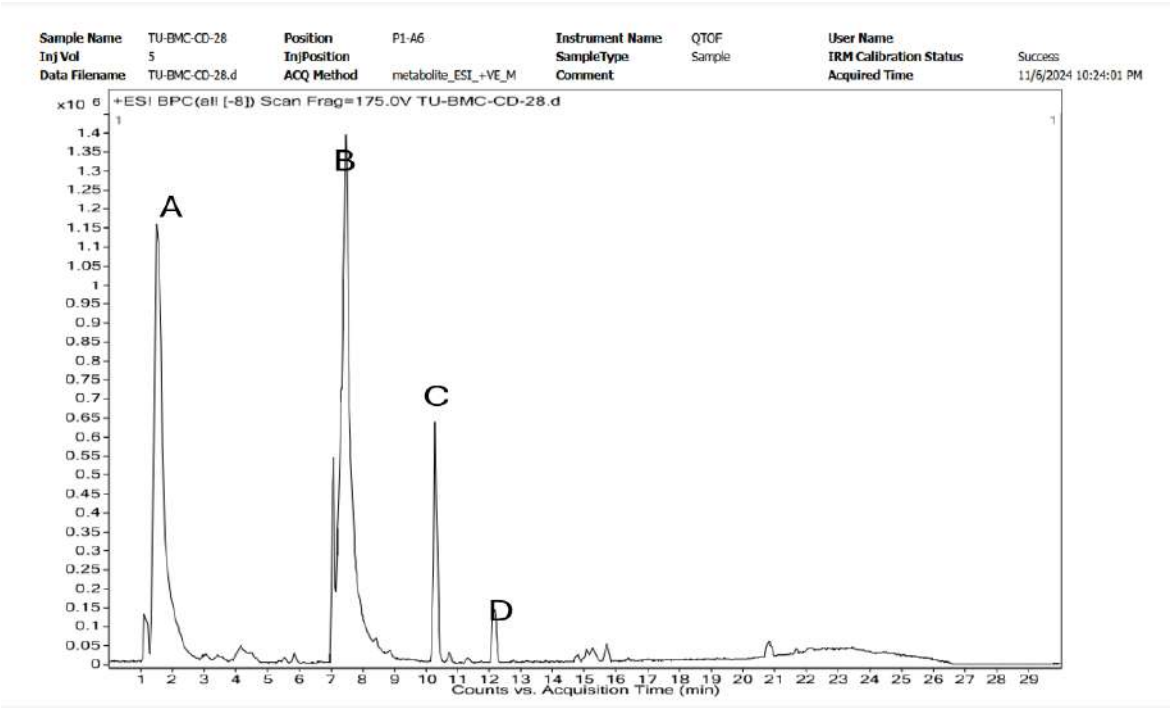


Figure 10: Base peak chromatogram of H7 in positive mode

A=Melosatin B

B=Elatine

C=Butyl (S)-3-hydroxybutyrate glucoside

D=5alpha-Ethoxy-6beta-hydroxy-5,6-dihydrophysalin B

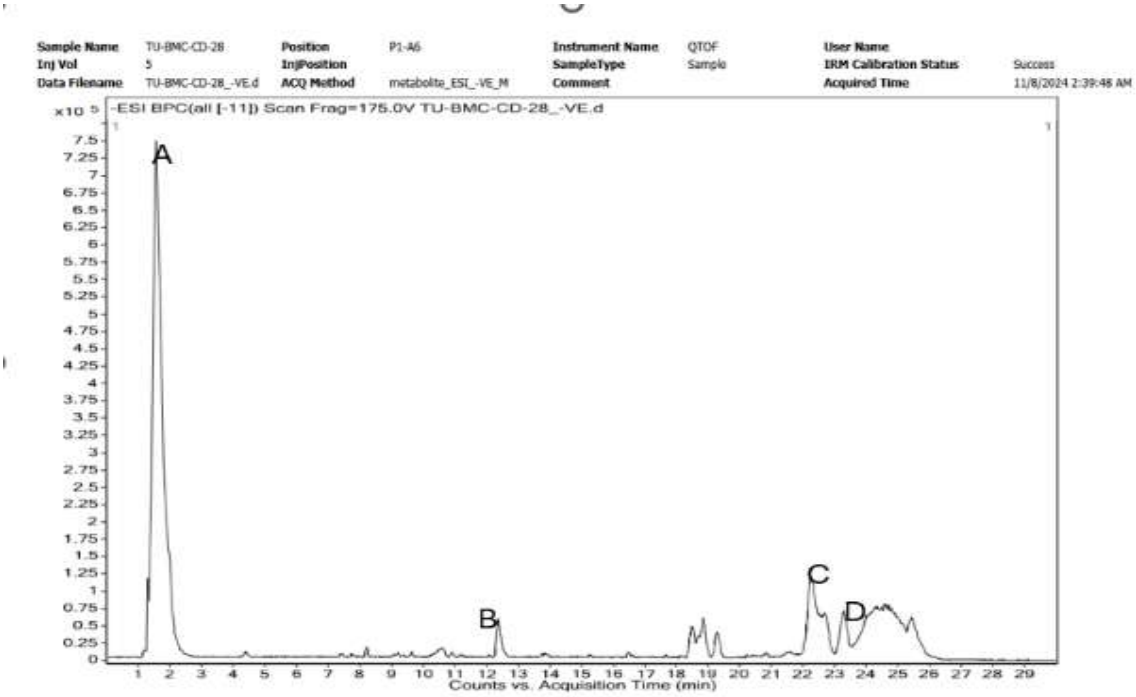


Figure 11: Base peak chromatogram of H7 in negative mode

A=Brompheniramine

B=6-Demethoxycapillarisin

C=5-Hydroxy-7-(4-hydroxyphenyl)-1-phenyl-3-heptanone

D=Canrenone

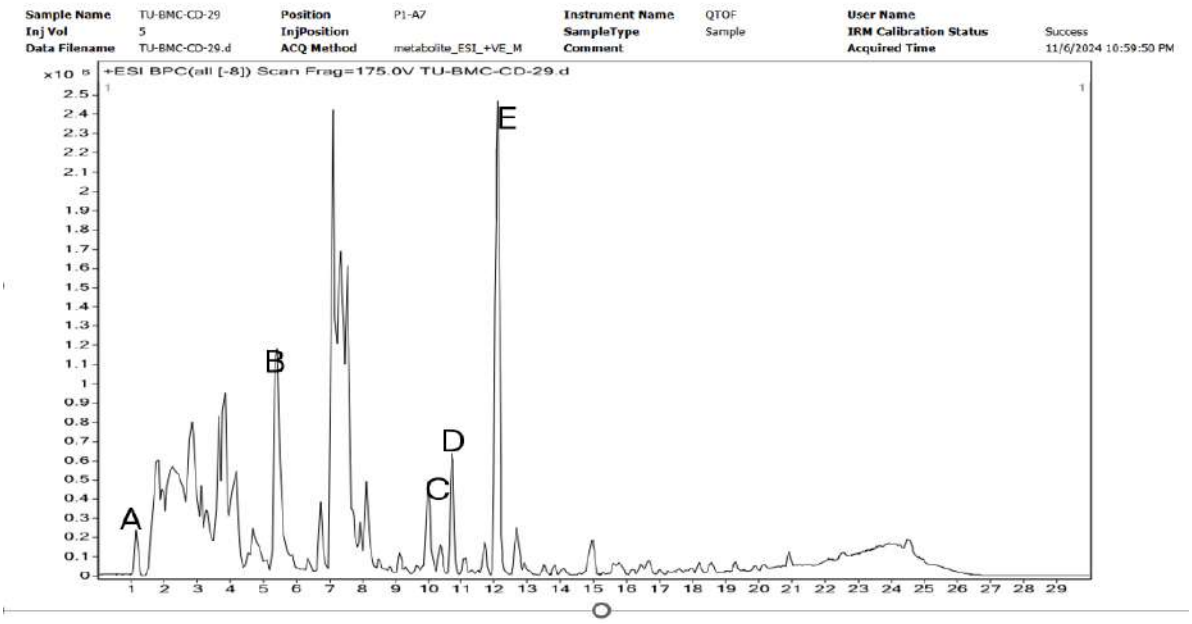


Figure 12: Base peak chromatogram of H12 in positive mode

A= Isopentyl gentiobioside

B= Myxochelin B

C= (Ac)2-L-Lys-D-Ala

D=Aspartyl-glutamate

E= Portulacaxanthin III

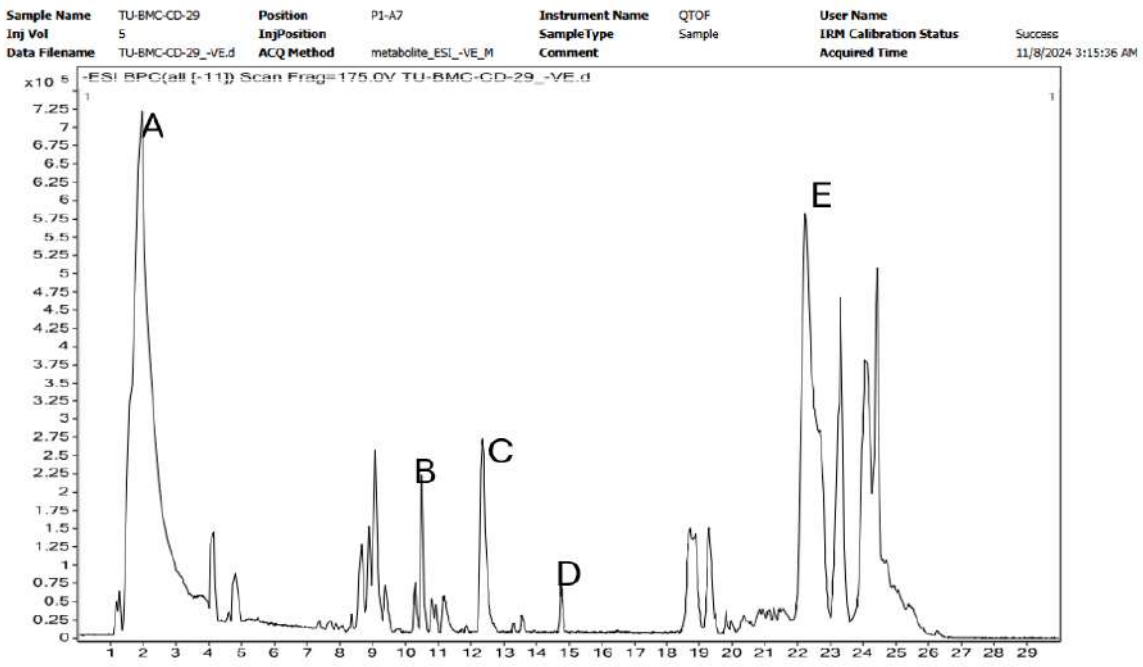


Figure 13: Base peak chromatogram of H12 in negative mode

A= Quercetin 3,7-dimethyl ether

B=Pinobanksin

C=luteolin

D=Emodin

E=4-dodeclybenzenesulfonic acid

Table 10 : HR-LCMS in honey

Compound name	H3	H7	H12	formula	m/z	ion	mass	RT (min)	Polarity	Identified	Properties
Naringenin-7-O-glucuronide	+			$C_{21}H_{20}O_{11}$	447.0924	[M-H]−	448.0999	9.614	-	flavonoid	Treatment of Alzheimer's Disease(Lawal et al., 2018)
Chlorogenic acid	+	+		$C_{16}H_{18}O_9$	353.0869	[M-H]−	354.0941	6.7	-	Phenolic	antioxidant and anticarcinogenic properties (Feng et al., 2005)
6-Demethoxycapillarisin		+		$C_{15}H_{10}O_6$	285.0394	[M-H]−	286.0466	12.382	-	Flavonoid	antidiabetic
Palmidin C	+			$C_{30}H_{22}O_7$	539.1382	(M+HCOO)-	494.1378	1.689	-	Flavonoid	member of anthracenes.
Canrenone	+	+	+	$C_{22}H_{28}O_3$	339.1998	[M-H]−	340.2066	24.274	-	Steroidal lactone	aldosterone antagonist, andcardioprotective agent.(Derosa et al., 2019a)
3-O-Methylquercetin		+		$C_{16}H_{12}O_7$	315.0505	[M-H]−	316.0577	12.443	-	Flavonoid	Phytopharmaceutical preparation.
5-Hydroxy-7-(4-hydroxyphenyl)-1-phenyl-3-heptanone		+		$C_{19}H_{22}O_3$	297.1527	[M-H]−	298.1597	18.77	-	Ketone Compound	antiviral effects against the influenza virus in vitro.(Sawamura et al., 2010)
Kaempferol			+	$C_{15}H_{10}O_6$	285.0407	[M-H]−	286.0477	12.358	-	Flavonoid	antioxidant, reduces inflammation, supports heart health, may help prevent cancer(Imran et al., 2019)

Compound name	H3	H7	H12	formula	m/z	ion	mass	RT (min)	Polarity	Identified	Properties
Emodin			+	$C_{15}H_{10}O_5$	269.0449	[M-H]−	270.0522	14.896	-	Anthraquinone	protect the kidneys in diabetes by preventing a type of cell damage called ferroptosis(Luo et al., 2021)
Caffeic acid			+	$C_9H_8O_4$	179.0352	[M-H]−	180.0422	7.259	-	Phenolic Acid	Antimicrobial Drugs toward Microbial Pathogens(Khan et al., 2021)
Pinobanksin			+	$C_{15}H_{12}O_5$	271.0605	[M-H]−	272.0677	10.606	-	Flavonoid	anti-oxidant, anti-bacterial, anti-inflammatory, anti-parasitic, anti-mutagenic, anti-proliferative, and anti-angiogenic agent
N-trans-Feruloyloctopamine			+	$C_{18}H_{19}NO_5$	328.1162	[M-H]−	329.1214	4.128	-	Phenolic Acid Derivative	prevent liver cancer cells from spreading by blocking signals.
Kinobeon A	+	+	+	$C_{20}H_{20}O_6$	379.1134	[M-H]+	356.1241	1.589	+	Phenolic compound	antioxidant, tyrosinase inhibition, and anti-inflammatory properties.
Benzosimuline	+	+	+	$C_{20}H_{19}NO_2$	328.1316	(M+Na)+	305.1427	3.147	+	Alkaloid	cytotoxic as well as antiplatelet aggregation activity
Phenindamine	+	+		$C_{19}H_{19}N$	284.1428	(M+Na)+	261.1534	4.538	+	Antihistamine	first-generation antihistamine with sedative, anticholinergic, and

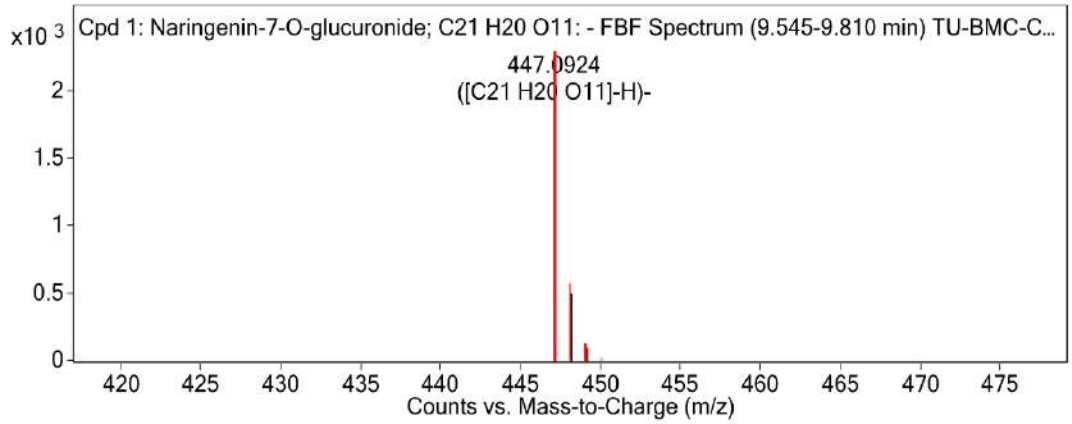
Compound name	H3	H7	H12	formula	m/z	ion	mass	RT (min)	Polarity	Identified	Properties
											local anesthetic properties(Wiiek et al., n.d.)
10-Hydroxyamitriptyline	+			$C_{20}H_{23}NO$	316.1677	(M+Na)+	293.1782	4.759	+	Amines	antidepressant, anticholinergic(Shimoda et al., 1997)
Cyclacillin	+	+	+	$C_{15}H_{23}N_3O_4S$	342.1472	[M-H]+	341.1399	5.399	+	Amino-derived	Treatment of infections of the respiratory system, urinary tract (UTIs), and skin. (Scheld et al., 1986)
Ophthalmic acid	+			$C_{11}H_{19}N_3O_6$	290.1325	[M-H]+	289.1254	6.244	+	Amino-derived	antioxidant and neuroprotective properties(Servillo et al., 2018)
Pemoline		+		$C_9H_8N_2O_2$	177.0661	[M-H]+	176.0559	0.872	+	oxazolidinone derivative)	CNS stimulant, stimulant ADHD(Riggs et al., 2004)
Gabapentin		+		$C_9H_{17}NO_2$	194.1134	(M+Na)+	171.1242	4.219	+	Amino compound	medication primarily used to treat neuropathic pain, epilepsy, and restless leg syndrome(Linde et al., 2013)
2-Aminoheptanedioic acid		+		$C_7H_{13}NO_4$	198.0718	(M+Na)+	175.0826	5.511	+	Amino compound	amino acid derivative involved in lysine metabolism(Barberini et al., 2019)
Acetohexamide		+		$C_{15}H_{20}N_2O_4S$	325.1188	[M-H]+	324.1117	10.679	+	sulfonylurea class of	treat type 2 diabetes mellitus by stimulating

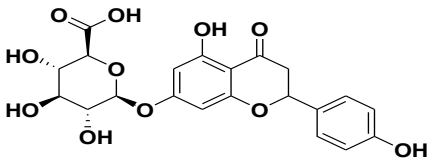
Compound name	H3	H7	H12	formula	m/z	ion	mass	RT (min)	Polarity	Identified	Properties
										antidiabetic drugs.	the release of insulin
3,4',5,6,8-Pentamethoxyflavone			+	$C_{20}H_{20}O_7$	395.1075	(M+Na)+	372.1185	1.867	+	Flavonoid	antioxidant, anticancer, and anti-inflammatory properties.
Melosatin B			+	$C_{19}H_{19}NO_2$	294.1489	[M-H]+	293.1415	2.943	+	Flavonoid	anticancer and antimicrobial activity.
Melochinone			+	$C_{22}H_{21}NO_2$	360.1571	(M+Na)+	293.1416	3.275	+	Quinone	cytotoxic and antimicrobial.
Capecitabine			+	$C_{15}H_{22}FN_3O_6$	354.1469	[M-H]+	359.1493	3.501	+	Antimetabolite (Prodrug of 5-FU)	chemotherapy drug for colon, breast, and gastric cancer.(Hoon et al., 2021)
Linezolid			+	$C_{16}H_{20}FN_3O_4$	337.1453	[M-H]+	338.1526	4.712	+	Oxazolidinone Antibiotic	treat bacterial infections, including MRSA and drug-resistant tuberculosis.(Singh et al., 2019)
Grandidentatin			+	$C_{21}H_{28}O_9$	424.1755	[M-H]+	425.1833	4.717	+	Phenolic Compound	antioxidant and anti-inflammatory properties.(Zhang et al., 2006)
Rutaecarpine			+	$C_{18}H_{13}N_3O$	287.1091	[M-H]+	288.1166	4.906	+	Alkaloid	traditional medicine, exhibits vasodilatory, anti-inflammatory, and anticancer effects.(Ueng et al., 2002)
Metyrapone			+	$C_{14}H_{14}N_2O$	226.1138	[M-H]+	227.1204	5.837	+	Steroido	Used in the diagnosis of adrenal insufficiency

Compound name	H3	H7	H12	formula	m/z	ion	mass	RT (min)	Polarity	Identified	Properties
										genesis Inhibitor	and treatment of Cushing's syndrome.(Fiad et al., 1994)
Lacidipine			+	$C_{26}H_{33}NO_6$	455.231	[M-H]+	456.2389	5.863	+	Dihydro pyridine Calcium Channel Blocker	treat hypertension.
Aspartyl-glutamate			+	$C_9H_{14}N_2O_7$	262.0803	(M+Na)+	285.0696	5.863	+	Amino Acid Derivative	neurotransmitter and plays a role in cognitive functions and neuroprotection.(Li et al., 2013)

4.12.1. Selective secondary metabolites found in honey with their structure.

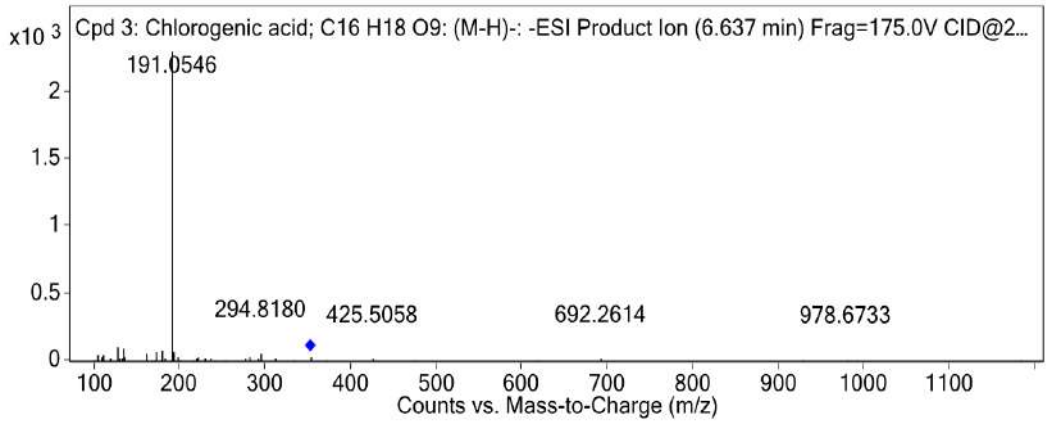
1. **Naringenin-7-O-glucuronide**: This flavonoid is primarily known for its potential in treating Alzheimer's disease. It possesses antioxidant properties and is a potent neuroprotective.(Lawal et al., 2018)

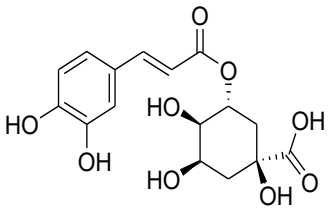




Naringenin-7-O-glucuronide

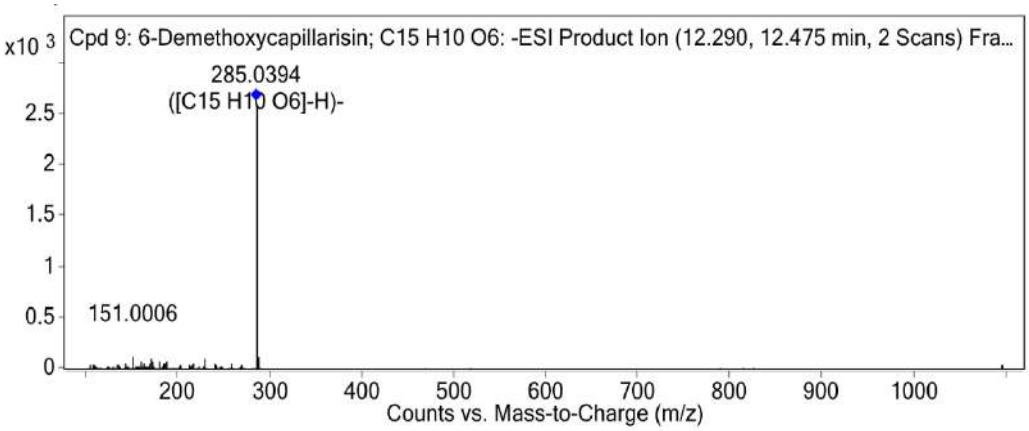
2. **Chlorogenic acid**: It is known for its antioxidant and anticarcinogenic properties, chlorogenic acid plays a role in reducing oxidative stress and may help in the prevention of cancer.(Feng et al., 2005)

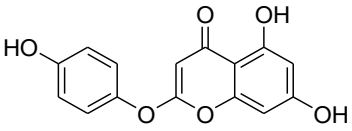




Chlorogenic acid

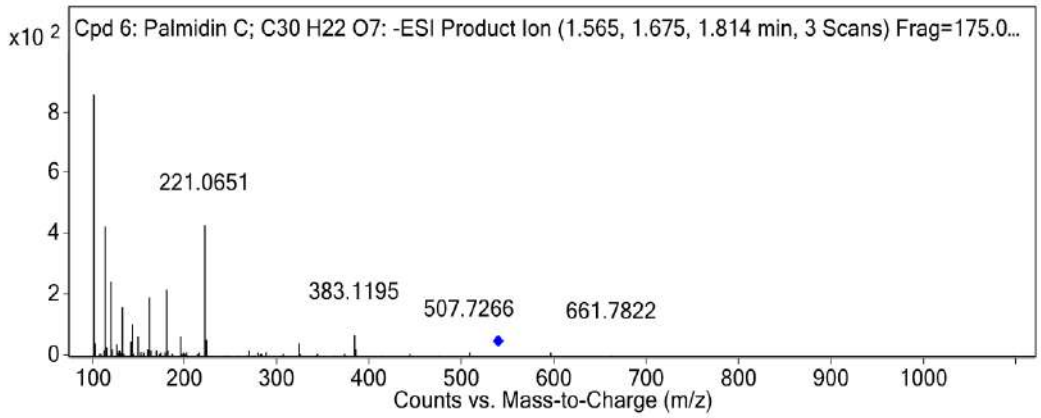
3. **6-Demethoxycapillarisin**: This flavonoid is praised for its antidiabetic properties. It can help in managing blood sugar levels and has potential therapeutic effects in diabetes treatment.

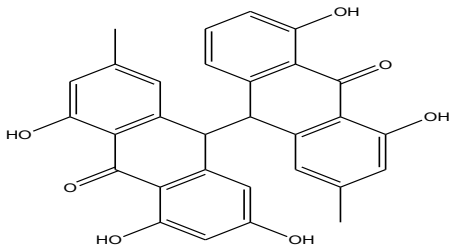




6-Demethoxycapillarisin

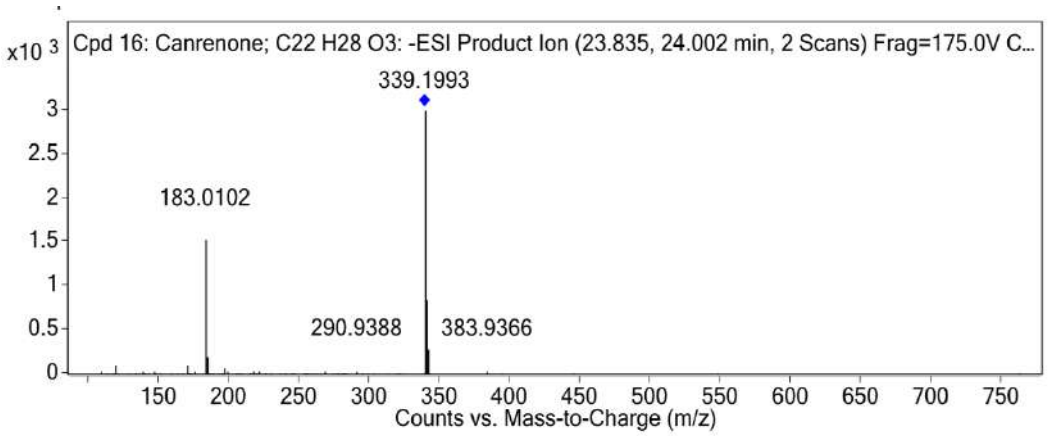
4. **Palmidin C**: Part of the flavonoid family, palmidin C is categorized as an anthracene derivative. It holds potential in various therapeutic applications due to its antioxidant and anti-inflammatory properties.

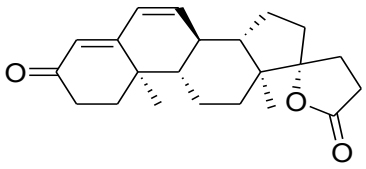




palmidin C

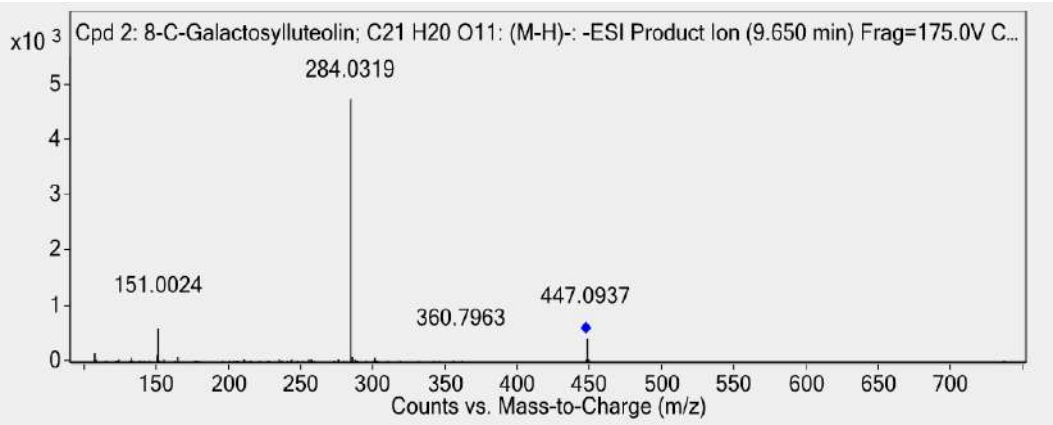
5. **Canrenone**: A steroidal lactone, canrenone functions as an aldosterone antagonist. It is utilized in managing hypertension and heart conditions by promoting fluid balance and preventing sodium retention.(Derosa et al., 2019b)

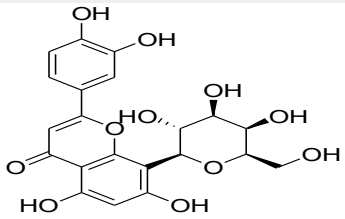




Canrenone

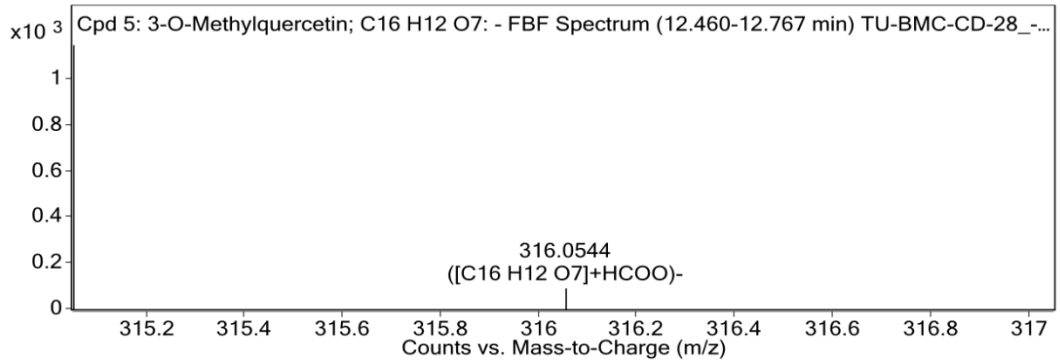
6. **8-C-Galactosylluteolin**: It is a flavonoid known for its anti-inflammatory and antioxidant and anti-inflammatory property. It contributes to reducing oxidative stress and might have therapeutic effects in chronic conditions. (Derosa et al., 2019b)

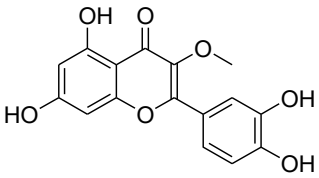




8-C-Galactosylluteolin

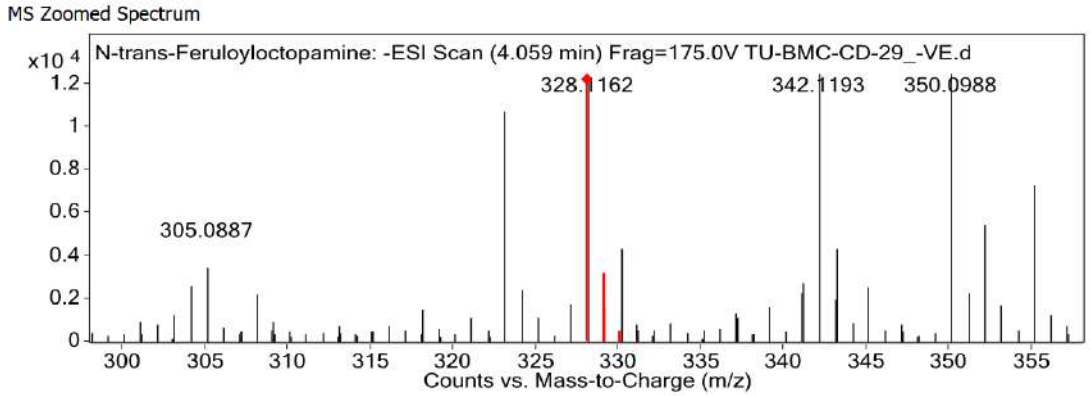
7. **3-O-Methylquercetin**: A flavonoid with potential medicinal value, including anti-inflammatory and antioxidant effects. It is often explored for its health benefits in phytopharmaceutical preparations

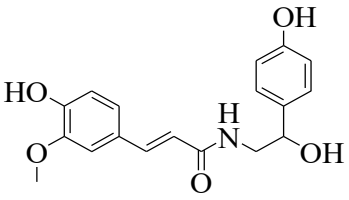




3-O-Methylquercetin

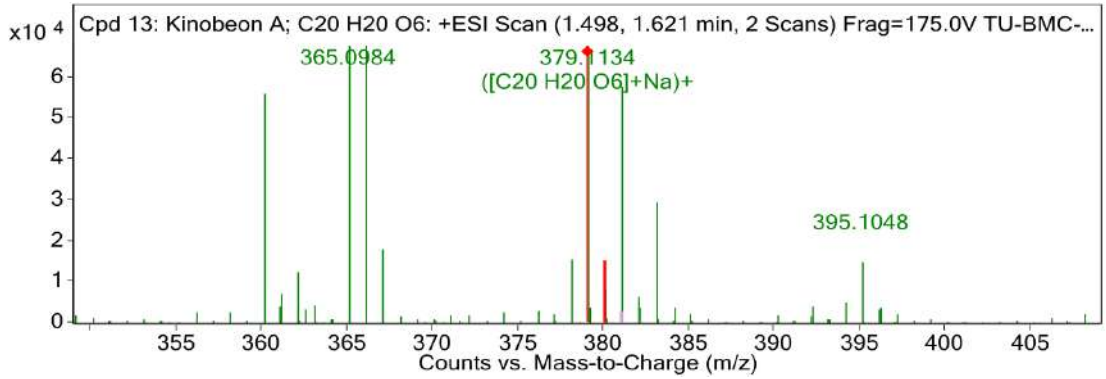
8. **N-trans-Feruloyloctopamine**: A phenolic acid derivative, it is known for its potential role in preventing liver cancer cell proliferation by blocking essential signaling pathways.

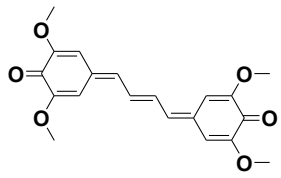




N-trans-Feruloyloctopamine

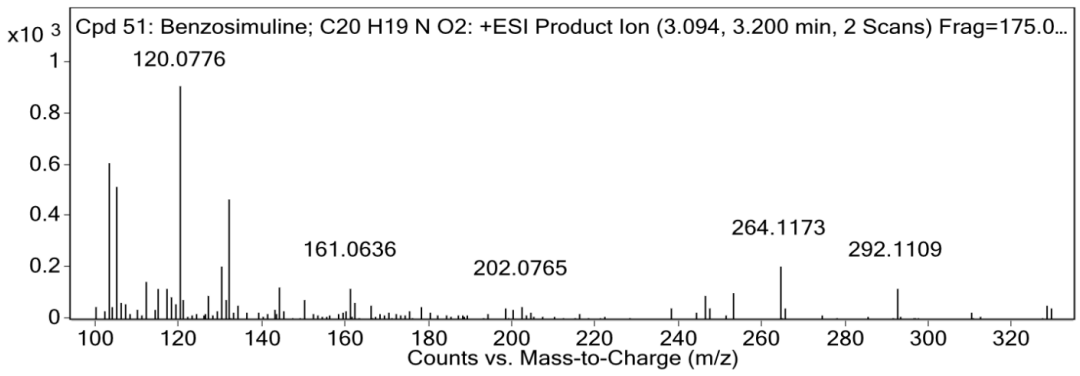
9. **Kinobeon A**: A phenolic compound with antioxidant and anti-inflammatory properties. It has shown promise in tyrosinase inhibition, which is beneficial for skincare treatments. .(Scheld et al., 1986)

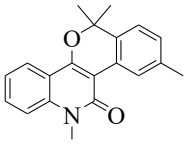




Kinobeon A

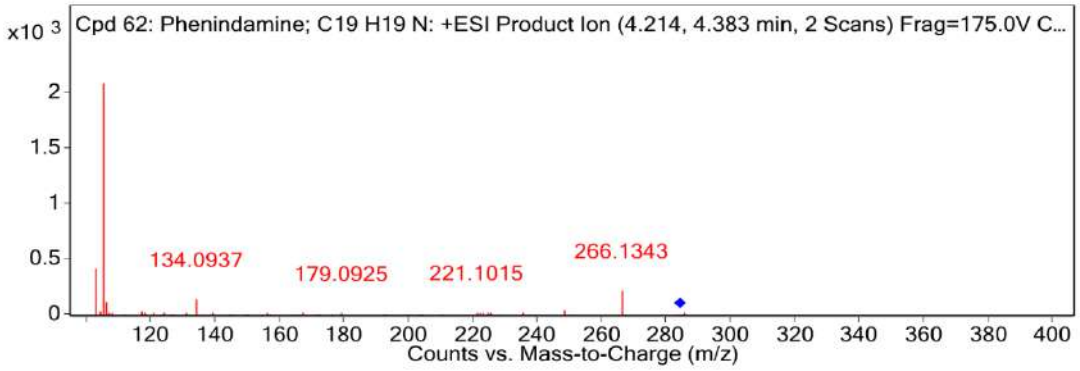
10. **Benzosimuline**: An alkaloid compound that displays cytotoxic and antiplatelet aggregation activity. It could be useful in treating platelet disorders or as a cancer therapeutic agent.(Riveira et al., 2016)

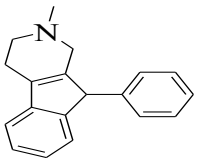




Benzosimuline

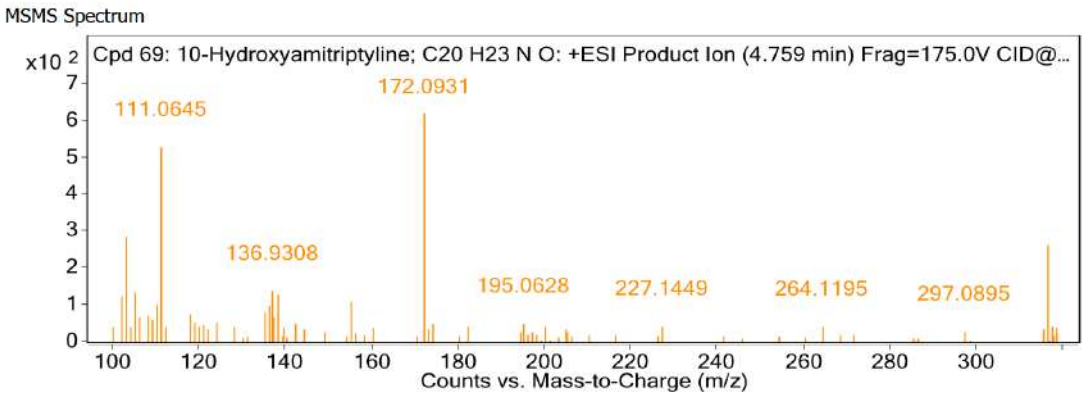
11. **Phenindamine**: An antihistamine that is used to treat allergic reactions. It has sedative, anticholinergic, and local anesthetic properties, making it useful in various therapeutic applications.(Wiiek et al., n.d.)

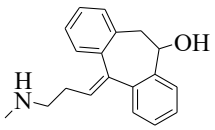




Phenindamine

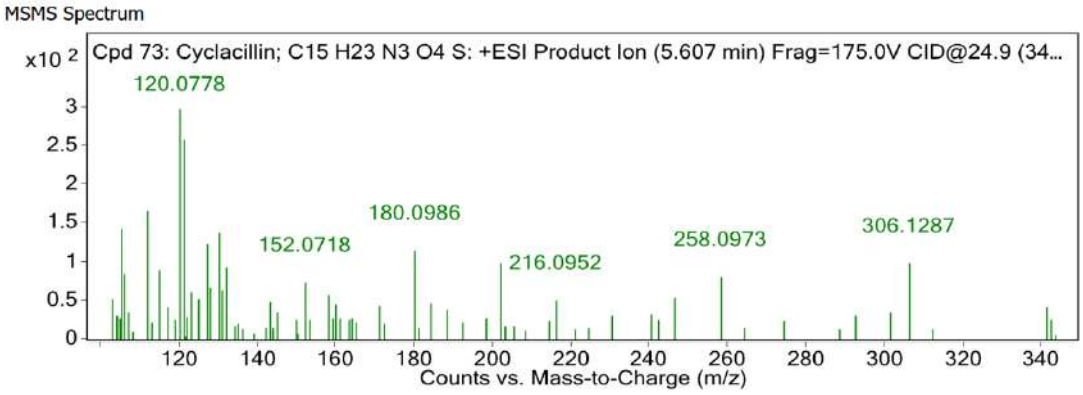
12. **10-Hydroxyamitriptyline**: An antidepressant with anticholinergic effects, it is used to treat mood disorders. It may also have some analgesic benefits in treating pain. .(Scheld et al., 1986)

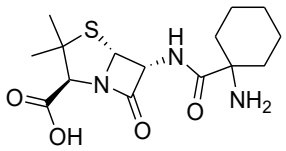




10-Hydroxyamitriptyline

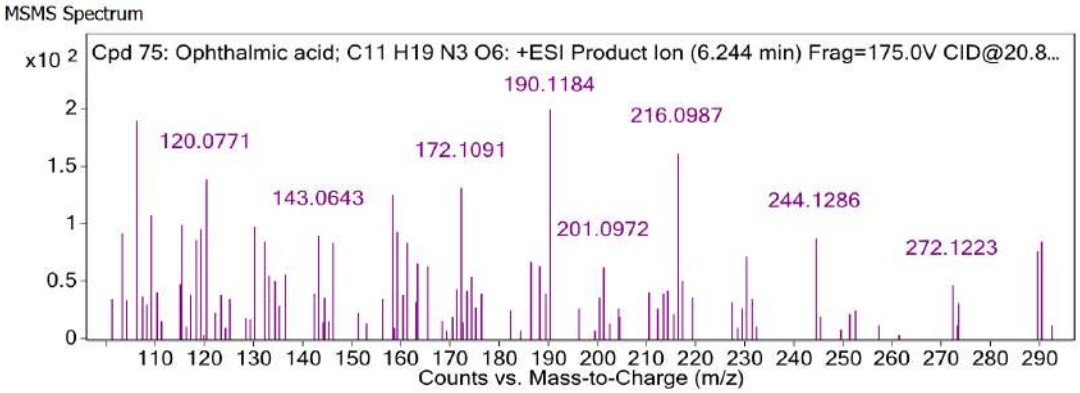
13. **Cyclacillin**: An antibiotic used to treat respiratory tract infections, urinary tract infections, and skin infections. It is part of the penicillin group and is used in various clinical treatments.(Scheld et al., 1986)

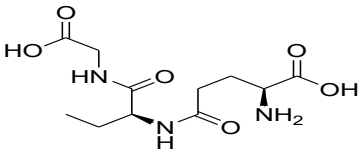




Cyclacillin

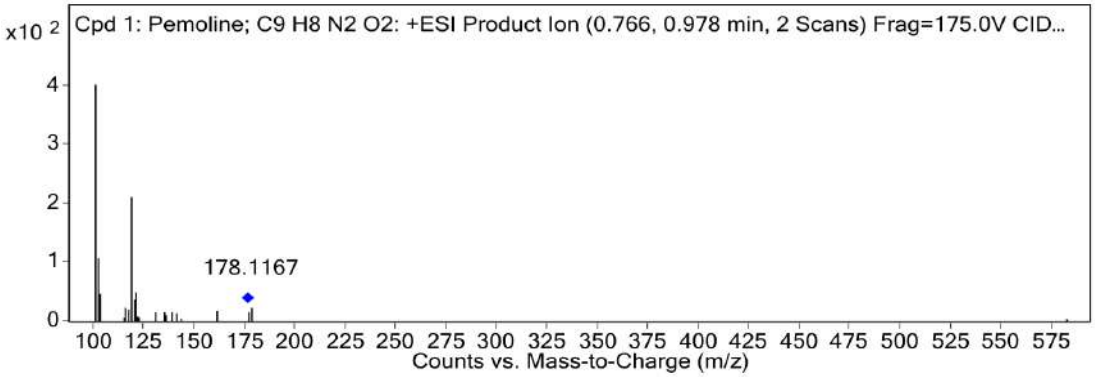
14. **Ophthalmic acid**: It is known for its neuroprotective properties, ophthalmic acid is an antioxidant that may play a role in protecting the brain and supporting cognitive function.

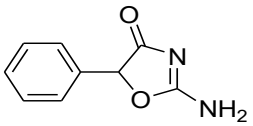




Ophthalmic acid

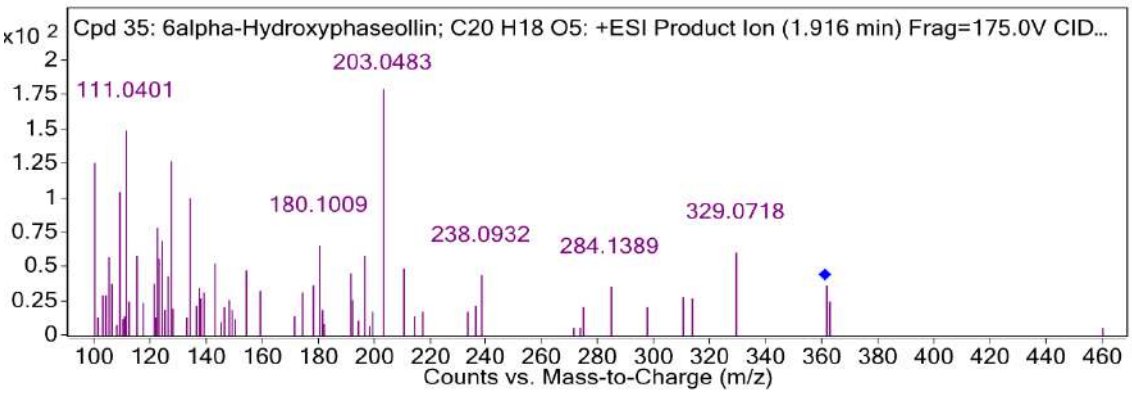
15. **Pemoline**: This CNS stimulant is used to treat ADHD. It has stimulant properties and can help improve focus and attention in individuals with ADHD.(Servillo et al., 2018)

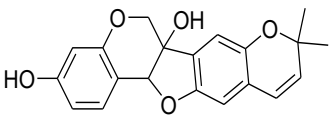




Pemoline

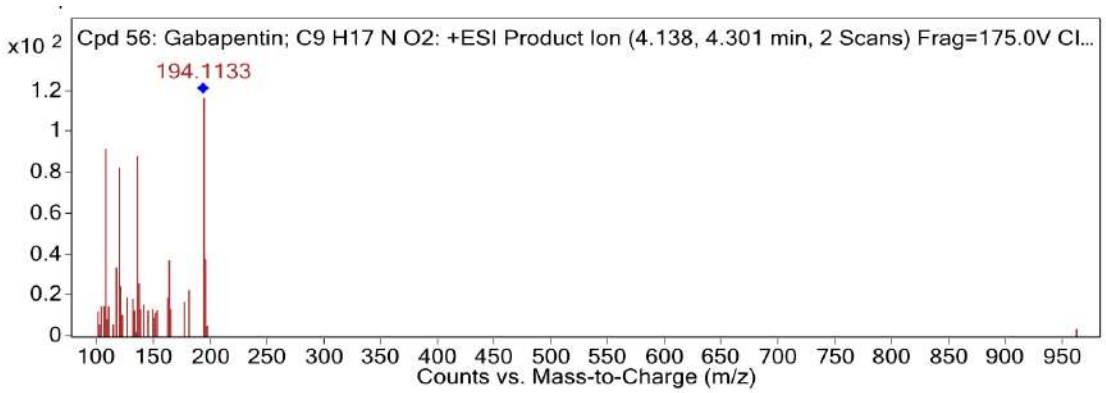
16. **6alpha-Hydroxyphaseollin**: An isoflavonoid with potential therapeutic benefits, including anti-inflammatory and antioxidant effects. It is studied for its role in managing chronic diseases.

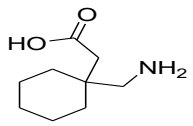




6alpha-Hydroxyphaseollin

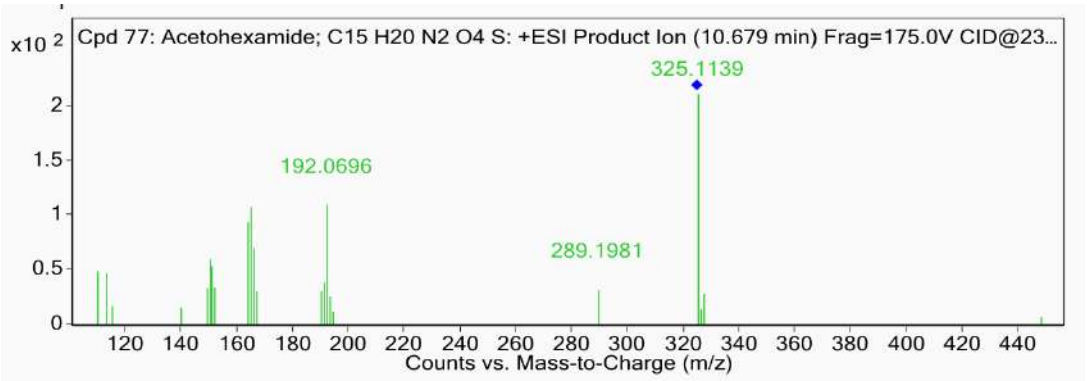
17. **Gabapentin**: A medication used to treat neuropathic pain, epilepsy, and restless leg syndrome. It works by stabilizing nerve activity in the brain and spinal cord.(Linde et al., 2013)

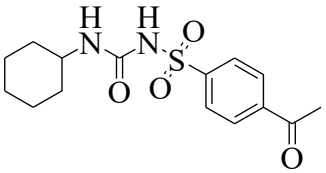




Gabapentin

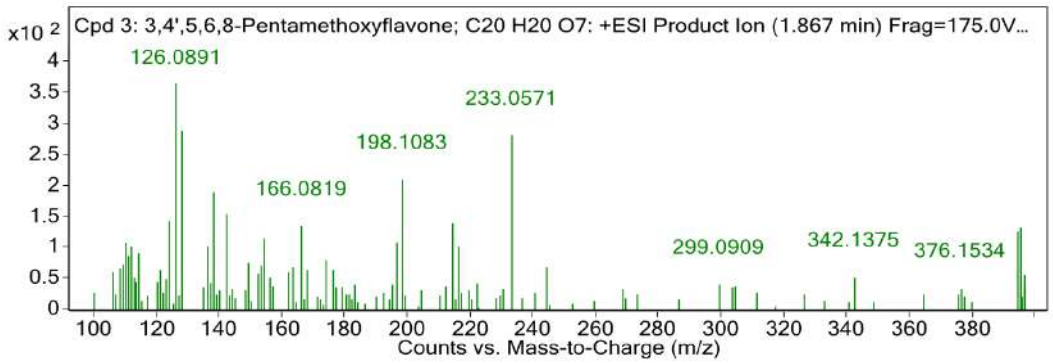
18. **Acetohexamide**: It is a sulfonylurea drug used to treat type 2 diabetes mellitus by stimulating insulin release from the pancreas.

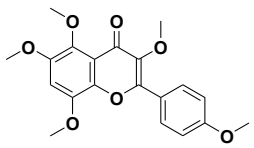




Acetohexamide

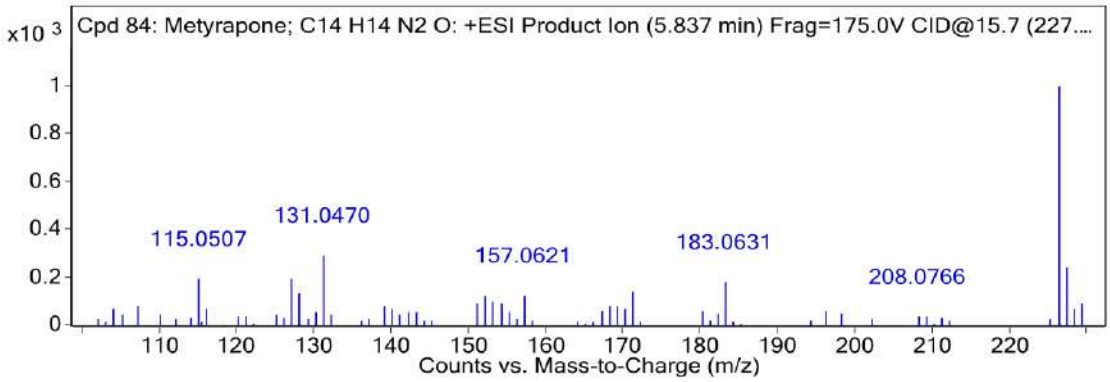
19. **3,4',5,6,8-Pentamethoxyflavone**: This flavonoid exhibit antioxidant, anticancer, and anti-inflammatory properties, contributing to its therapeutic potential in various chronic conditions.

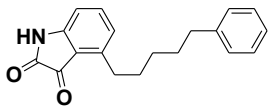




3,4',5,6,8-Pentamethoxyflavone

20. **Melosatin B**: A flavonoid known for its anticancer and antimicrobial activities. It is studied for its potential in treating cancers and preventing infections.



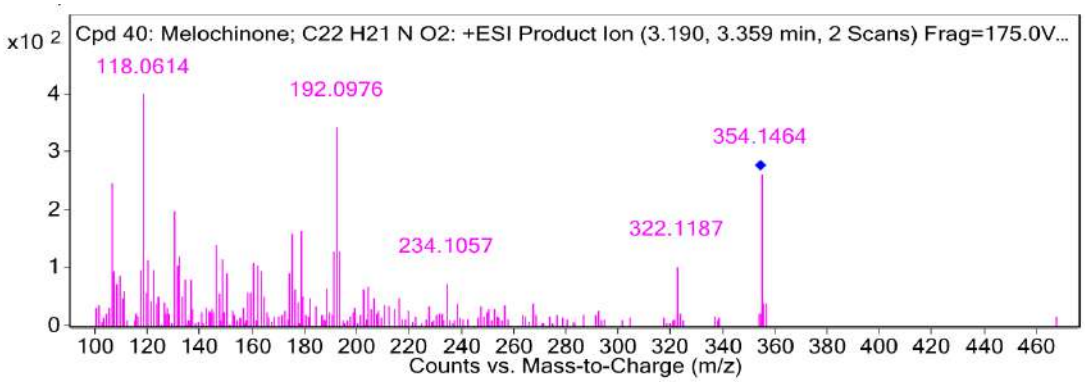


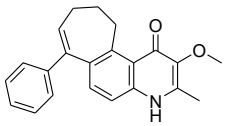
Melosatin B

O

O

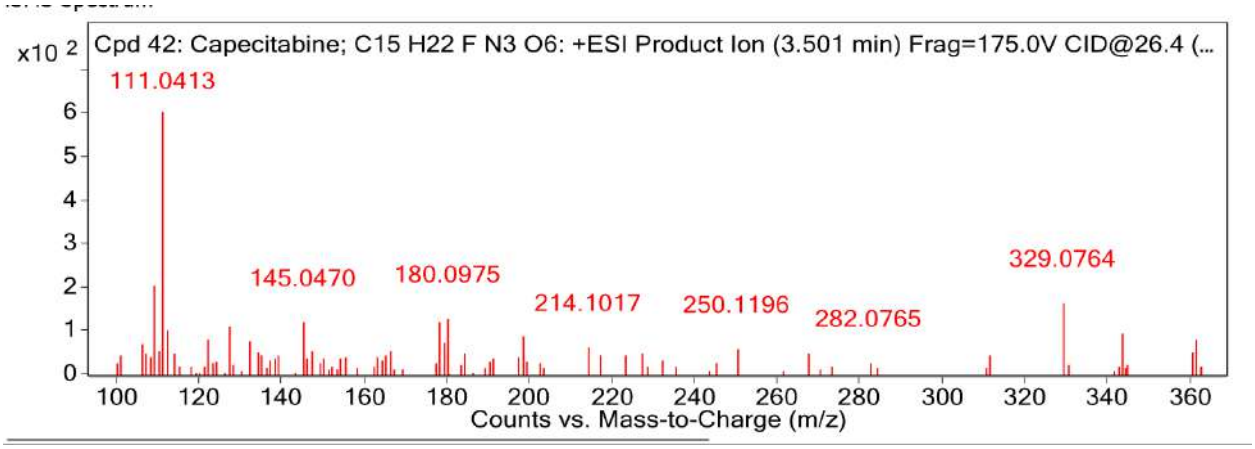
21. **Melochinone**: A quinone compound with cytotoxic and antimicrobial properties, melochinone is explored for its therapeutic potential in cancer treatment and infection management.Click or tap here to enter text.

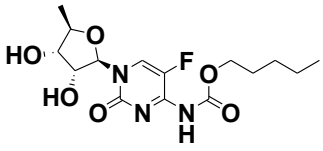




melochinone

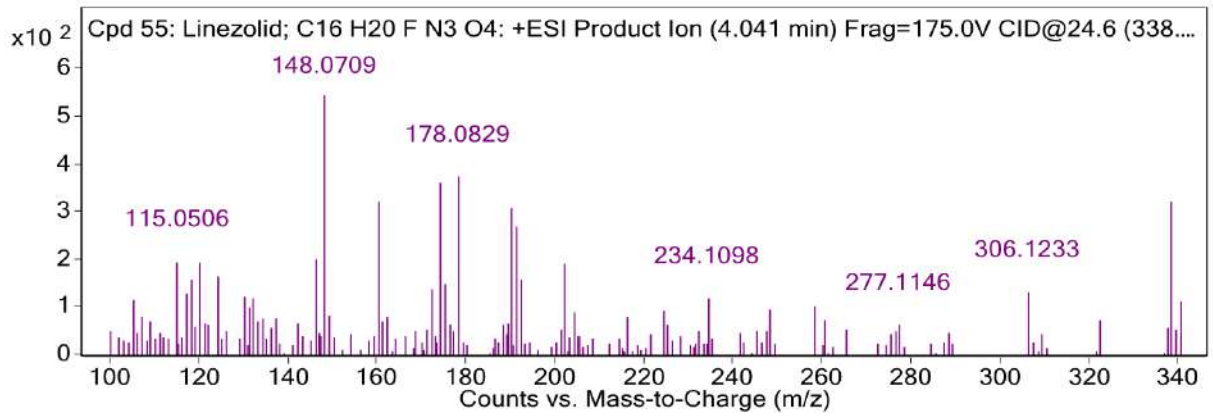
22. **Capecitabine**: An antimetabolite chemotherapy drug that is a prodrug of 5-FU, used to treat colon, breast, and gastric cancer.

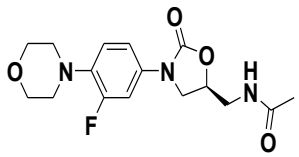




Capecitabine

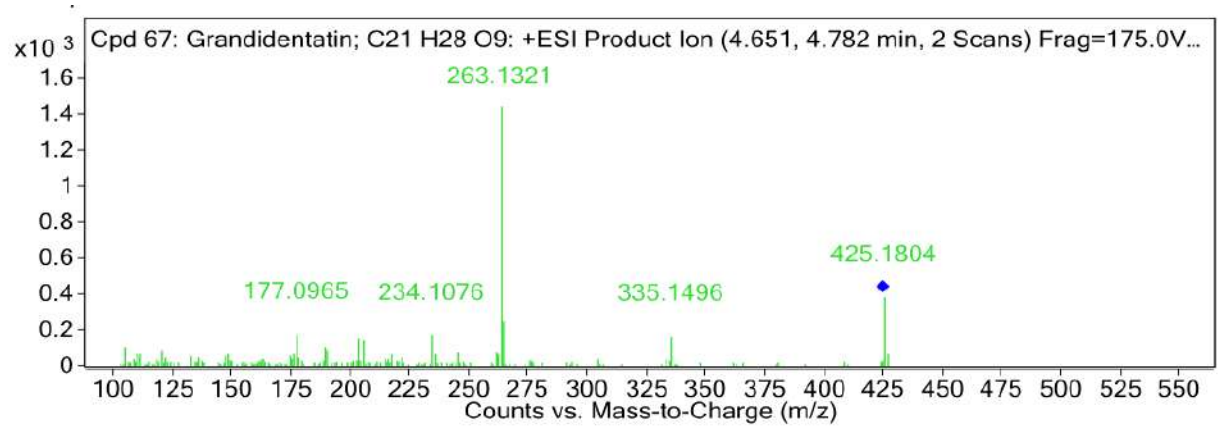
23. **Linezolid**: An oxazolidinone antibiotic used to treat bacterial infections, including MRSA and drug-resistant tuberculosis. It is effective in combating multi-drug-resistant infections.(Singh et al., 2019)

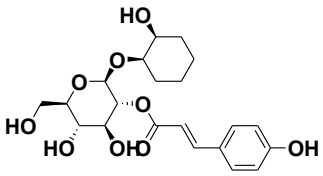




Linezolid

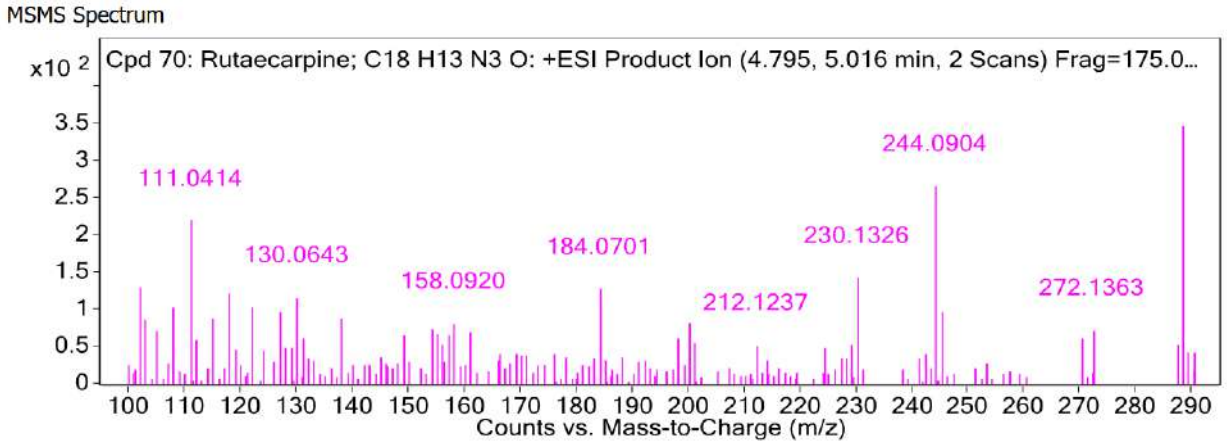
24. **Grandidentatin**: A phenolic compound known for its antioxidant and anti-inflammatory properties.

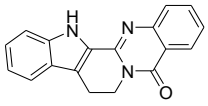




Grandidentatin

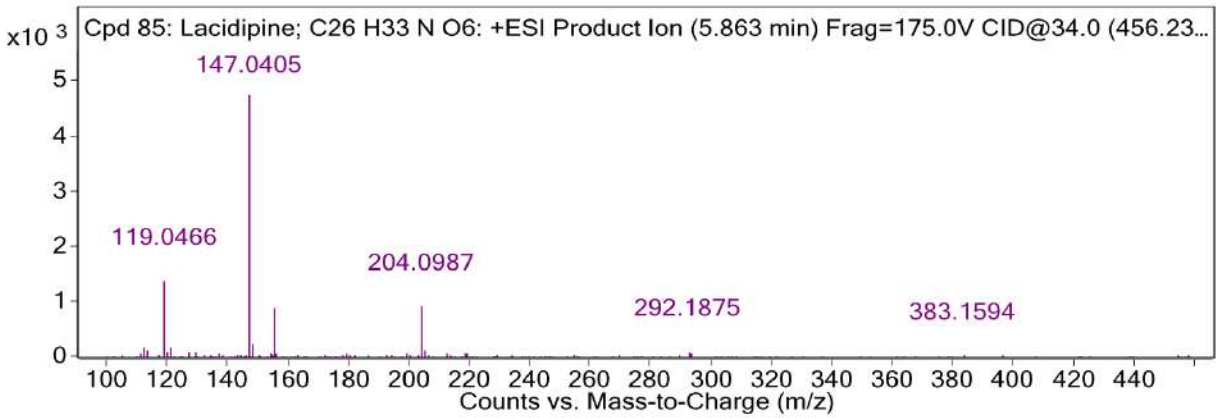
25. **Rutaecarpine**: An alkaloid found in traditional medicine, rutaecarpine has vasodilatory, anti-inflammatory, and anticancer effects, making it valuable in cardiovascular health and cancer prevention.(Ueng et al., 2002)

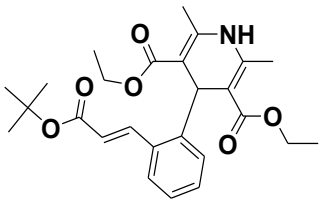




rutaecarpine

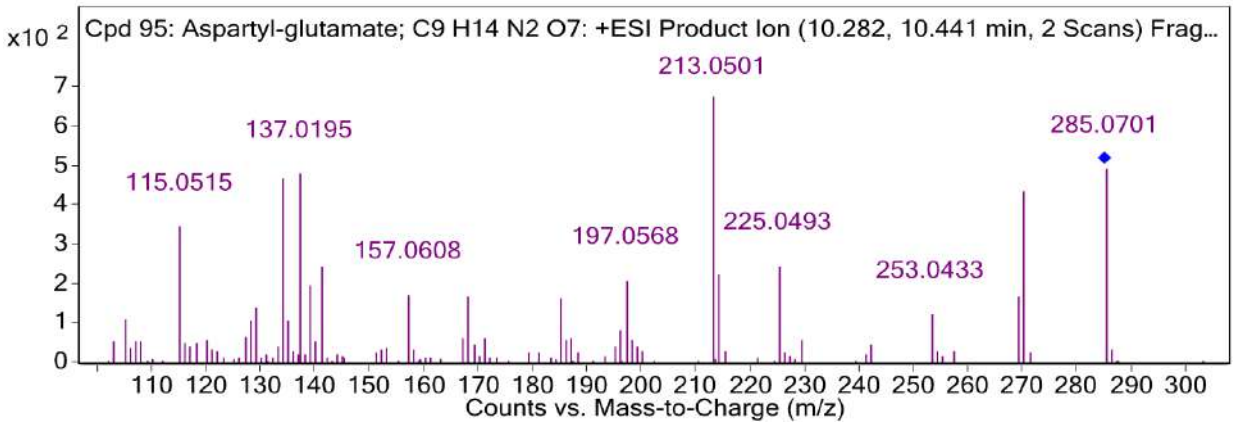
26. **Lacidipine**: A dihydropyridine calcium channel blocker used to treat hypertension. It helps reduce blood pressure by relaxing the blood vessels.Click or tap here to enter text.

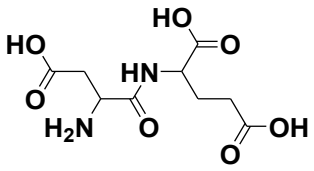




Lacidipine

27. **Aspartyl-glutamate**: An amino acid derivative involved in neurotransmission, it plays a role in cognitive functions and neuroprotection, making it important in managing neurological health.(Li et al., 2013)





Aspartyl-glutamate

CHAPTER 5

CONCLUSIONS

5.1. Conclusions

This study presents a detailed examination of the physicochemical and bioactive characteristics of honey samples sourced from different floral origins in Nepal. The moisture content of the analyzed honey samples varied between 13.86±0.02% to 19.89±0.03%, with sample H2 exhibiting the highest moisture content (19.89±0.03%). Electrical conductivity varied between 0.20±0.02 and 1.72±0.02mS/cm, with sample H12 showing the highest value (1.72 mS/cm). All analyzed honey samples exhibited acidic properties, with pH values ranging from 3.86±0.02 to 4.95±0.03, aligning with the standard characteristics of honey. Sample H6 displayed a notably higher pH (4.95±0.02), while sample H5 had the lowest pH (3.86±0.02), correlating with its higher citric acid content (68.64 mg/100g). Lactic acid content ranged from 12.05±0.02 to 19.04±0.02mg/100g, with sample H5 again showing the highest concentration (19.04±0.02 mg/100g). Riboflavin content varied between 0.01±0.001 to 0.09±0.004 mg/100g, with sample H7 representing a typical level (0.0187 mg/100g) for *Apis cerana* honey.

Antioxidant activity, as measured by DPPH, ranged from 33.24% to 53.10%, with sample H5 exhibiting the highest activity (53.10%). The IC_{50} values for antioxidant activity ranged from 1158.08 µg/mL to 1662.63 µg/mL.Total phenolic content (TPC) ranged from 21.71±3.42 to 72.02±2.103 mg GAE/100mg/ml, with sample H13 showing significantly higher levels. Similarly, total flavonoid content (TFC) ranged from 9.43±1.23 to 39.69±1.67 GAE/100mg/ml, with sample H13 again exhibiting the highest levels.

HR-LCMS analysis revealed the presence of numerous bioactive compounds, including Naringenin-7-O-glucuronide, Chlorogenicacid, Kaempferol, Caffeic acid, Emodin, Pinobanksin, 6-Demethoxycapillarisin, Isomaltulose, Palmidin C, Canrenone, 8-C-Galactosylluteolin, 3-O-Methylquercetin, 5-Hydroxy-7-(4-hydroxyphenyl)-1-phenyl-3-heptanone, N-trans-Feruloyloctopamine, Benzosimuline, Phenindamine, 10-Hydroxyamitriptyline, Cyclacillin, Ophthalmicacid, Pemoline, 6alpha-Hydroxyphaseollin, Gabapentin, 2-Aminoheptanedioic acid, and

Acetohexamide. These compounds are known for their diverse therapeutic properties, such as antioxidant, antimicrobial, anti-inflammatory, neuroprotective, and antidiabetic effects. Naringenin-7-O-glucuronide and Kaempferol are associated with neuroprotection and anti-inflammatory activities, making them potential candidates for treating neurodegenerative diseases like Alzheimer's. Chlorogenic acid and Caffeic acid demonstrated significant antioxidant and antimicrobial properties, supporting honey's role in combating oxidative stress and microbial infections. Emodin and Pinobanksin exhibited protective effects against kidney damage and cancer cell proliferation, respectively, further emphasizing honey's therapeutic activity. Acetohexamide highlights honey's potential in managing metabolic disorders, including diabetes, due to their antidiabetic properties. Canrenone, a steroidal lactone, showed cardioprotective and aldosterone antagonist properties, which could be beneficial for cardiovascular health.3-O-Methylquercetin and 6-Demethoxycapillarisin are known for their antioxidant and antidiabetic effects, contributing to honey's health benefits.5-Hydroxy-7-(4-hydroxyphenyl)-1-phenyl-3-heptanone exhibited potential antiviral activity, particularly against influenza virus, suggesting honey's role in viral infection management and Kinobeon A demonstrated anti-inflammatory and antioxidant properties, further supporting honey's therapeutic applications. Gabapentin and Acetohexamide are associated with neuropathic pain relief and diabetes management, respectively, highlighting honey's potential in addressing chronic health conditions.

5.2. Recommendations

Unidentified compound appeared in the HR-LCMS should isolate and characterize them.

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APPENDIX.

1 Preparation of reagents for phytochemical screening

i) Wagner reagent: 2g of iodine and 6g of potassium iodide was dissolved in 100 mL of water.

ii) Mayer reagent: 1.36gms of mercuric chloride and 5 gm of potassium iodide were dissolved in 100 mL of distilled water.

iii) Ferric chloride solution (5% w/v): 5gms of ferric chloride was dissolved in 100 mL of distilled water.

3.6.1.2 Qualitative analysis of bioactive compounds

i) Tests for alkaloids :

a) Mayer Test: A few drops of Mayer's reagent were introduced to 1 mL of the sample solution, resulting in the formation of a yellowish or white precipitate, which signified the presence of alkaloids.

b) Wagner Test:1 mL of Wagner's reagent was mixed with 1 mL of the sample solution, leading to the formation of a reddish-brown precipitate, which suggests the presence of alkaloids.

ii) Tests for flavonoids:

a) Alkali Test: Two to three drops of sodium hydroxide were added to 2 mL of the sample solution. A deep yellow color appeared initially, which gradually turned colorless upon the addition of a few drops of dilute HCl, indicating the presence of flavonoids.

b) Lead Acetate Test: To 1 mL of the sample solution, 3-4 drops of 10% lead acetate solution were added. The formation of a yellow precipitate indicates the presence of flavonoids.

iii) Tests for Carbohydrates:

a) Molisch's Test:1 mL of the sample solution was mixed with 2-3 drops of 1% alcoholic alpha-naphthol, followed by the addition of 2 mL of concentrated H_2SO_4 along the side of the test tube. A violet ring appeared at the junction of the two layers, indicating the presence of carbohydrates.

b) Fehling Test: equal volumes ofFehling's A (copper sulfate in distilled water) and Fehling's B (potassium tartrate and sodium hydroxide in distilled water) were combined. A few drops of the sample were added and the mixture was boiled. The formation of a brick-red precipitate of cuprous oxide indicates the presence of reducing sugars.

iv) Test for tannins

a) Ferric Chloride Test: A few drops of ferric chloride reagent were added to the test solution. Formation of an intense green, purple, blue, or black color indicated the presence of tannins.

v) Test for Phenolic compounds

a) Ferric Chloride Test: A few drops of neutral 5% ferric chloride solution were added to the test solution. The appearance of a dark green color signified the presence of phenolic compounds.

b) Lead Acetate test: A few drops of 10% lead acetate solution were added to the test solution. The formation of a white precipitate indicates the presence of phenolic compounds.

Vi) Test for polyphenols

a) Ferric Chloride Test: A few drops of 10% $FeCl_3$ solution were added to the sample solution and mixed. The development of a blue or dark green color, or the formation of a precipitate, indicates the presence of phenolic compounds.

b) Lead Acetate Test: 2 mL of lead acetate solution were added to the sample solution. A white precipitate formed.

Vii) Test for Proteins

a) Ninhydrin Test

A mixture of 5 mL of honey extract and 2 mL of a 0.2% Ninhydrin solution was boiled. The appearance of a violet color indicated the presence of proteins.

viii) Test for saponins

A drop of $Na_2 CO_3$ solution was added to 5 mL of the extract in a test tube. After shaking vigorously and letting it sit for five minutes, the formation of foam indicated the presence of saponins.

ix) Test for starch

a) Iodine Test: 2 milliliters of iodine solution with potassium iodide were added to 2 mL of the test extract. The appearance of a blue color indicated the presence of starch**.**

x) Test for Steroids

Two ml of sample solution, 10 mL of chloroform, and 1 ml of acetic anhydride were combined in a test tube. Then, 2 mL of concentrated sulfuric acid (H_2SO_4) was carefully added along the side of the tilted test tube. A ring formed at the boundary between the solutions.

PLAGIARISM REPORT

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जो जस सँग सम्बन्धित छ ।

तपाई **RANJANA KHANAL** 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 **PHYSICOCHEMICAL AND HR-LCMS ANALYSIS OF *SECONDARY METABOLITES* PRESENT IN HONEY COLLECTED *FROM DIFFERENT FARMS OF NEPAL*** शिर्षकको ***M.Sc. in*** **CHEMISTRY** तहको उपाधिका लागि तयार गरिएको Dissertation / Thesis मा प्लेजारिजम परीक्षण पछिको समानता सूची (Similarity Index)१९(उन्नाइस)प्रतिशत रहेको व्यहोरा प्रमाणित गरिन्छ ।

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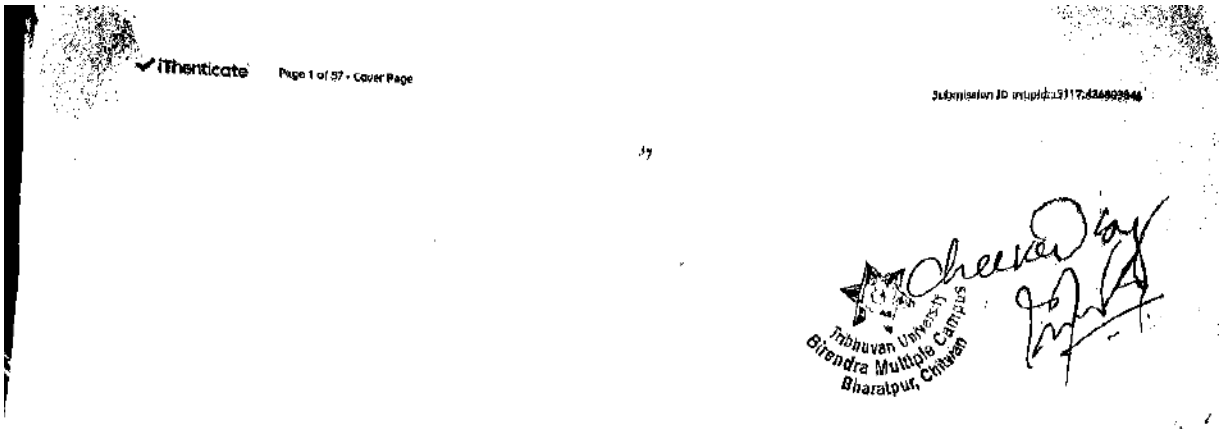

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