ANTIOXIDANT, ANTIBACTERIAL AND HR-LCMS ANALYSIS OF SECONDARY METABOLITES PRESENT IN *Psidium guajava* L. (GUAVA) LEAVES

A THESIS SUBMITTED TO THE

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INSTITUTE OF SCIENCE AND TECHNOLOGY

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BY

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DECLARATION

I declare that this dissertation entitled "**Antioxidant, Antibacterial and HR-LCMS Analysis of Secondary Metabolites Present in *Psidium guajava* L. (Guava) Leaves,**" are my own research work. This work has not been published or accepted and submitted for any degree award. Plagiarism checked at Birendra Multiple Campus Library also confirmed that the work is original and genuine

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RECOMMENDATION

The dissertation entitles **"Antioxidant, Antibacterial and HR-LCMS Analysis of Secondary Metabolites Present in *Psidium guajava* L. (Guava) Leaves,"** is submitted by **Mr. Bipin Khanal** for the partial fulfilment of M.Sc. degree in Chemistry at Birendra Multiple Campus. The entire work is completed under our supervision. All the reports presented here are his finding. We confidently recommend this thesis for final evaluation.

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FOREWORD

The thesis work "**Antioxidant, Antibacterial and HR-LCMS Analysis of Secondary Metabolites Present in *Psidium guajava* L. (Guava.) Leaves**," submitted by **Bipin 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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Thank you all

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LIST OF ABBREVIATIONS

AAC	:	Ascorbic acid Concentration
AAE	:	Ascorbic acid Equivalent
ABTS	:	2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
DMSO	:	Dimethyl Sulphoxide
DPPH	:	1,1- Diphenyl-2-Picrylhydrazyl
DPPH	:	2, 2-diphenyl-1-picrylhydrazyl
FCR	:	Folin - Ciocalteu Reagent
FRAP	:	ferric reducing antioxidant power
GAC	:	Gallic acid concentration
GAE	:	Gallic Acid Equivalent
GAE	:	Gallic acid equivalent
GC	:	Gas Chromatography
GC-MS	:	Gas Chromatography-Mass Spectrometry
IC_{50}	:	Inhibitory Concentration for 50% Inhibition
HR-LCMS	:	High Resolution Liquid Chromatography Mass Spectrometry.
Mg AAE/G	:	Milligram Ascorbic acid equivalent per gram
Mg GAE/g	:	Milligram Gallic acid equivalent per gram
MHA	:	Mueller Hinton Agar
ORAC		Oxygen Radical Absorbance Capacity
PPM	:	Parts per million
RF	:	Retention factor
TFC	:	Total Flavonoid Content
TPC	:	Total Phenolic Content
ZOI	:	Zone of Inhibition

LIST OF SYMBOLS

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

ABSTRACT

Antioxidant, antibacterial properties and secondary metabolites present in *Psidium guajava* L. (Guava) fresh leaves are still to explore fully. This study aimed to evaluate the phytochemical constituents present in fresh *Psidium guajava* L. (Guava) leaves to validate their medicinal potential.

The samples were collected (GPS point 27˚68'33" N 84˚43'33" E) from Bharatpur Metropolitan City-11, Chitwan, Nepal. Spectrophotometric methods were used to determine the phenolic, flavonoid, and tannin contents as well as the phosphomolybdenum and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assays. The Folin-Ciocalteu method was used to quantify the phenolic content, aluminium chloride colorimetric method was used to measure the flavonoid content and Folin-Ciocalteu method was used to calculate the tannin content. Antioxidant activity was assessed using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay and the phosphomolybdenum assay. Antibacterial activity was determined using the agar diffusion method and extract was characterised using HR-LCMS.

The qualitative analysis revealed the presence of terpenoids, glycosides, polyphenols, alkaloids, flavonoids and tannins. However, for quantitative evaluation maceration showed higher concentrations of total phenolic content (316.1±7.4 mg gallic acid equivalent (GAE)/g), total flavonoid content (257.9±4.4 mg/g quercetin equivalent (QE)), phosphomolybdenum assay (125.0±2.1mg ascorbic acid equivalent (AAE)/g), and DPPH free radical scavenging capacity (IC_{50}=6.08 µg/mL). Soxhlet extract showed highest tannin content consisting of 563.6±4.5µg/mL. The antimicrobial assay showed good activity against five bacterial strains. A total of 144 different metabolites were observed in HR-LCMS out of which 104 were known compounds and other 40 compounds were unidentified compounds. Among them four compounds were reported for the first time in *Psidium guajava* L. (Guava) fresh leaves.

Among the extraction methods used, maceration was the most effective for phenolic, flavonoid and antioxidant contents whereas sonication was the least effective. Furthermore, the secondary metabolites observed in HR-LCMS analyses possess significant impact on the antioxidant, phenolic, flavonoid, alkaloid, tannin, and antimicrobial properties. These results offer scientific support for the traditional use of fresh *Psidium guajava* L. leaves for medicinal purposes.

Keywords: *Alkaloid, Flavonoid, Phytochemicals, Phenolic, Tannin.*

सारांश

एन्टिअक्सिडेन्ट, एन्टिब्याक्टेरियल गुणहरू र *Psidium guajava* L. (गुवा) का ताजा पातहरूमा उपस्थित दोस्रो श्रेणीका मेटाबोलाइटहरू अझै पूर्ण रूपमा अन्वेषण गर्न बाँकी छन्। यस अध्ययनले ताजा *Psidium guajava* L. (गुवा) पातहरूमा रहेका फाइटोकिमिकल संघटकहरूको मूल्याङ्कन गर्ने लक्ष्य राखेको थियो ताकि तिनको औषधीय सम्भावनालाई पुष्टि गर्न सकियोस्।

नमूनाहरू भरतपुर महानगरपालिका-११, चितवन, नेपाल (GPS बिन्दु २७°६८'३३" N ८४°४३'३३" E) बाट सङ्कलन गरिएका थिए। फेनोलिक, फ्लाभोनोइड र ट्यानिन सामग्रीको निर्धारण गर्न साथै फोस्फोमोलिब्डेनम र १,१-डाइफेनाइल-२-पिक्राइलहाइड्राजाइल (DPPH) स्वतन्त्र रेडिकल स्काभेन्जिङ परिक्षण गर्न स्पेक्ट्रोफोटोमेट्रिक विधिहरू प्रयोग गरियो। फोलिन-सिओकाल्ट्यु विधि फेनोलिक सामग्रीको मात्रा निर्धारण गर्न प्रयोग गरियो, एलुमिनियम क्लोराइड रङ्गमित विधि फ्लाभोनोइड सामग्रीको मापन गर्न प्रयोग गरियो, र फोलिन-सिओकाल्ट्यु विधि ट्यानिन सामग्रीको गणना गर्न प्रयोग गरियो। एन्टिअक्सिडेन्ट गतिविधि १,१-डाइफेनाइल-२-पिक्राइलहाइड्राजाइल (DPPH) स्वतन्त्र रेडिकल स्काभेन्जिङ परिक्षण र फोस्फोमोलिब्डेनम परिक्षण प्रयोग गरी मूल्याङ्कन गरियो। एन्टिब्याक्टेरियल गतिविधि एगार डिफ्युजन विधि प्रयोग गरी निर्धारण गरियो र HR-LCMS प्रयोग गरेर एक्स्ट्रयाक्टको विशेषता अध्ययन गरियो।

गुणात्मक विश्लेषणले टरपेनोइड, ग्लाइकोसाइड, पॉलीफेनोल, एल्कलाइड, फ्लाभोनोइड र ट्यानिनको उपस्थिति देखायो। यद्यपि मात्रात्मक मूल्याङ्कनका लागि मेसेरेशन विधिले कुल फेनोलिक सामग्री (३१६.१±७.४ mg gallic acid equivalent (GAE)/g), कुल फ्लाभोनोइड सामग्री (२५७.९±४.४ mg/g quercetin equivalent (QE)), फोस्फोमोलिब्डेनम परिक्षण (१२५.०±२.१ mg ascorbic acid equivalent (AAE)/g), र DPPH स्वतन्त्र रेडिकल स्काभेन्जिङ क्षमता (IC_{50}=6.08 µg/mL) उच्च देखायो। सोक्सलेट एक्स्ट्रयाक्टले सबैभन्दा उच्च ट्यानिन सामग्री (५६३.६±४.५ µg/mL) देखायो। एन्टिमाइक्रोबियल परिक्षणले पाँचवटा ब्याक्टेरियल स्ट्रेन्स विरुद्ध राम्रो गतिविधि देखायो। HR-LCMS मा कुल १४४ विभिन्न मेटाबोलाइटहरू पहिचान गरिए, जसमध्ये १०४ चिनिएका कम्पाउन्डहरू थिए र अन्य ४० अज्ञात कम्पाउन्डहरू थिए। तीमध्ये चार कम्पाउन्डहरू *Psidium guajava* L. (गुवा) का ताजा पातहरूमा पहिलो पटक रिपोर्ट गरिएका थिए।

प्रयोग गरिएका एक्स्ट्रयाक्सन विधिहरूमध्ये, मेसेरेशन विधि फेनोलिक, फ्लाभोनोइड र एन्टिअक्सिडेन्ट सामग्रीका लागि सबैभन्दा प्रभावकारी थियो भने सोनिकेशन विधि सबैभन्दा कम प्रभावकारी थियो। साथै, HR-LCMS विश्लेषणमा अवलोकन गरिएका दोस्रो श्रेणीका मेटाबोलाइटहरूले एन्टिअक्सिडेन्ट, फेनोलिक, फ्लाभोनोइड, एल्कलाइड, ट्यानिन, र एन्टिमाइक्रोबियल गुणहरूमा महत्वपूर्ण प्रभाव पार्ने देखिएको छ। यी नतिजाहरूले *Psidium guajava* L. (गुवा) का ताजा पातहरूको औषधीय उपयोगको परम्परागत प्रयोगलाई वैज्ञानिक समर्थन प्रदान गर्छन्।

कीवर्डहरू: *एल्कलॉइड, फ्लाभोनोइड, फाइटोकिमिकल्स, फिनोलिक र ट्यानिन*

CHAPTER ONE: INTRODUCTION

1.1 Introduction

Nepal is a landlocked nation of stunning mountains, higher Himalayan region, unique wildlife and diverse cultures. From the top of Mount Everest to the Terai lowland regions, which are home to a multitude of human cultures and natural environments. China lies to the north, while India lies to the west, south and east. Its total area is 1,47,181 square kilometres, with its longitude ranging from 80°40' east to 88°12' east and its latitude ranging from 26°22' north to 30°27' north. Its east to west length measures around 885 km, and its north to south width is around 195 km. Around 86 percent of land area consists of green hills and high mountains and remaining 14% comprising Terai flatlands. The Terai region is only about 60 meters higher than sea level, while Mount Everest, the highest point on Earth, stands at 8848.86 meters above sea level (Chaudhary et al., 2016).

Distinct geographic location along with fluctuations in temperature and altitude, are reflected in its rich biodiversity. Nepal provides an enormous diversity of wildlife and plants due to its unique geography, which includes dramatic elevation changes and high levels of eco-climatic variability (Goverment of Nepal, 2023).

Chitwan is a central valley in Province No. 3, is one potential site for the establishment of native tropical plants that are medicinal and aromatic. Due to the favourable geophysical and ecological conditions, most tropical medicinal plant species are found Chitwan district, according to Joshi et al., (2019).

Plants are capable of producing a large variety of organic compounds with distinct and complex structures that can be divided into primary and secondary metabolites. Primary metabolites, which are compounds derived from fundamental inorganic molecules found in nature are necessary for the development of living things. Sugars, proteins, fatty acids, nucleosides, acetyl-CoA, and fatty acids are examples of primary metabolites.

Primary metabolites are used in the biosynthesis of secondary metabolites, which include terpenoids, alkaloids, flavonoids, quinones, polyphenols, and steroids. The

study of these secondary metabolites and their medicinal applications is known as natural product chemistry (Considine, 1984).

Maceration, soxhlet extraction, Sonication and other popular extraction techniques are some of the techniques used to acquire the crude mixture of phytochemicals. The obtained phytochemicals are grouped various classes, including, flavonoids, alkaloids and polyphenols (Abegaz et al., 2006). The specific kind of solvent employed during the extraction process is primarily responsible for the successful detection of biologically active plant components (Singh et al., 2012).

A group of molecules with a widely recognised possible therapeutic use are the polyphenols. Polyphenols are naturally occurring small molecular weight compounds (molecular weight 200–400 g/mol). They are produced as secondary metabolites that defend the plant from damaging bacteria and sunlight. The plant host initiates one of the synthesis pathways in reaction to an environmental threat, which causes the synthesis and subsequent secretion of polyphenol structures. The specific kind of polyphenol that generated depend on the host, the region of origin, and the surrounding conditions all play a major role (Dhale et al., 2014).

Flavonoids are linked to numerous health-promoting benefits and are an essential component of many pharmaceutical, medical, and cosmetic applications. This is because of their ability to change essential cellular enzyme functions as well as their antioxidative, anti-mutagenic, anti-inflammatory and anti-carcinogenic qualities (Panche et al., 2016).

Food manufacturers are using food-grade antioxidants to maintain the nutritional content and stop the products quality from deteriorating. Because they protect the body from chronic illnesses and damage from reactive oxygen species, antioxidants have attracted the interest of biochemistry and medical researchers (Guney et al., 2011). It is an easy, fast, and precise way to find out how much antioxidant activity is in a particular chemical or in extracts from plants. Because of its odd electron, DPPH (1,1-diphenyl-2-picrylhydrazyl) is stable radical to find antioxidant.

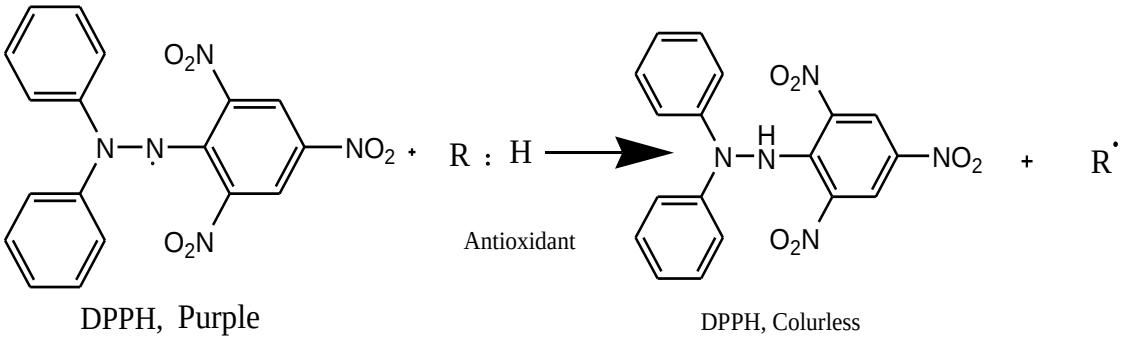


Figure 1: Mechanism of DPPH free radical scavenging.

Taxonomic Classification

Kingdom	:	Plantae
Sub-division	:	Spermatophyte
Division	:	Magnoliophyta
Order	:	Myrtales
Family	:	Myrtaceae
Genus	:	*Psidium*
Species	:	*guajava*

(Lone, Z. & Jain, N., 2022)

1.2 Objectives

1.2.1 General Objective

Phytochemical analysis and biological activity analysis of fresh *Psidium guajava* L. leaves are the main objectives of the study.

1.2.2 Specific Objectives

The following specific objectives are highlighted based on the above-mentioned general objective.

1. To prepare methanolic extract by maceration, soxhlet and sonication.
2. To detect TPC, TFC and tannin content.

3. To evaluate the antioxidant activity.
4. To determine the antibacterial properties.
5. To analyze function group present in secondary metabolites using FTIR.
6. To characterizes secondary metabolites using HR-LCMS.

CHAPTER TWO: LITERATURE REVIEW

The Medline index includes seven in vitro studies with extracts from *Psidium guajava* L. leaves. These seven studies by Sato et al., (2010) imply the possibility of anticancer activity.

The findings of Lee and Park suggest that acetone extract of guava branch (GBA) have anticancer effects by causing apoptosis and inhibiting the cell cycle and the total flavanol content (TFC) and total phenolic content (TPC) of GBA are displayed 35.99±1.6 and 575.94±1.3 in mg per g. The findings imply that GBA may represent a viable new therapeutic agent in the fight against cancer (Lee & Park, 2010).

Jiang et al., (2020) study showed that 66 chemical components identified in *Psidium guajava* L. leaves and it may play a key role in gene-targeting therapy because they slowed the growth of tumours via a gene regulatory network. By using network pharmacology. (Jiang et al., 2020) discovered that *Psidium guajava* L. leaves were possible targets that interacted with different types of tumours, cancer, and signalling pathways.

The Yan et al., (2006) study discovered that the *Psidium guajava* L. fruit has a low potential for secondary antioxidants but a high potential for primary antioxidants. Antioxidants are present in significant concentration. The ascorbic acid content rises and the total phenolic content falls as the fruit ripens. The *Psidium guajava* L. fruit has a high potential for primary antioxidants but a low capacity for secondary antioxidants. Overall, the study finds that it has a high antioxidant content and safe to eat and may have health benefits.

He & Venant, (2004) found that the total phenolic contents of Psidium guajava L. leaves extracts in ethanol and water were extremely high at 575.3±15.5 and 511.6±6.2 mg of GAE/g of dried weight material respectively. The lyophilised extracts antioxidant activity was evaluated using room-temperature DPPH colorimetry. The outcomes demonstrated that ascorbic acid was a more potent antioxidant than *Psidium guajava* L. leaves extracts. However, water leaves extracts demonstrated lower antioxidant activity.

Thaipong et al., (2006) conducted a comparison of antioxidant activity in *Psidium guajava* L. fruit extracts. The results showed a strong correlation between the ABTS, DPPH, FRAP, and ORAC assays, but a negative correlation between ascorbic acid levels and total carotenoids. The results of the study showed how crucial it is to employ several assays in order to accurately evaluate fruit extract antioxidant activity. Additionally, the study found that the different assays used to assess the antioxidant activity of fruit extracts from *Psidium guajava* L. showed strong correlations with one another. It has been discovered that the main sources of antioxidant activity in *Psidium guajava* L. fruit are ascorbic acid and phenolic. The study also discovered that the FRAP assay was a good way to assess antioxidant activity in *Psidium guajava* L. fruit extracts and that antioxidant activity measured in dichloromethane extract was lower than that measured in methanol extract.

The study by Nantitanon et al., (2010) examined the effects of specific parameters on the yield, total phenolic content (TPC), and antioxidant activity (AA) of the *Psidium guajava* L. leaves extract. The best way to extract *Psidium guajava* L. leaves is by ultra-sonication. The results demonstrated, as ultra-sonication method produced an extract with much higher TPC and AA. Young leaves displayed the highest activity in a study on leaves maturity. The research disclosed that hot water was most effective solvent for removing the active components.

Seo et al., (2014) investigated the impact of extracting *Psidium guajava* L. leaves with various concentrations of hydroethanolic solvents, water, ethanol, methanol, and other solvents on phenolic, flavonoids compounds and antioxidant qualities. Reduction power, nitric oxide and nitrate scavenging activities, DPPH radical and ABTS radical-scavenging abilities were used to assess the antioxidant capability. *Psidium guajava* L. leaves extracts antioxidant capacity was more strongly correlated with their phenolic compound content than with their flavonoid content. However, the hydro ethanolic solvent extracted more phenolic compounds than water carried out and 50% hydro ethanolic was found to be the most effective solvent with strong antioxidant properties.

According to Bisht et al., (2016) the crude extract of *Psidium guajava* L. leaves was a potent antimicrobial, as demonstrated by the minimum inhibitory concentration of 3.75 mg/mL for both *P. aeruginosa* and *B. subtilis*. The extracts contained bioactive

compounds like tannins, alkaloids, flavonoids, terpenoids, and saponins, according to phytochemical analysis.

A study was carried out by Zaminur Rahman et al., (2013) to investigate the potential of treated *Psidium guajava* L. leaves meal in broiler diets and determine the impact of dietary treatment at various levels on broiler production and quality attributes. 180-day-old broiler chicks (Cobb 500) were used in this study. They were split into four treatments at four or one day of age, and each treatment had three replications (15 birds per replication). 0%, 2.5%, 3.5%, and 4.5% *Psidium guajava* L. leaves meal was added to the diets that were manually prepared after being treated with different physical and chemical processes. The results revealed that, except for antibiotic sensitivity, mortality rate, and fat content, feed intake, body weight gain, and feed conversion ratio were almost identical at various dietary treatments and that the differences were not statistically significant. Increases in *Psidium guajava* L. leaves meal were linked to lower fat content and death rates up to a 4.5% level.

In their 2007 study, Chen and Yen utilised extracts from *Psidium guajava* L. to show strong antioxidant activity. The antioxidant properties of *Psidium guajava* L. extracts are additionally believed to be attributed to phenolic compounds. According to the finding *Psidium guajava* L extracts have a chemo-preventive applications (Chen & Yen, 2007).

CHAPTER THREE: MATERIALS AND METHODS.

3.1 Sample Collection

The samples were gathered from Bhojad-11, Chitwan, Bagmati Province (No. 03), Nepal, during the flowering phase in mid-summer, from June 2 to June 12, 2022. It was collected from the southern plains at an altitude of 208 meters above sea level, located at GPS point 27˚68'33" N 84˚43'33" E, and placed in a clean Gripper bag.

3.2 Identification of the Plant

Research Officer Ganga Dutta Bhatta carried out the taxonomic identification of the plants at "The National Herbarium and Plant Laboratories (NHPL)", Godawari, Nepal.

3.3 Sample Preparation

After the collection of fresh *Psidium guajava* L. leaves, the contaminants were eliminated by wash them a tap water and then a distilled water rinse. The plant parts were cut into small pieces using stainless steel scissors and then allowed to air dry at room temperature. The samples were left at room temperature (24 to 30 degrees Celsius) to dry for three to four weeks without exposure to the sun, or until a consistent sample weight was reached. Following the process of drying until the samples reached a uniform weight, they were sealed in a neat polythene zipper bag and kept inside a desiccator to avoid absorbing moisture.

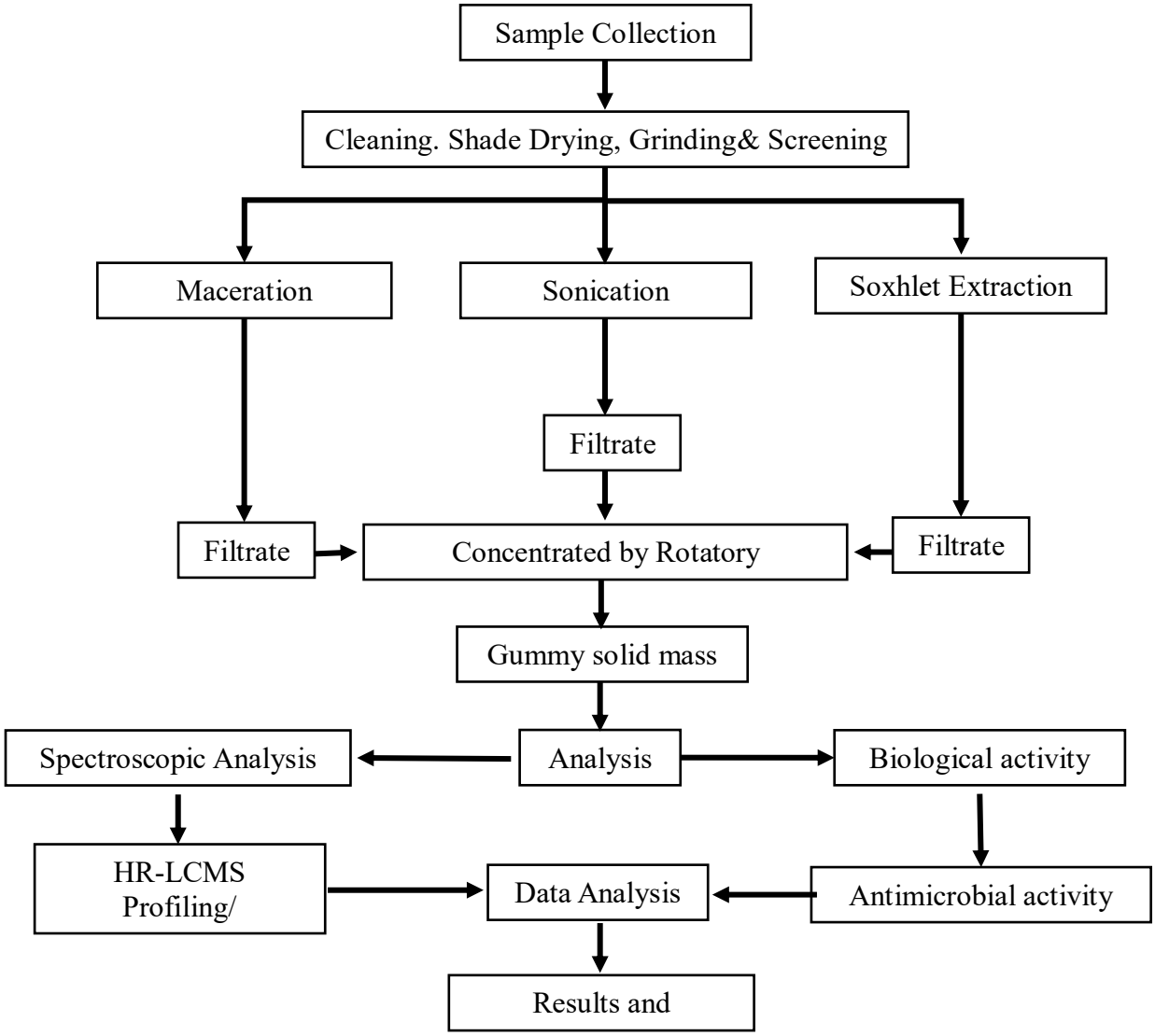


Figure 2: Scheme for extraction, fraction and analysis of fresh *Psidium guajava* L. leaves extract.

3.4 Extraction Process

Three extraction methods were employed: maceration, sonication and Soxhlet extraction. Methanol was used as the solvent to extract phytochemicals from powdered *Psidium guajava* L. leaves. Phytochemicals from powdered *Psidium guajava* L. fresh leaves were extracted using methanol as the solvent.

Separate dry and clean conical flasks were filled with 50 gram of powdered *Psidium guajava* L. fresh leaves for the maceration process. 500 mL of methanol was added to each flask, and it was left to macerate for 7 days while being shaken periodically. During this time, the mixture was filtered several times.

The sonication method involved mixing 500 mL of methanol with 50 gram of powdered *Psidium guajava* L. leaves. 20 minutes of 20 kHz frequency and 23 k rpm of sonication were applied to the mixture.

50 grams of powdered *Psidium guajava* L. leaves and 500 millilitres of methanol were added to a Soxhlet apparatus for the Soxhlet extraction. The extraction process took 16 hours to complete.

At the Birendra Multiple Campus of the Department of Chemistry, the mixtures were decanted and filtered using cotton plugs. The resulting filtrates were then concentrated using a rotary evaporator through a distillation process at a temperature lower than 40ºC. Aluminium foil with tiny pores was used to keep the concentrated filtrates in a beaker so that the solvent could evaporate more easily. There were (semi) solid methanol extracts after the solvent had completely evaporated. The following formula is used to calculate yield.

$$\text{Yield (\%)} = \frac{\text{Wt.of the extract (in g)}}{\text{Wt.of the powdered leaves or fruits (in g)}} \times 100\ \%$$

3.5 Phytochemical Analysis

The Atoui et al., (2005) protocol was applied in order to analyse the phytochemicals identified in *Psidium guajava* L extracts.

3.6 Total Phenolic Content (TPC)

The Folin Ciocalteu colorimetric method is used to determine TPC its depends on the oxidation-reduction reaction (Singleton & Rossi, 1965).

3.6.1 Preparation of the Standard Gallic Acid Solution

1000 µg/mL stock solution was produced by dissolving 10 mg of gallic acid in 10 mL of methanol. The stock solution was serially diluted to produce different gallic acid concentrations, comprising 5, 10, 20, 40, and 100 µg/mL.

3.6.2 Construction of the Calibration Curve

Briefly, to 0.5ml of each sample in graduated glass vial, 2.5ml of 1/10 dilution of Folin Ciocalteu reagent and 2ml of 7.5% Na_2CO_3 (w/v) were added, shaken vigorously and

incubated at 45°C for 15 minutes under light protected environment in hot water bath. After incubation, the absorbance having λmax at 765nm was determined using UV-vis spectrophotometer (T80+, PG Instrument, UK). Using the calibration curve generated with gallic acid as a standard, the total phenolic content was expressed mgGAE/g DS.

3.6.3 Preparation of the Sample Solution

A 1000 µg/mL stock solution of the extract was prepared by diluting 10 mg of the extract in 10 mL of methanol. Then, using the same method as for gallic acid, the extract was made in triplicate using serial dilution at a concentration of 100µg/mL, and its absorbance values were determined.

3.6.4 Calculation of the TPC

A standard calibration curve for gallic acid was developed and the straight line equation was used to calculate the concentration of total phenolic content.

$$\text{TPC} = \frac{C \times V}{m} \ldots\ldots\ldots\ldots\ldots \quad (1)$$

Where,

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

V = final volume of extract (mL)

m = weight in gram of extract

3.7 Total Flavonoid Content (TFC)

Aluminium chloride method was used to measure total flavonoid content. Quercetin was used as a standard (Fruit et al., 2015).

3.7.1 Preparation of the Standard Quercetin Stock Solution

The aluminum-chloride assay was used to determine the total flavonoid content, with quercetin serving as a standard. To make a quercetin stock solution, 10 mg of quercetin was dissolved in 10 millilitres of 80% methanol. 4, 16, 40, 80, 120 and 150µg/mL concentration of quercetin were prepared from stock solution and used for calibration. 1.5ml of 80% methanol was added to standards and samples (0.5ml) followed by addition of 100µl $AlCl_3$, 100µl Potassium acetate and 2.8ml distilled water with

vigorous shaking and left for incubation at 30°C for 30 minutes protected under light free condition. After incubation, a UV-VIS spectrophotometer (T80+, PG Instrument, UK) was applied to measure the absorbance at 410 nm. The mg QE/g DS were used to represent the total flavonoid content.

3.7.2 Preparation of the Sample Solutions

To prepare the stock solution (1000 µg/mL), 10 mg of the extract were dissolved in 10mL of methanol. Then, triplicate concentrations of the extract at 100 µg/mL were prepared by serial dilution and their absorbance were observed.

3.7.3 Calculation of the TFC

Same as mention in 3.6.4.

3.8 Total Tannin content

Total tannin content was determined by using folin-coicalteu phenol reagent (Labu et al., 2015).

Briefly, 0.1mL of the different sample extract (200 µg/mL)/standard tannin (20, 40, 80, 100, and 200µg/mL) was mixed with 0.5mL reagent and 7.5mL of distilled water. After that, 1 mL of 35% Na_2CO_3 was added and make final volume 10mL with distilled water. The mixture was mix and allowed to stand for 30min. at room temperature and measure absorbance at 725nm using spectrophotometer. Distilled water is used as a blank. The tannin content expressed as mg of tannic acid equivalent per gram of extract and a calibration curve was generated using a standard tannic acid. Using the calibration curve the tannin concentration was determined.

3.9 Antioxidant Activity using DPPH.

The following formula was applied to calculate the samples ability to scavenge free radicals (Jadid et al., 2017).

$$\text{Percentage scavenging (RSA)} = \frac{\text{Ao} - \text{A}}{\text{Ao}} \times 100\%$$

Where,

A_o= absorbance of control

A = absorbance of sample

Solutions of varying concentrations of ascorbic acid as standard was used to evaluate the 50% of radical scavenging capacity (IC_{50}) and graph was constructed to correlate the % RSA vs. concentrations of samples.

3.9.1 Preparation of 0.2mM DPPH Solution

A 0.2 mM DPPH solution was prepared by weighing out 0.005 g of DPPH, dissolved it in methanol in a 250 mL volumetric flask, and then adjusting the final volume to the proper level.

3.9.2 Preparation of Ascorbic Acid Solution.

To prepared 1000µg/mL, 10 mg of ascorbic acid were weighed out and dissolved in 10 mL of methanol to produce the stock solution, Ascorbic acid solutions with concentrations of 2, 4, 10, 20 µg/mL were then created by serial dilution.

3.9.3 Preparation of Sample Solutions

First, 10 mg of methanol extracts were weighed out and dissolved in 10 mL of methanol in order to produce a stock solution of 1000 µg/mL. Then by serial dilution, extract solutions having concentration 2, 4, 10, and 20 µg/mL were prepared.

3.9.4 Measurement of DPPH Radical Scavenging Activity.

1ml of varying concentrations of ascorbic acid standard and sample extracts were mixed with 2ml of 0.004% of methanolic DPPH and incubated at room temperature for 30 minutes in dark. After incubation, a UV-VIS spectrometer was applied to evaluate the absorbance of the standard and samples at 517 nm. A blank of 1ml methanol and 2ml 0.004% methanolic DPPH was used as control.

3.10 Antioxidant Activity Using the Phosphomolybdenum Method.

The phosphomolybdate method was implemented for estimation of total antioxidant capacity of samples using ascorbic acid as standard (Ajaib et al., 2017). In brief, 1ml of extract (200 µg/mL) and standard (5, 20, 40, 60 and 80 µg/mL) were separately mixed

with 4ml of phosphomolybdenum reagent. The phosphomolybdenum reagent was prepared by mixing equal parts of 4 mM ammonium molybdate, 28 mM Na_3PO_4, and 0.6 M H_2SO_4. The extract and phosphomolybdenum reagent mixture was incubated for 90 minutes at 95°C and it was allowed to cool to room temperature. After cooling, the absorbance having λ max at 695nm using spectrophotometer (T80+, PG Instrument, UK). The total antioxidant capacity of sample extract was expressed as mg AAE/g DS in comparision to the standard ascorbic acid calibration curve. A blank of 1ml reagent and 4ml methanol was implemented for the estimation of TAA.

Blank = Reagent + Solvent

3.11. Statistical Analysis

The average absorbance from triplicate measurement of each concentration and the concentration was determined by regression equation.

$$y = mx + c \quad \text{.....(2)}$$

Where,

y= absorbance of extract

m=slope

c=intercept

The regression equation was implemented to calculate the total phenolic ($y = 0.0099x + 0.0183$, $R^2 = 0.999$), flavonoid ($y = 0.0013x + 0.0078$, $R^2 = 0.992$), and tannin ($y = 0.0011x + 0.012$, $R^2 = 0.992$) contents as well as the phosphomolybdenum antioxidant content ($y = 0.0078x - 0.0074$, $R^2 = 0.999$).

3.12 Antimicrobial Assay

The antimicrobial susceptibility test of extracts was carried out by agar well diffusion protocol. The potency of plant extracts against bacterial activity was ascertained zone of inhibition. Clinical laboratories standard institute 2018 guidelines were followed to conduct the antimicrobial susceptibility test (El Astal et al., 2005).

3.12.1 Collection of Test Organisms

In the Bharatpur Hospital Chitwan, Nepal provided samples of every common pathogenic microbe. To achieve maximum microorganism growth, nutrient agar was used. Two separate bacterial species were included the strains under study. *E.coli, P. aeruginosa, K. pneumonia, and S. typhirium* are of gram-negative bacteria. gram-positive bacteria include *S. aureus*.

3.12.2 Preparation of Working Solution

A sterile vial containing 5ml of 50% DMSO solvent was aseptically filled with 2.5g of each crude extract to create a 50 mg/mL working solution. In DMSO, the extract was dissolved. The test tubes were prepared with a stock solution, sealed, and refrigerated (2–8°C) until needed.

3.12.3 Evaluation of Antibacterial Activity

45.6g of Mueller Hinton Agar was dissolved completely in 1200 ml distilled water and stirred with autoclaved glass rod. Then the mixture was transferred equally into three 500ml conical flasks. The agar mixture was then autoclaved at 121°C for 20 minutes. The pressure of autoclave was 15atm. Thus, prepared agar solution was left for few minutes under cooling. The agar plates of 90 mm diameter were incubated at 180°C and left under laminar flow to avoid cross contamination. The agar solution was poured into the agar plates and covered with lid. The lid-covered plates were left for 45 minutes to cool. Thus, cooled agar plates were left under refrigeration for 24 hrs. The refrigerated agar plates were left under laminar flow for few minutes. Mueller Hinton Agar (MHA) plates, previously prepared were dried to eliminate excess water on the agar surface. The agar plates were labelled with date of experiment and organisms. The turbid solution of micro-organism was prepared using normal saline purchased from a local pharmacy, in different test-tubes for different micro-organisms. The bacterial solution turbidity was measured by comparing it to the 0.5 McFarland standard. After immersing a sterile cotton swab into the prepared inoculum, any extra inoculum was forced out by rotating and gently pressing the swab against the test tube upper inner wall, just above the liquid level. After each swabbing, the plates were rotated at 60-degree and final swabbing was done along the periphery of agar surface. The cross contamination was avoided by leaving the inoculated plates to dry inside the laminar

flow with covered lids. A sterile corks borer having 7mm of diameter was employed to punch the wall on the dried agar plate and marked accurately. 80µl plant stock solution was gently loaded into respective wells with the help of micropipette. The solvent (50% DMSO) was used as negative control while different antibiotics were placed as positive control. The agar plates with controls, samples were covered with lid and left about 45 minutes to diffuse the extracts and controls. The plates were uprightly placed in an incubator at 37°C for 24 hours for appropriate incubation. The clear zone of inhibition around each well in the agar plates were examined and measured using ruler. The average value of zone of inhibition was presented to disclose the strength of plant extract against pathogenic bacteria.

3.13 HR-LCMS Analysis

The detail procedure for HR-LCMS analysis was as given in Magar, *et al.* (2024).

3.14 Software

Microsoft Word, Excel, and PowerPoint were used for the analysis and interpretation of all study-related data. Mendeley was used as the reference manager, Google Dock was used as the plagiarism detector, and Chem Draw 12 pro was used to draw the chemical structure. The software called Origin Lab Origin pro was used to analyse the data.

CHAPTER FOUR: RESULTS AND DISCUSSIONS

4.1 Yield of Extract

Table 1: Table with percentage yield of methanol extract

Extraction method	Dry weight of plant(g)	Weight of methanolic extract(g)	Yield
Maceration	50	5.7	11.5%
Sonication	50	7.4	14.9%
Soxhlet	50	10.5	21.2%

It was discovered that the methanol extract of *Psidium guajava* L. fresh leaves had a percentage yield for maceration, sonication and soxhlet was found to be 11.5%, 14.9%, and 21.2%, respectively. According to the results, maceration resulted in lowest while soxhlet extraction was highest yield.

4.2 Phytochemical screening

The three extracts of *Psidium guajava* L. fresh leaves undergo phytochemical screening. The following table shows the phytochemical outcomes of the extract.

Table 2: *Psidium guajava* L. leaves extract is subjected to phytochemical screening.

S. N.	Phytochemicals	Colours	Maceration	Sonication	Soxhlet
1 .	Terpenoids	Reddish grey	+	+	+
2.	Flavonoids				
	a. Alkaline reagent test	Intense yellow color	+	+	+
	b. Lead acetate test	ppt of yellow	+	+	+
3 .	Polyphenols	Greenish blue	+	+	+
4 .	Quinones	Red	+	+	+
5 .	phlobatannins	Red ppt	-	-	-
6 .	Anthocyanins	Pink red color	-	-	-
7 .	Anthraquinone	Pink red color	-	-	-
8 .	Alkaloids				
	a. Hager's test	Yellow color ppt	+	+	+
	b. Wagner's test		+	+	+
	c. Dragendorff's test	Orange ppt	+	+	+
9 .	Glycosides	Brown ring	+	+	+
10.	Tannins	Blue-black	+	+	+

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

There is some variance between the findings of the phytochemical screening test carried out on the plant three extract. This is a result of different variables that can affect the chemical composition of the plant, including the pH, composition, altitude of the vegetation, sample collection season, and environmental conditions. The amount of

chemicals in the extract depends on the solvent purity, the extraction technique, and the chemical grades.

4.3 Quantitative Analysis

4.3.1 Estimation of Phenolic Content

Because of their hydroxyl groups, phenolic and flavonoid contents are highly significant plant constituents and important indicators of potential antioxidant activity. It also has significant effects on human nutrition and health. These compounds function as antioxidants, lowering the risk of diseases carried due to oxidative stress.

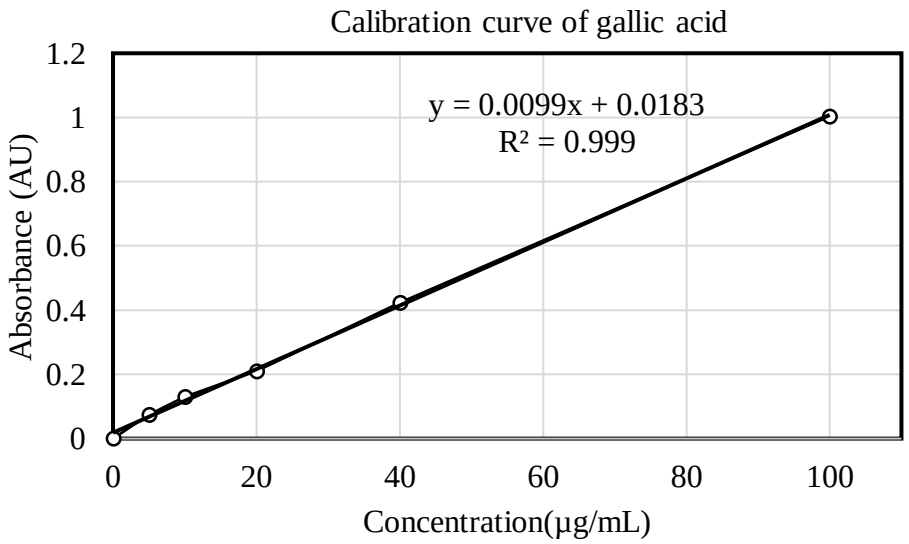


Figure 3: Absorbance vs. Concentration curve for Gallic acid

The study examined the calibration curve that relates to the absorbance values of different methanol extracts from fresh *Psidium guajava* L. leaves concentration and absorbance of gallic acid. As shown in the table, using the calibration curve the total phenolic content was found to be 316.1±7.4, 234.7±4.9, and 280.8±16.3mg GAE/g in the maceration, sonication, and Soxhlet extracts, respectively. The result shows that the maximum phenolic content was produced in maceration process.

Table 3: Total Phenolic Content of fresh *Psidium guajava* L. leaves Extracts.

S.N.	Extraction process	(mg GAE/g) ± S.D
1	Maceration	316.1±7.4
2	Sonication	234.7±4.9
3	Soxhlet	280.8±16.3

All measurements were done in triplicate.

4.3.2 Estimation of Total Flavonoid Content

Quercetin provides a standard for quantifying their content.

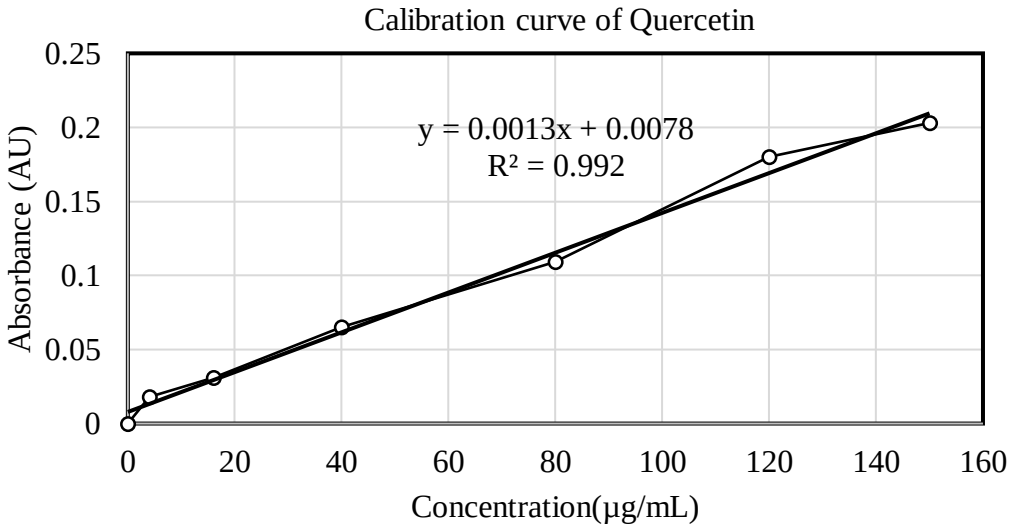


Figure 4: Absorbance vs. concentration curve for quercetin.

The absorbance values of several methanol extracts from fresh *Psidium guajava* L. leaves were compared to the calibration curve that connected the absorbance and concentration of gallic acid. On the basis of calibration curve, the total flavonoid content in maceration, sonication and soxhlet was observed as 257.9±4.4, 242.5±2.7, and 224.7±2.1mg QE/g, respectively.

Table 4: Total Flavonoid Content of *Psidium guajava* L. Extracts**.**

S.N.	Extraction process	(mg QE/g) ± S.D
1	Maceration	257.9±4.4
2	Sonication	242.5±2.7
3	Soxhlet	224.7±2.1

All measurements were done in triplicate.

With respect to the sonication and Soxhlet extracts, the maceration extracts show a significantly greater concentration of flavonoid compounds. In general, phenolic compounds eliminate different kinds of oxidising enzymes. Phenolic compounds found in *Psidium guajava* L. leaves have several kinds of biological functions that offer potential for identifying specific phytochemicals and the health benefits associated with them.

4.3.3 Evaluation of Total Tannin Content

The total tannin content was expressed as tannin acid equivalent. In comparison to the maceration (404.5±8.3µg/mL) and sonication (468.2±8.3µg/mL) methods, the total tannin content in the Soxhlet extraction (563.6±4.5µg/mL) of tannic acid equivalents) was found to be significantly higher.

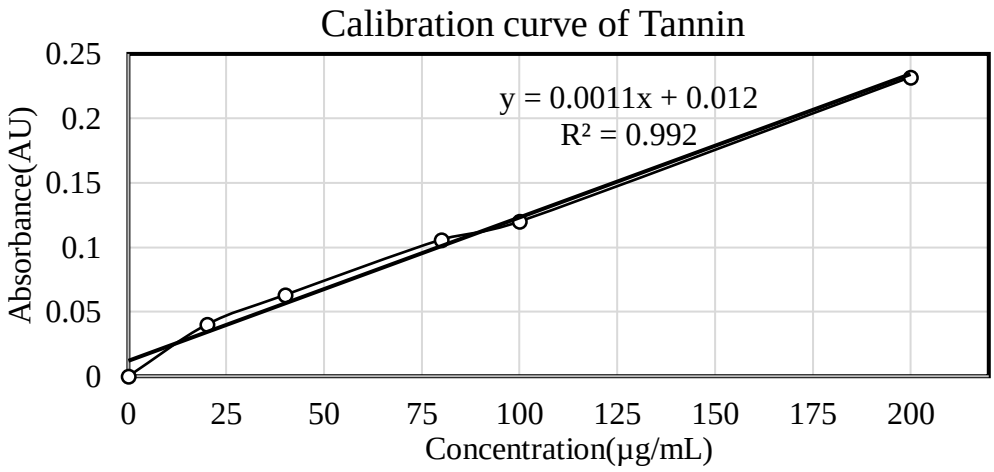


Figure 5: Absorbance vs concentration of tannic acid standard.

Table 5: Total Tannin Content of *Psidium guajava* L. fresh leaves.

S.N	Process	Tannic acid equivalent (mg/g) ± SD
1	Maceration	404.5±8.3
2	Sonication	468.2±8.3
3	Soxhlet	563.6±4.5

All measurements were done in triplicate.

4.4 Antioxidant Activity

4.4.1 Antioxidant Activity using DPPH

The IC_{50} value for three extract were measured by plotting percentage of free radical scavenging vs concentration to determine the antioxidant activity of the different extracts. DPPH was utilised as a reference chemical during the in vitro free radical scavenging studies.

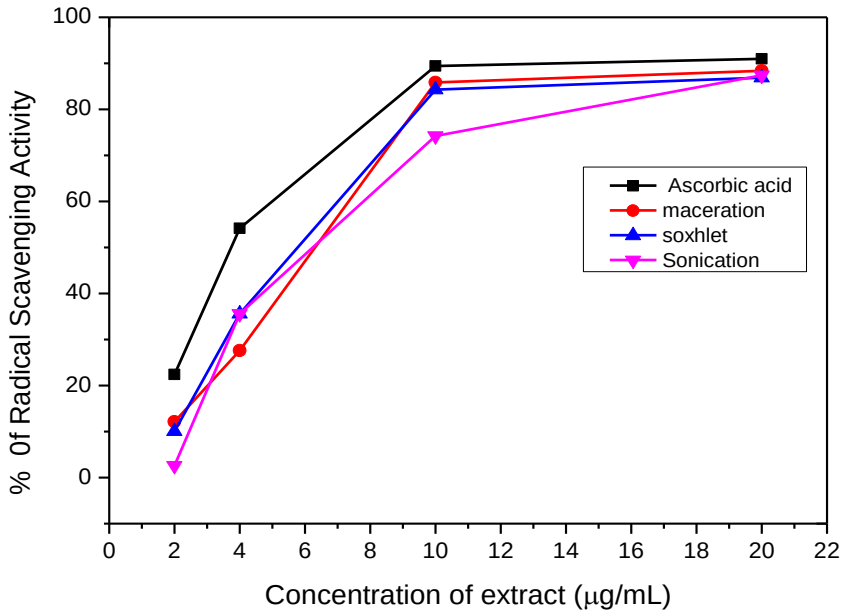


Figure 6: Antioxidant activity of Ascorbic Acid, Maceration extract, Sonication extract and Soxhlet extract of the fresh *Psidium guajava* L. leaves.

The Food and Drug Administration defines IC_{50} as the minimum drug concentration needed to result in 50% inhibition when tested in vitro. Each extract's potential antioxidant activity was determined by calculating its IC_{50} value and comparing it to that of ascorbic acid. To determine each extract potential antioxidant activity, the IC_{50} value was calculated and compared to the IC_{50} value of ascorbic acid (Taylor et al., 2014). The antioxidant potency of an extract was determined by its IC_{50} values; lower IC_{50} values correspond to higher antioxidant activity than higher IC_{50} values. The IC_{50} values for maceration, sonication, and Soxhlet extracts were 6.0, 6.9, and 6.1 µg/mL, respectively. However, the antioxidant values for the sonication extract are lower for the standard ascorbic acid (3.1 µg/mL) as compare the corresponding extracts. The

fresh *Psidium guajava* L. leaves maceration extract exhibited the highest power or effectiveness of antioxidants among all the extracts with an IC_{50} value of 6.08 µg/mL, slightly higher than that of standard ascorbic acid (3.1µg/mL).

4.4.2 Phosphomolybdenum antioxidant activity

Methanolic maceration extract exhibited maximum antioxidant activity of 125.0±2.1mM of ascorbic acid eqvt/g of dry wt.

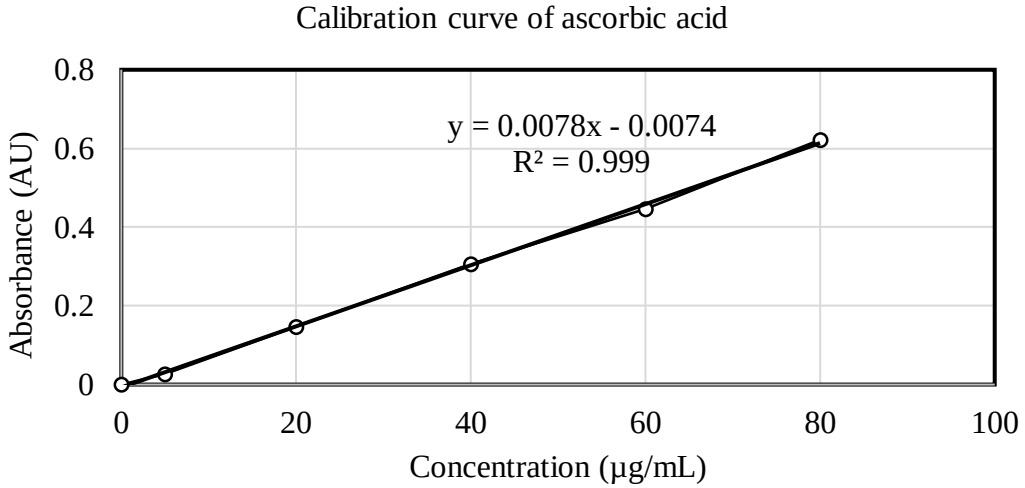


Figure 7: Absorbance vs. Concentration curve for Ascorbic acid.

Table 6: Antioxidant activity of different extracts of fresh leaves of *Psidium guajava* L. by Phosphomolybdenum method.

S.No	Extract Process	Total antioxidant activity (mM of ascorbic acid eqvt/g of sample) ± SD.
1	Maceration	125.0±2.1
2	Sonication	77.3±0.7
3	Soxhlet	81.8±0.9

All measurements were done in triplicate.

When evaluated using the phosphomolybdenum assay, two extract including sonication and Soxhlet extraction, exhibited relatively lower antioxidant activities with values of

77.35±0.7mM and 81.85±0.9mM of ascorbic acid equivalents per gram of sample respectively. The maceration extract observed the highest antioxidant activity with a value of 125.0±2.1mM ascorbic acid equivalents per gram of sample.

4.5 Antimicrobial Activity

The antimicrobial properties of different extract of fresh Psidium guajava L. was observed at 50mg/mL concentration. The following table summarizes the result for five bacteria with their zone of inhibition (including the well diameter 6mm).

Table 7 : 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	*S. aureus*	17.667±0.57
2	Amikacin	5 ppm	*k. pneumonia*	28±1.0
3	Amikacin	5 ppm	*P. aeruginosa*	24.67±0.57
4	Ciprofloxacin(CIP)	10ppm	*E. Coli*	24.5± 0.57
5	Ciprofloxacin(CIP)	10ppm	*S. typhirium*	24.67±0.57

All measurements were done in triplicate.

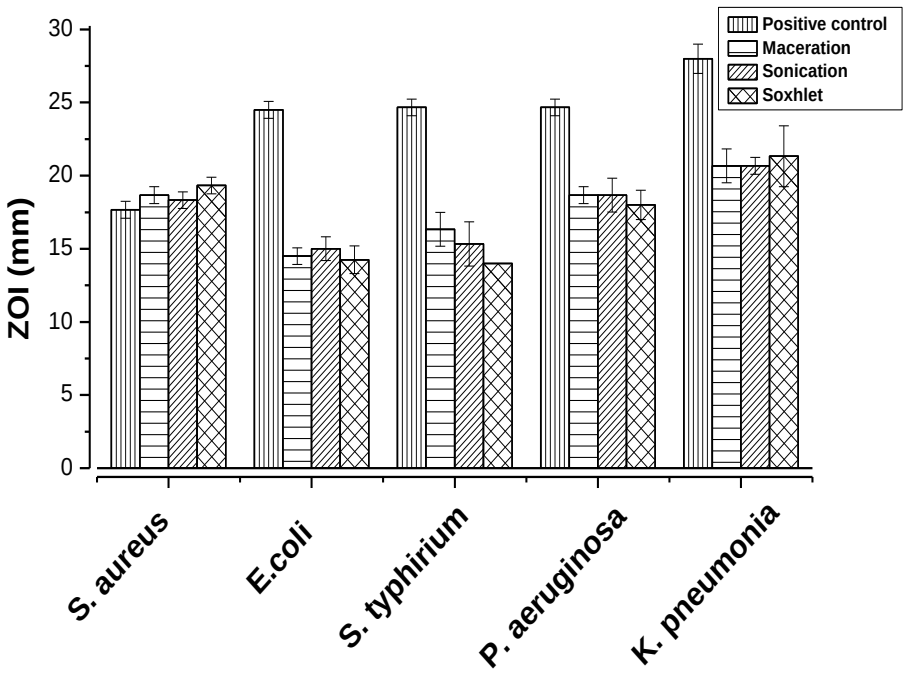


Figure 8: Examination of antibacterial activity.

The fresh leaves maceration extract showed an inhibition zone of 18.67 ± 0.57 mm against the gram-positive bacteria *B. anthracis* and 17.33 ± 0.57 mm against the gram-positive bacteria *S. aureus*. Likewise, the extract demonstrated inhibition zones of 14.5 ± 0.57 mm, 18.67 ± 0.57 mm, 25.33 ± 1.15 mm, 20.67 ± 1.15 mm, and 16.33 ± 1.15 mm against the gram-negative bacteria *E. coli*, *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, and *S. typhimurium*, respectively. Gram-negative bacteria *A. baumannii* was especially susceptible to the extract maximum zone of inhibition.

The sonication extract of fresh leaves showed inhibition zones of 18.33 ± 0.57 mm for *S. aureus* and 17.33 ± 1.15 mm for B. anthracis, two gram-positive bacteria. The sonication extract also demonstrated inhibition zones of 15 ± 0.81 mm, 18.67 ± 1.15 mm, 25 ± 0.0 mm, 20.67 ± 0.57 mm, and 15.33 ± 1.52 mm against the gram-negative bacteria *E. coli*, *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*, and *S. typhimurium*, respectively. The highest zone of inhibition was observed in the gram-negative bacterium *A. baumannii*.

Utilising the Soxhlet method, fresh leaves were extracted and the resulting inhibition zones against Gram-positive bacteria *B. anthracis* and *S. aureus* measured 19.33 ± 0.57

mm and 17.33 ± 0.57 mm, respectively. Similarly, using the Soxhlet extract against the gram-negative bacteria *S. typhimurium*, *P. aeruginosa*, *A. baumannii*, *E. coli*, and *K. pneumoniae*, in that order, showed inhibition zones of 14.25 ± 0.95 mm, 18 ± 1.0 mm, 25.33 ± 0.57 mm, 21.33 ± 2.08 mm, and 14 ± 0.0 mm. The extract exhibited the largest zone of inhibition against *A. baumannii*, a gram-negative bacteria.

4.6 HR-LCMS analysis.

Psidium guajava L. fresh leaves was subjected to HR-LCMS to better interpret the diversity of available phytochemical. Summarise all the compounds that were characterised in *Psidium guajava* L. fresh leaves including retention time (RT), molecular formula, experimental m/z, and properties of compound. Similar Prakoso & Nita, (2023) study carried out in *Psidium guajava* L. leaves. Thus, HR-LCMS analysis was conducted in both positive and negative modes which revealed the presence of 144 distinct compounds. Among them, 104 compounds are successfully identified, while the remaining 40 compounds needs to be characterization.

Thus *Psidium guajava* L. fresh leaves contains a wide variety of molecules having medicinal importance for human health. The traditional use of this plant as a medicine paid attention for 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, alkanoid compound and its derivative and other compound in both ESI mode.

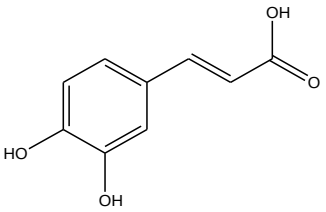
Table 8: Selective secondary metabolites (Phenolic compound).

S. N	Compounds Name	Formula	Ion	Mass	RT(min)	m/z (Cal.)	Properties
1.	Quinic acid	$C_7 H_{12} O_6$	(M-H)-	192.0633	1.456, 1.533	191.056	Cyclohexanecarboxylic acid derivative acts as Antibioflm agent (Lu et al., 2021).
2.	Gallic acid	$C_7 H_6 O_5$	(M-H)- (M+CH3COO)-	170.0212	4.554 4.4	169.0139	Anticancer, antimicrobial, antimutagenic, antiangiogenic and anti-inflammatory (Choubey et al., 2015).
3.	Caffeic acid	$C_9 H_8 O_4$	(M-H)- (M+HCOO)- (M+CH3COO)-	180.0418	6.838	239.0557	Phenolic acid, Hydroxycinnamic acid, Antioxidant, Antimicrobial activity and use as treatment of dermal diseases
4.	Chlorogenic acid	$C_{16} H_{18} O_9$	(M-H)-	354.0941	6.703	353.0868	It is used as a neuroprotective, antiviral, anti-inflammatory, cardioprotective, antioxidant, and antihypertensive agent (Naveed et al., 2018).
5.	Ferulic acid	$C_{10} H_{10} O_4$	(M+HCOO)-	194.0575	6.838	239.0557	It serves as an antimicrobial, anti-inflammatory, anti-cancer, and antithrombotic agent (Ou & Kwok, 2004).
6.	Ellagic acid	$C_{14} H_6 O_8$	(M-H)- (M+HCOO)-	302.0069	8.693, 9.147 8.782	300.9997	Used as neuroprotective effect, anti-inflammatory, anti-atherogenic and anti-cancer (Ríos et al., 2018).
7.	Epigallocatechin gallate	$C_{22} H_{18} O_{11}$	(M-H)- (M+HCOO)-	458.0851	7.642	457.0774	Chemo preventive agent (Fujiki et al., 1992).
8.	2-O-Galloylpunicalin	$C_{41} H_{26} O_{26}$	(M-2H)-2	934.073	5.684	466.0293	Antioxidant activity, anticancer (Yoshida et al., 2010).

S. N	Compounds Name	Formula	Ion	Mass	RT(min)	m/z (Cal.)	Properties
9.	Punicacortein B	$C_{27}H_{22}O_{18}$	(M-H)-	634.0826	6.501	633.0753	Antioxidant, anti-inflammatory and anticancer (Tanaka et al., 1986).
10.	1-O-Galloylpedunculagin	$C_{41}H_{28}O_{26}$	(M-H)-	936.0895	6.624, 6.688	935.0828	Hydrolysable tannin, anti-inflammatory, anticancer and ant oxidant effect (Snarska et al., 2024).
11.	Palmidin B	$C_{30}H_{22}O_{7}$	(M+HCOO)-	494.138	21.877 22.137	539.1364	Antioxidant, antimicrobial and antitumor activity (Cao et al., 2017).
+ve mode	Trichocarposide	$C_{22}H_{24}O_{9}$		432.1477	18.828	433.1551	Phenolic glycosides derivative of cinnamic derivatives. Anti-inflammatory and antipyretic activities (Sobeh et al., 2019).

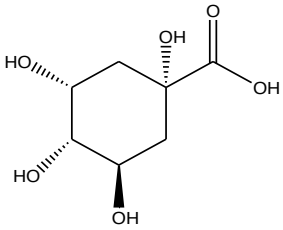
Note: RT, retention time.

Structures of phenolic compound and its derivative

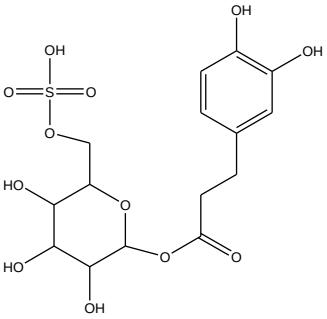


Caffeic acid

$C_9H_8O_4$
Mol. Wt.: 180.2

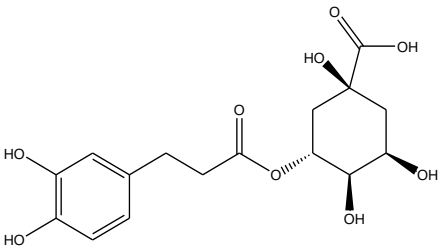


Quinic acid
$C_7H_{12}O_6$
Mol. Wt.: 192.2

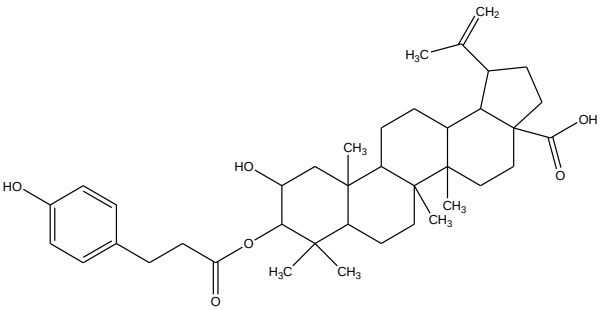


-Caffeoyl-(b-D glucose 6-O-sulfate)

$C_{15}H_{20}O_{12}S$
Mol. Wt.: 424.4

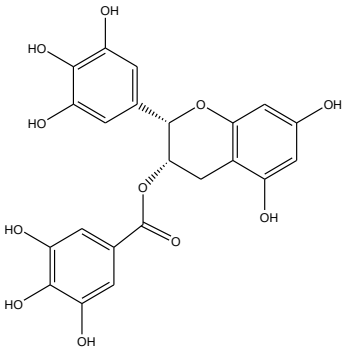


Chlorogenic acid
$C_{16}H_{20}O_9$
Mol. Wt.: 356.3



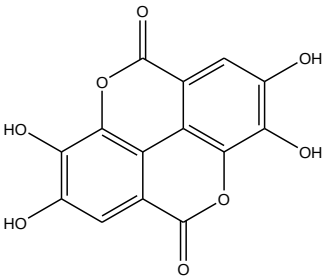
3-O-p-trans Coumaroylalphitolic acid

$C_{39}H_{56}O_6$
Mol. Wt.: 620.9

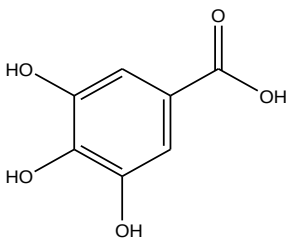


Epigallocatechin gallate

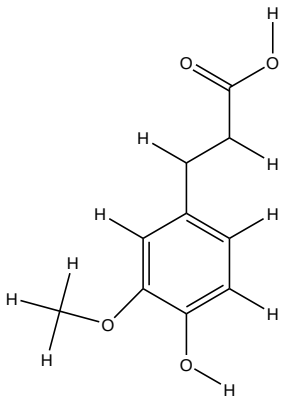
$C_{22}H_{18}O_{11}$
Mol. Wt.: 458.4



Ellagic acid
$C_{14}H_6O_8$
Mol. Wt.: 302.2



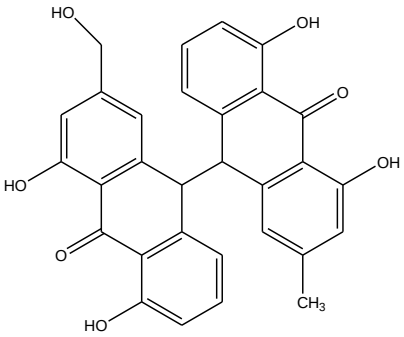
Gallic acid
$C_7H_6O_5$
Mol. Wt.: 170.1



Ferulic acid

$C_{10}H_{12}O_4$

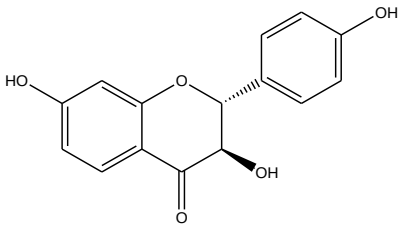
Mol. Wt.: 196.2



Palmidin B

$C_{30}H_{22}O_7$

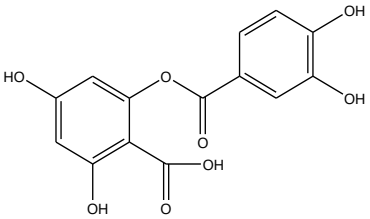
Mol. Wt.: 494.5



Garbanzol

$C_{15}H_{12}O_5$

Mol. Wt.: 272.3



2-Protocatechoyl phloroglucinol carboxylate

$C_{14}H_{10}O_8$

Mol. Wt.: 306.2

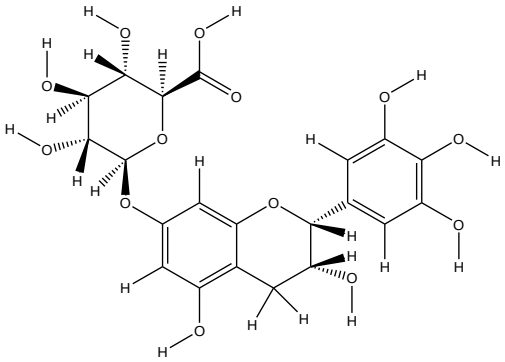
Table 9: Selective secondary metabolites (flavonoids compound).

S. N	Compounds Name	Formula	Ion	Mass	RT(min)	m/z (Cal.)	Properties
1.	Quercitrin	$C_{21}H_{20}O_{11}$	$(M-H)^-$	448.1013	9.353	447.0944	Antioxidant and anti-inflammation and antimicrobial immunomodulation, analgesia, wound healing and vasodilation (Chen et al., 2022).
2.	Luteolin 4'-O-glucoside	$C_{21}H_{20}O_{11}$	$(M-H)^-$	448.1013	9.352	447.0944	It act as antioxidant, anti-microbial, anti-inflammatory and anti-anti-cancer activity (López-Lázaro, 2009).
3.	Genistein	$C_{15}H_{10}O_5$	$(M-H)^-$	270.0529	7.319	329.067	Strong anticancer durg (Polkowski & Mazurek, 2000).
4.	(-)-Catechin	$C_{15}H_{14}O_6$	$(M-H)^-$	290.0786	5.200	289.0713	It increase protective strength of Uv radiation, antimicrobial, anti-viral, anti-allergenic and anti-cancer durg (Bae et al., 2020).
5.	Gossypetin 8-glucoside	$C_{21}H_{20}O_{13}$	$(M-H)^-$	480.0911	8.062	479.0841	Antimicrobial activity and cardiovascular disease (Gadotti et al., 2015).
6.	Myricetin 3-arabinoside	$C_{20}H_{18}O_{12}$	$(M-H)^-$	450.0806	8.335	449.0734	Flavonoid glycoside derived from myricetin, Antioxidant, anti-inflammatory and anticancer properties (Ong & Khoo, 1997).
7.	Quercetin 3-(2-galloylglucoside)	$C_{28}H_{24}O_{16}$	$(M-H)^-$	616.1081	8.337	615.1011	Flavonoid glycoside derivative of quercetin.
8.	Myricitrin	$C_{21}H_{20}O_{12}$	$(M-H)^-$	464.0975	8.681	463.0903	Hepatoprotective activity, antioxidant potential (Domitrović et al., 2015)
9.	8-Hydroxyluteolin 8-sulfate	$C_{15}H_{10}O_{10}S$	$(M-H)^-$	382.0002	8.684, 9.021	380.993	Antioxidant, anti-inflammatory and anticancer activities (Olatunde et al., 2021).
10.	2",4",6"-Triacetylglycitin	$C_{28}H_{28}O_{13}$	$(M-H)^-$	572.1538	10.192 10.654	571.1471	Flavonoid glycoside derivative of glycitin, antioxidant and function of typrosinase inhibitor (Wu et al., 2021)
11.	Herbacetin	$C_{15}H_{10}O_7$	M-H)$^-$	302.0428	11.167	301.0356	Used to treatment of hearing loss, chemopreventive and antitumor activities, antioxidant and antiinflammatory properties (Kim et al., 2016).

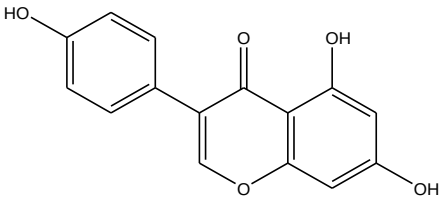
12.	Sophoranone	$C_{30}H_{36}O_4$	(M+HCOO)$^-$	460.2622	24.86	505.2604	Used as anti-inflammatory, anti-cancer and immunomodulatory properties (Long et al., 2022; Isogai et al., 1977).
13.	Mammeisin	$C_{25}H_{26}O_5$	(M-H)-	406.1795	23.04	405.1723	Antifungal activity (Marcondes et al., 2015).

Note: RT, retention time.

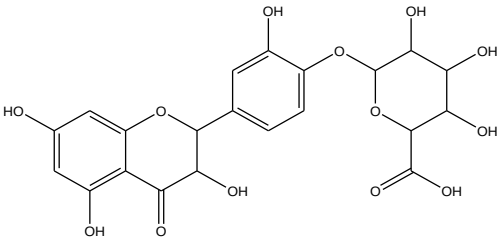
Structure of flavonoid and its derivative



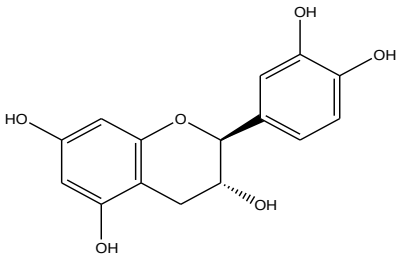
(-)-Epigallocatechin 7-glucuronide
$C_{21}H_{22}O_{13}$
Mol. Wt.: 482.4



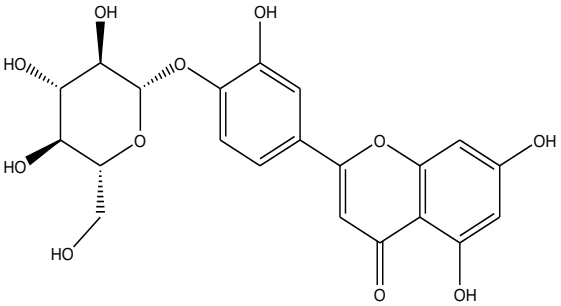
Genistein
$C_{15}H_{10}O_5$
Mol. Wt.: 270.2



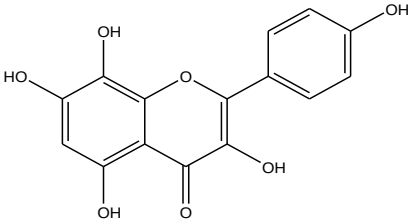
Quercetin-4'-glucuronide
$C_{21}H_{20}O_{13}$
Mol. Wt.: 480.4



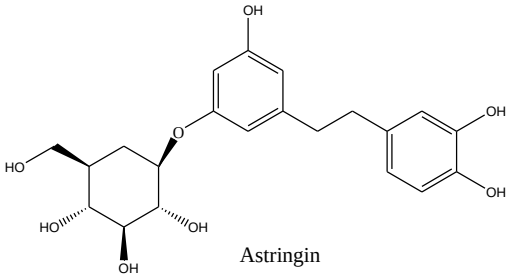
(-)-Catechin
$C_{15}H_{14}O_6$
Mol. Wt.: 290.3



Luteolin 4'-O-glucoside
$C_{21}H_{20}O_{11}$
Mol. Wt.: 448.4



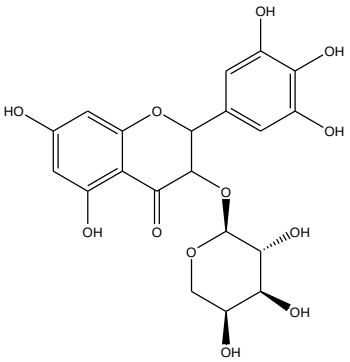
Herbacetin
$C_{15}H_{10}O_7$
Mol. Wt.: 302.2



Astringin

$C_{21}H_{26}O_8$

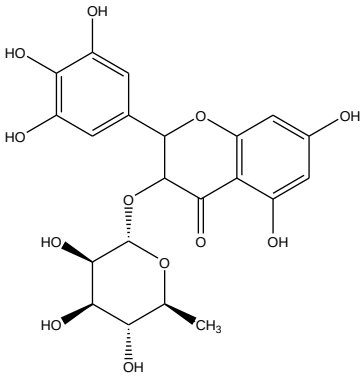
Mol. Wt.: 406.4



Myricetin 3-arabinoside

$C_{20}H_{20}O_{12}$

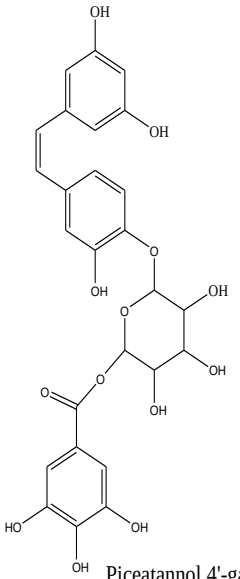
Mol. Wt.: 452.4



Myricitrin

$C_{21}H_{22}O_{12}$

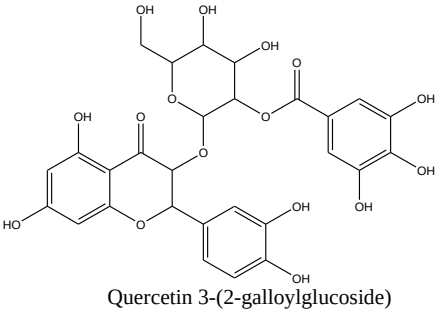
Mol. Wt.: 466.4



Piceatannol 4'-galloylglucoside

$C_{26}H_{24}O_{13}$

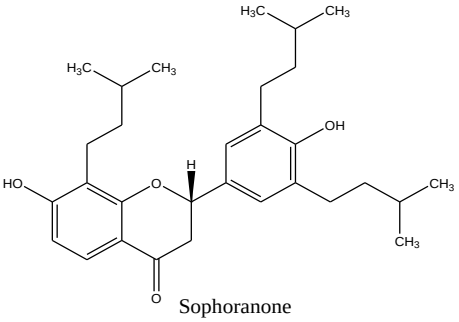
Mol. Wt.: 544.5



Quercetin 3-(2-galloylglucoside)

$C_{28}H_{26}O_{16}$

Mol. Wt.: 618.5



Sophoranone

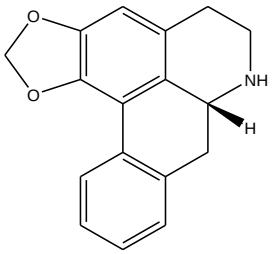
$C_{30}H_{42}O_4$

Mol. Wt.: 466.7

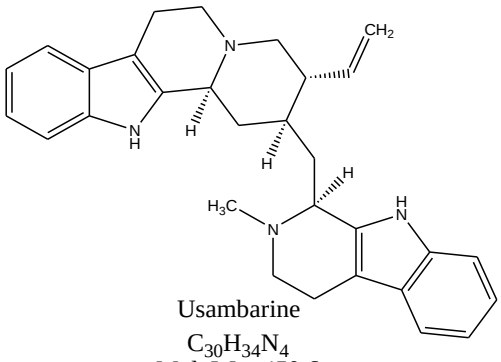
Table 10: Selected secondary metabolites (alkaloids compound).

S.N	Compounds Name	Formula	Ion	Mass	RT(min)	Measured (m/z)	Properties
1.	(-) Annonaine	C_{17} H_{15} N O_2	(M+H)+	265.1104	1.297	266.1177	Antiplasmodial, antibacterial, antifungal, antioxidation, anticancer, anridepression and vasorelaxant agent (Li et al., 2013).
2.	Usambarine	C_{30} H_{34} N_4	(M+Na)+	450.2809	19.467,	473.2705	Antiplasmodial activites (Frédérich et al., 2002).
3.	Somniferine	C_{36} H_{36} N_2 O_7	(M+H)+	608.2516	23.777	609.2587	Anti-viral agent (Joshi et al., 2022).
4.	Delphinine	C_{33} H_{45} N O_9	(M+Na)+	599.3097	24.42	622.2976	Cardiovascular action (Desai et al., 1998).
5.	Delcorine	C_{26} H_{41} N O_7	(M+Na)+	479.295	25.041	502.2844	Anesthetic properties, antiarrhythmic and antifibbrillatory action (Dzhakhangirov et al., 1997).
6.	Cepharanthine	C_{37} H_{38} N_2 O_6	(M+H)+	606.2743	26.065 26.438	607.2819	Treatment of snake bites, xerostomia, leukopenia and alopecia (Bailly, 2019).
7.	Americine	C_{31} H_{39} N_5 O_4	(M+H)+	545.3031	26.253	546.3103	Alkaloids (Klein & Rapoport, 1968).
8.	Cinnzeylanine	C_{22} H_{34} O_8	(M+HCOO)-	426.2205	24.971 25.556	471.2186	Diterpenoid compound, Killed larvae of silkworm (Isogai et al., 1 Insecticidal substances (Isogai et al., 1977).

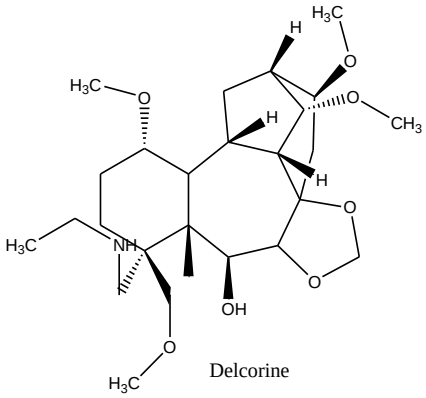
Structures of alkaloids compound and its derivative



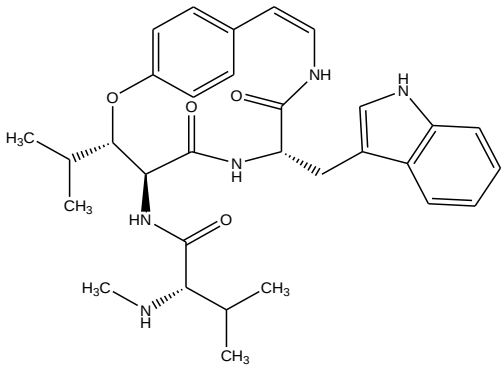
(-)-Annonaine
$C_{17}H_{15}NO_2$
Mol. Wt.: 265.3



Usambarine
$C_{30}H_{34}N_4$
Mol. Wt.: 450.6



Delcorine
$C_{26}H_{45}NO_7$
Mol. Wt.: 483.6



Americine
$C_{31}H_{39}N_5O_4$
Mol. Wt.: 545.7

Table 11: Secondary metabolite other than TPC, TFC and alkaloid.

For +ve mode

S.N	Compounds Name	Formula	Ion	Mass	RT(min)	m/z	Properties
1.	Patientoside A	C_{19} H_{21} Cl O_8	(M+Na)+	412.0933	8.178	435.0825	Antioxidant and Antimicrobial activity (Kowalczewski & Zembrzuska, 2023).
2.	O-Carbamoyl-deacetylcephalosporin C	C_{15} H_{20} N_4 O_8 S	(M+H)+	416.1018	8.491	417.1086	Cephalosporin family, Antimicrobial activity (Martín & Liras, 2006).
3.	Isolimonic acid	C_{26} H_{34} O_{10}	(M+Na)+	506.2096	17.82	529.199	Triterpenoid, Antimicrobial activity (Vikram et al., 2012).
4.	Plantaricin BN	C_{24} H_{36} O_{10}	(M+Na)+	484.2278	17.856	507.2173	Bacteriocin, Antimicrobial peptide produced by certain bacteria, Antimicrobial agent used as food preservative (Abdulhussain Kareem & Razavi, 2020).
5.	Tobramycin	C_{18} H_{37} N_5 O_9	(M+Na)+	467.2578	20.727	490.2488	Aminoglycoside and Antibiotic similar to gentamicin (Brogden et al., 1976).
6.	3-O-Protocatechuoylceanothic acid	C_{37} H_{50} O_8	(M+Na)+	622.3486	18.819	645.3404	Used as antioxidant anti-inflammatory, anti-hyperglycemic and antiapoptotic drug (Semaming et al., 2015).
7.	L-Olivosyl-oleandolide	C_{26} H_{44} O_{10}	(M+H)+	516.2872	18.918	517.2951	Antibiotic properties (Rodríguez et al., 2001).
8.	Perindopril	C_{19} H_{32} N_2 O_5	(M+Na)+	368.2285	19.018	391.2172	Angiotensin-converting enzyme and reduce cardiovascular event (Campbell, 2006).
9.	Inuline	C_{32} H_{46} N_2 O_8	(M+H)+	586.329	21.486,	587.3374	Fructans, Dietary fiber (Van Loo et al., 1995).
10.	23-trans-p-Coumaroyloxytormentic acid	C_{39} H_{54} O_8	(M+Na)+	650.3825	22.412 22.75,	673.3728	Active against human leukaemia cell (Alicja et al., 2012).
11.	3-O-trans-Feruloyleuscaphic acid	C_{40} H_{56} O_8	M+Na)+	664.3976	22.848 23.365	687.3878	Derivative of Ferulic acid, Anti-inflammatory, antioxidant and anticancer properties (Ito et al., 2001).

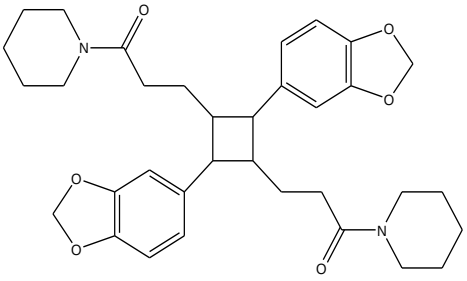
S.N	Compounds Name	Formula	Ion	Mass	RT(min)	m/z	Properties
12.	Dipiperamide A	C_{34} H_{38} N_2 O_6	(M+Na)+	570.277	24.239 24.514	593.2664	Piperidine group, Antifungal, antidiarrhoeal and anti-inflammatory activities (Tsukamoto et al., 2002).
13.	Isomigrastatin	C_{27} H_{39} N O_7	(M+Na)+	489.2794	25.529	512.269	Analogue of migrastatin, Anticancer drug (Lo Re et al., 2015).

For -ve mode

S.N	Compounds Name	Formula	Ion	Mass	RT(min)	m/z	Properties
14.	Brompheniramine	C_{16} H_{19} Br N_2	(M+CH3COO)-	318.0731	1.442	377.0871	Alkylamine class, Antihistaminic effect in the skin, antihistamine used extensively for prevention and control of allergic reaction (Schlesier et al., 2002; Bruce et al., 1968)
15.	Bromadiolone	C_{30} H_{23} Br O_4	(M+CH3COO)-	526.078	9.797	585.0917	Rodenticide used to kill rats and mice, anticoagulant, toxic to mammals (Grobosch et al., 2006).
16.	Azukisapogenol	C_{30} H_{48} O_4	(M-H)-	472.3563	19.179	471.3492	Triterpene glycosides, Anti-inflammatory, antiviral, cytotoxic and anti-fungal properties (Wang et al., 2018).
17.	Paramethasone acetate	C_{24} H_{31} F O_6	(M+HCOO)-	434.2111	19.244	479.2095	Anti-inflammatory and immunosuppressant activities (Cortés-Gallegos et al., 1984).
18.	Caribenolide I	C_{33} H_{52} O_{11}	(M+HCOO)-	624.3575	20.202	669.3584	Strong Antitumor activities (Bauer et al., 1995)
19.	Kenposide B	C_{21} H_{36} O_{10}	(M-H)-	448.2272	24.436 24.79	447.22	Glycoside, Ant oxidative and cytoprotective, anti-inflammatory (Lu et al., 2022).
20.	Flumethasone	C_{22} H_{28} F_2 O_5	(M+CH3COO)-	410.1911	25.19	469.2029	Anti-inflammatory activity (Dougherty et al., 1973) .
21.	Enterocin 900	C_{31} H_{33} N O_2	(M+CH3COO)-	451.2522	25.453	510.2661	Bacteriocin, Antimicrobial properties (Park et al., 2003).
22.	Trigofoenoside A	C_{45} H_{74} O_{18}	(M+HCOO)-	902.4784	26.021	947.4769	Glucoside derivative, Anti-inflammatory and analgesic properties (Vyas et al., 2008)
23.	Irbesartan	C_{25} H_{28} N_6 O	(M+HCOO)-	428.237	26.206	473.2352	Antihypertensive agents (Gillis & Markham, 1997).

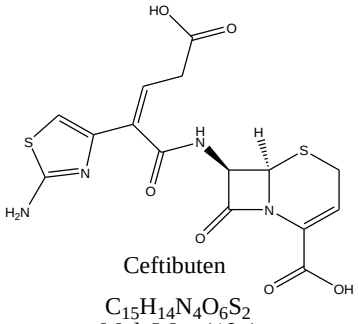
Note: RT, retention time.

Structure of other compound having antimicrobial and anticancer properties



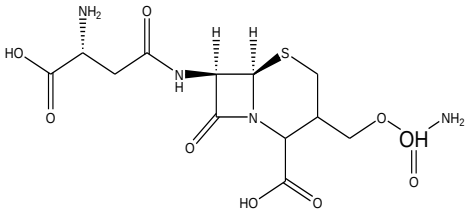
Dipiperamide A

$C_{34}H_{42}N_2O_6$
Mol. Wt.: 574.7



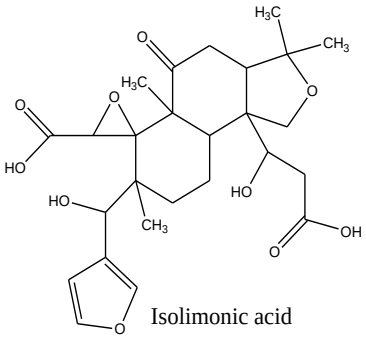
Ceftibuten

$C_{15}H_{14}N_4O_6S_2$
Mol. Wt.: 410.4



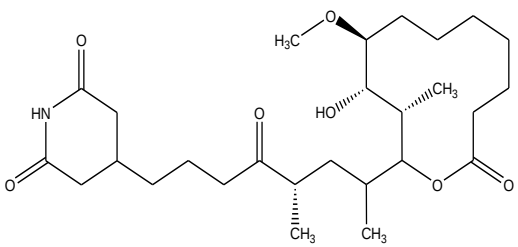
O-Carbamoyl deacetylcephalosporin C

$C_{12}H_{19}N_4O_9S$
Mol. Wt.: 395.4



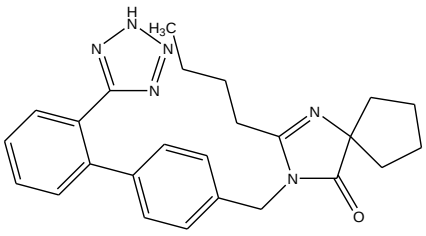
Isolimonic acid

$C_{26}H_{34}O_{10}$
Mol. Wt.: 506.5



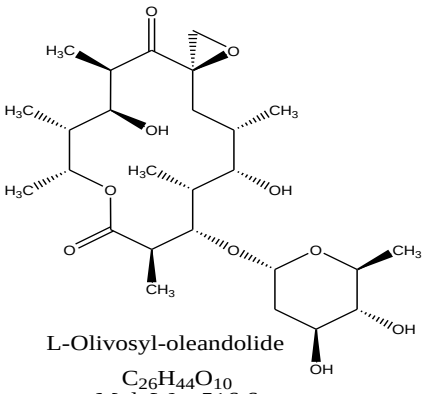
Isomigrastatin

$C_{27}H_{45}NO_7$
Mol. Wt.: 495.6



Irbesartan

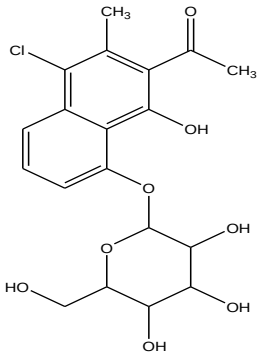
$C_{25}H_{28}N_6O$
Mol. Wt.: 428.5



L-Olivosyl-oleandolide

$C_{26}H_{44}O_{10}$

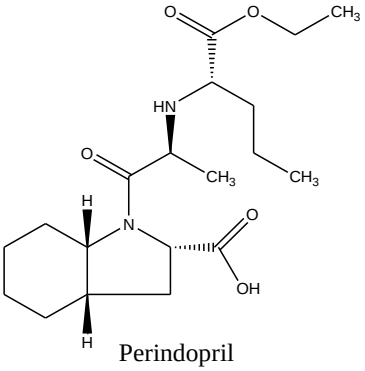
Mol. Wt.: 516.6



Patientoside A

$C_{19}H_{21}ClO_8$

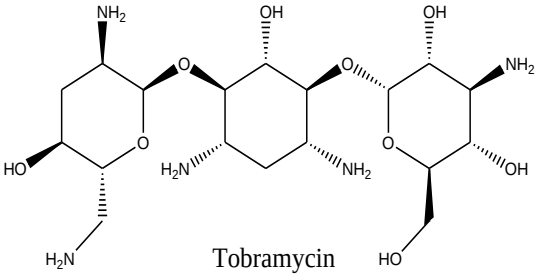
Mol. Wt.: 412.8



Perindopril

$C_{19}H_{32}N_2O_5$

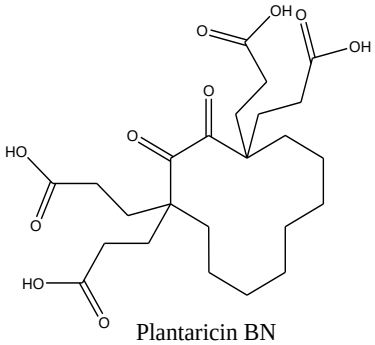
Mol. Wt.: 368.5



Tobramycin

$C_{18}H_{37}N_5O_9$

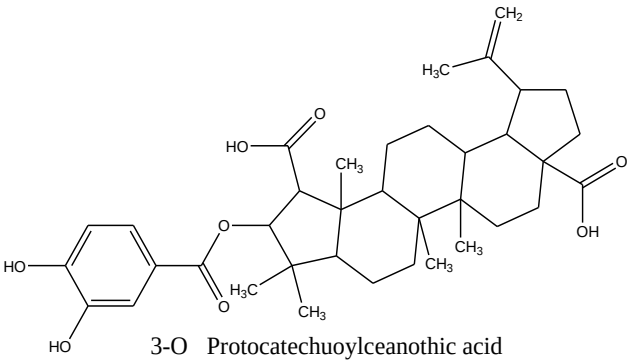
Mol. Wt.: 467.5



Plantaricin BN

$C_{24}H_{36}O_{10}$

Mol. Wt.: 484.5



3-O Protocatechuoylceanothic acid

$C_{37}H_{50}O_8$

Mol. Wt.: 622.8

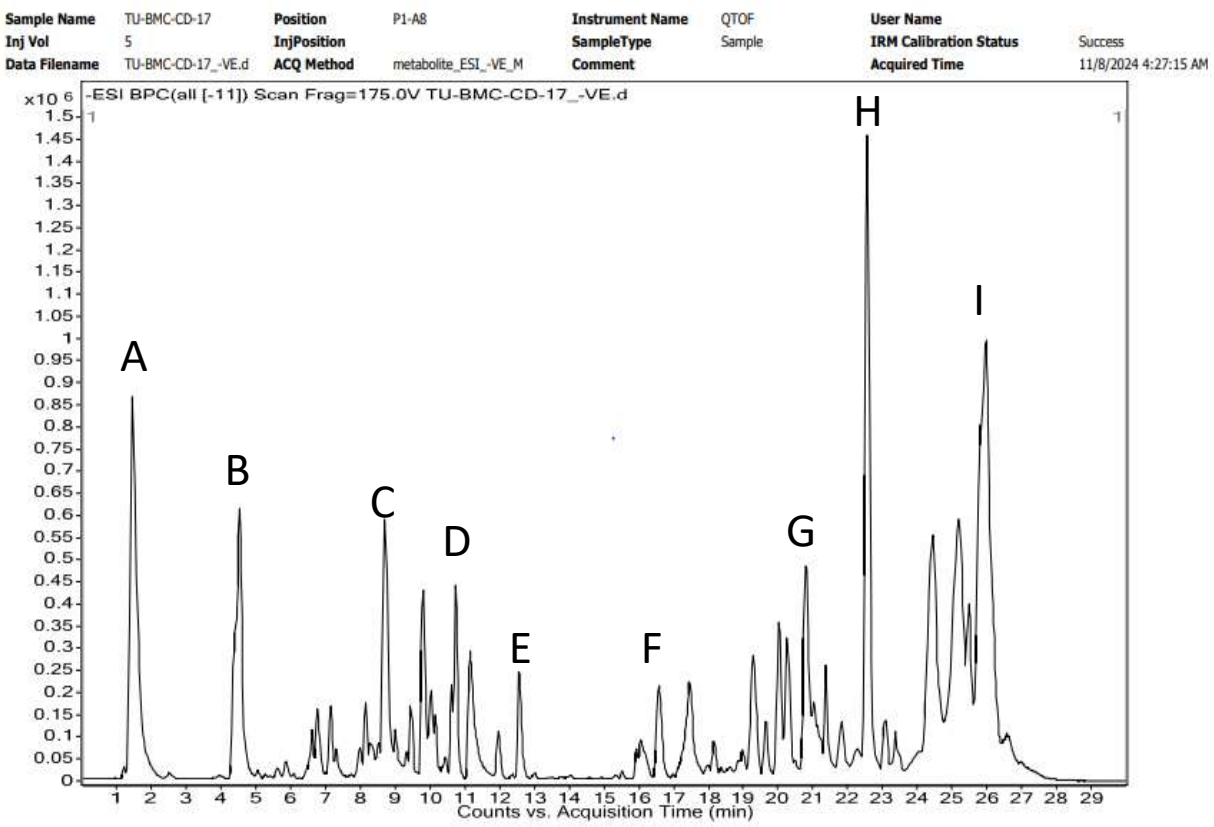


Figure 9 : Base peak chromatograms of *Psidium gaujava* L. fresh leaves extract negative mode

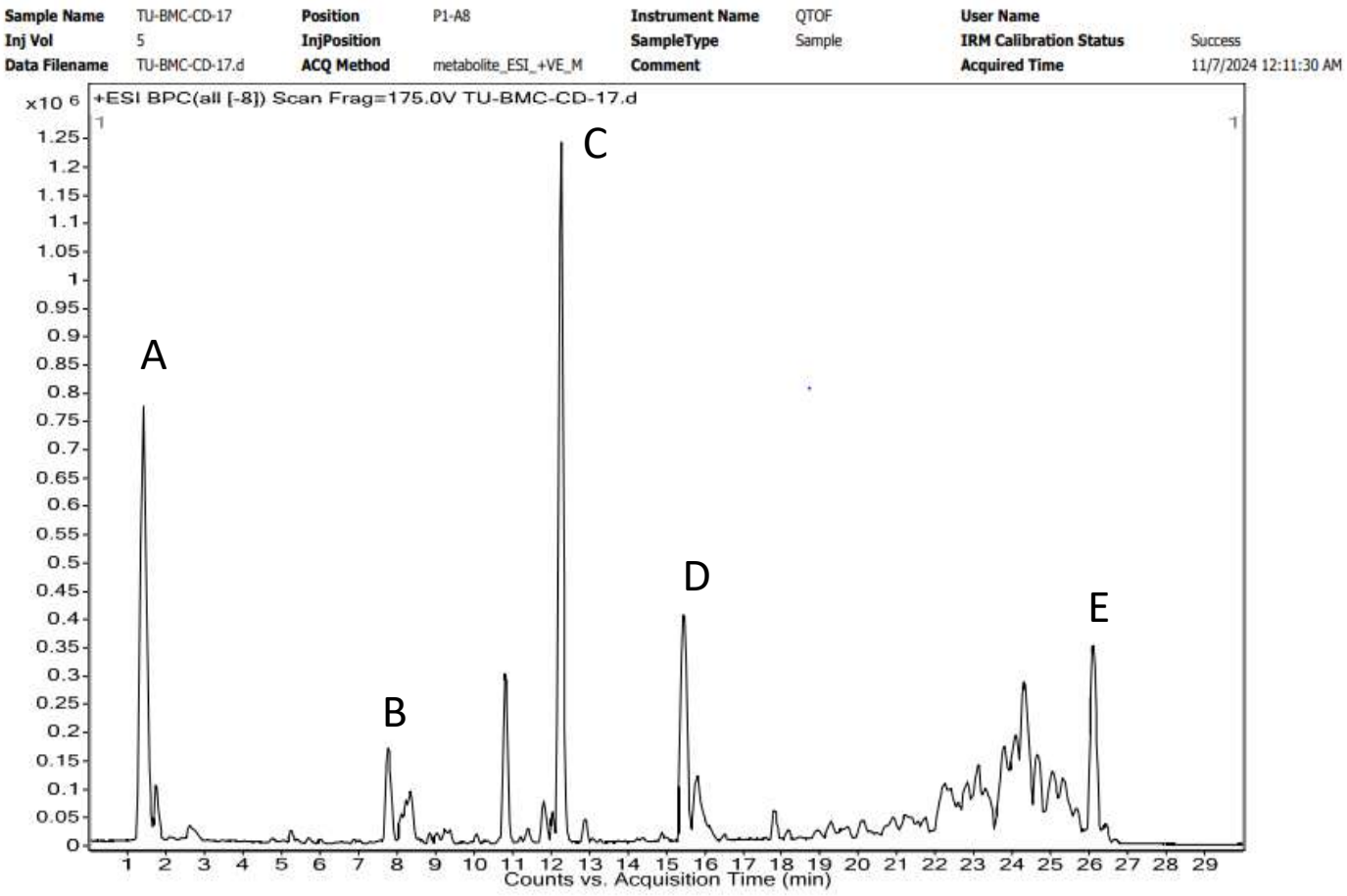


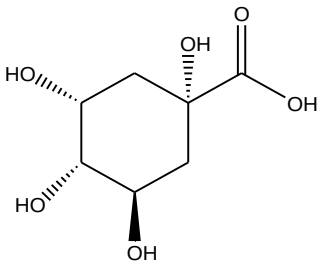
Figure 10: Base peak chromatograms of *Psidium gaujava* L. fresh leaves extract positive mode.

4.7 Selective secondary Metabolites

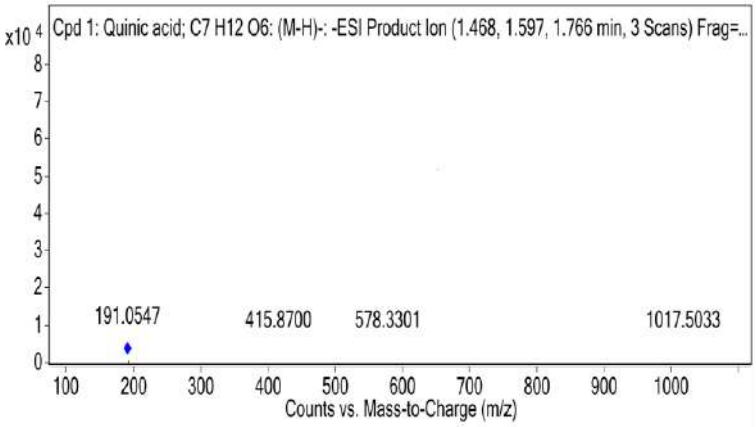
Quinic acid.

The chromatogram having retention time of 1.46, 1.59, 1.76 minutes in HR-LCMS is of quinic acid. The extract of fresh *Psidium gaujava* L. leaves contains substantial amount of this molecules which is represented "A" in negative ESI mode. In 1818, French chemists Joseph Bienaimé Caventou and Pierre Joseph Pelletier first isolated quinic acid. It is used in anti-viral, anti-inflammatory and antioxidant properties (Clifford, 2000). Recent research shows that quinic acid used to synthesis of bioactive

compounds like antiviral and used in green chemistry (Valanciene & Malys, 2022).

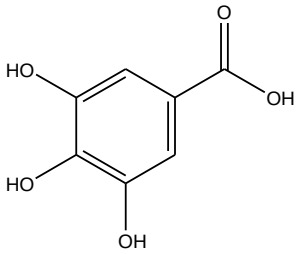


Quinic acid
$C_7H_{12}O_6$
Mol. Wt.: 192.2

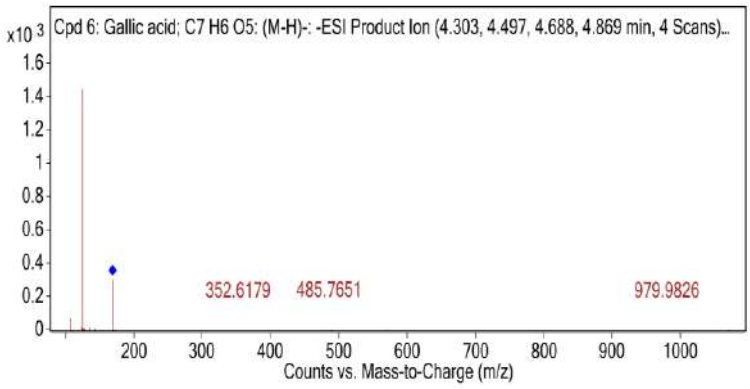


Gallic acid

Gallic acid (GA) has the molecular formula $C_7H_6O_5$ appears "B" in negative mode in chromatogram at retention time of 4.30, 4.49, 4.68, 4.86 minutes in HR-LCMS analysis of extract in negative ESI mode. In 1786, Carl Wilhelm Scheele first discovered gallic acid. GA finds applications in various industries, including food, ink, dye, and pharmaceuticals (Schaechter, 2009). Medicinally, GA exhibits antiviral, anticancer, antimicrobial, and antioxidant properties, with its strong antioxidative capacity enabling it to neutralize free radicals, prevent oxidative stress, and protect cellular integrity. Additionally, GA demonstrates anti-inflammatory activity by inhibiting inflammatory cytokines and enzymes, positioning it as a potential therapeutic for inflammatory diseases (Hadidi et al., 2024).

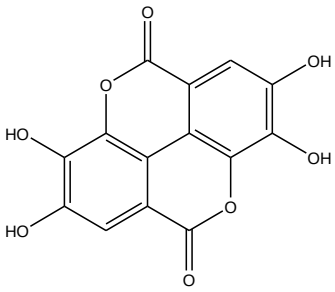


Gallic acid
$C_7H_6O_5$
Mol. Wt.: 170.1

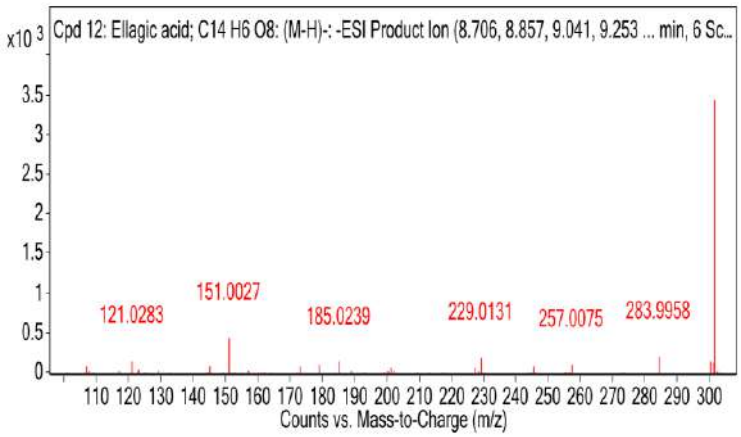


Ellagic acid

Ellagic acid is a phenolic compound that are present in the fresh *Psidium gaujava* L. leaves extract at retention time of 8.70, 8.85, 9.04, 9.29 minutes in HR-LCMS as shown in “C” in negative mode chromatogram. Ellagic acid (EA) was first identified in 1831 by French chemist Henri Braconnot, who named it "acide ellagique," derived from the reversed spelling of "galle." (Grasser, 1922). Structurally it is a derivative of dimeric gallic acid, and is primarily produced in plants through the hydrolysis of ellagitannins (Sharifi-Rad et al., 2022).

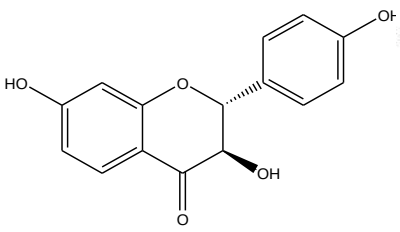


Ellagic acid
$C_{14}H_6O_8$
Mol. Wt.: 302.2

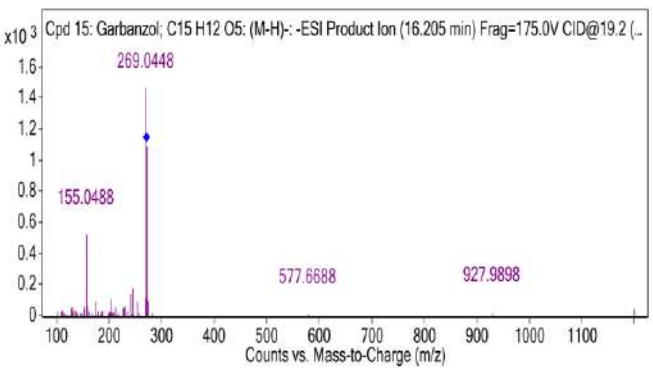


Garbanzol

The naturally occurring isoflavonoid compound that are present in the fresh *Psidium gaujava* L. leaves extract at retention time of 16.20 minutes in HR-LCMS as shown in “F” in negative mode chromatogram. Garbanzol was initially discovered in leguminous plants, especially chickpeas (Cicer arietinum). Potent antioxidants especially garbanzol can scavenge free radicals and protect cells from oxidative damage. Garbanzol has antimicrobial properties and works well against a range of bacterial and fungal infections (Guardado-Félix et al., 2017).

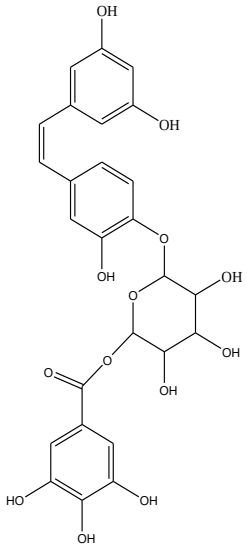


Garbanzol
$C_{15}H_{12}O_5$
Mol. Wt.: 272.3



Piceatannol 4'-galloylglucoside

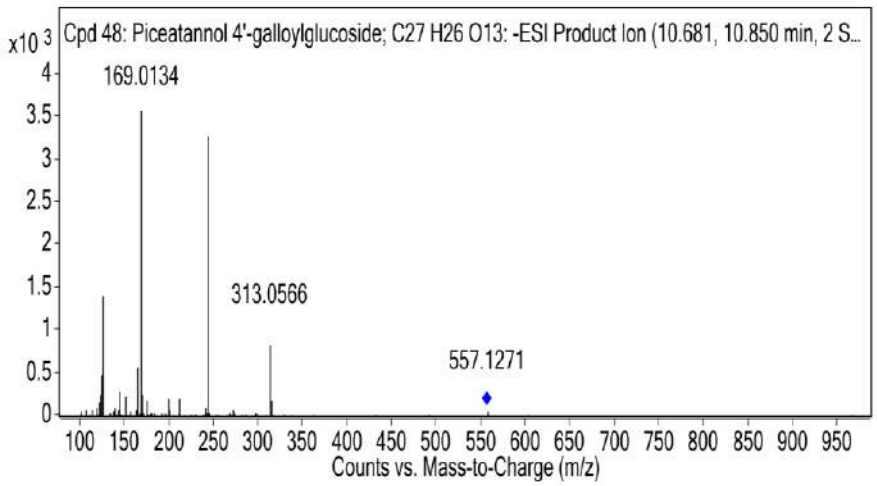
Piceatannol 4'-galloylglucoside has complex structure. Its presence in the fresh *Psidium gaujava* L. leaves extract which proved by its negative ESI mode chromatogram present at "D" of retention time 10.68, 10.85 minutes. The stilbenoid family of polyphenolic compounds includes piceatannol 4'-galloylglucoside, a derivative of piceatannol. The phenolic structure of piceatannol 4'-galloylglucoside is responsible for its strong antioxidant qualities. Which is related to long-term conditions include diabetes, cardiovascular disease and neurological disorders (Zheng et al., 2018).



Piceatannol 4'-galloylglucoside

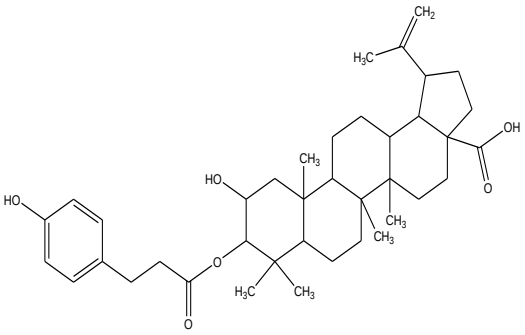
$C_{26}H_{24}O_{13}$

Mol. Wt.: 544.5



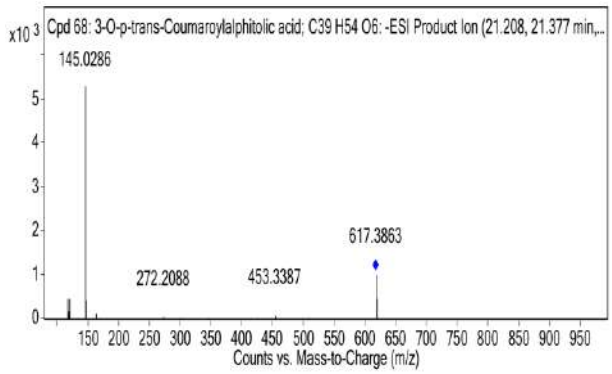
3-O-p-trans-Coumaroylalphitolic acid.

The molecule 3-O-p-trans-Coumaroylalphitolic acid has found in fresh *Psidium gaujava* L. leaves extract at retention time of 21.20, 21.37 minute represented as "G" in negative ESI mode. It is triterpenoid ester primarily discovered in a variety of plant species that are well-known for their therapeutic qualities and anti-inflammatory (Ríos et al., 2000). It has shown cytotoxic activity against specific cancer cell (Ma et al., 2020).



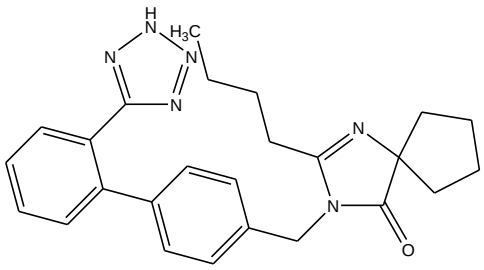
3-O-p-trans Coumaroylalphitolic acid

$C_{39}H_{56}O_6$
Mol. Wt.: 620.9



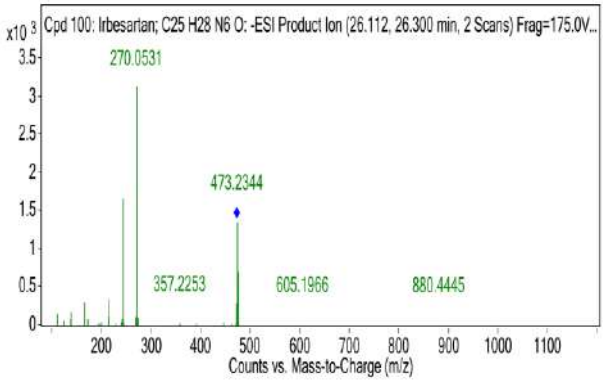
Irbesartan

Irbesartan has the molecular formula $C_{25}H_{28}N_6O$ appears "I" in negative mode at retention time of 26.11, 26.30 minutes in HR-LCMS analysis of extract of fresh *Psidium gaujava* L. leaves. In 1997, the Food and Drug Administration authorised the medicine ues in the United States. The primary reason that irbesartan is prescribed is to treat hypertension. It minimises blood pressure and improves cardiovascular outcomes by blocking the AT_1 receptor (Gillis & Markham, 1997).



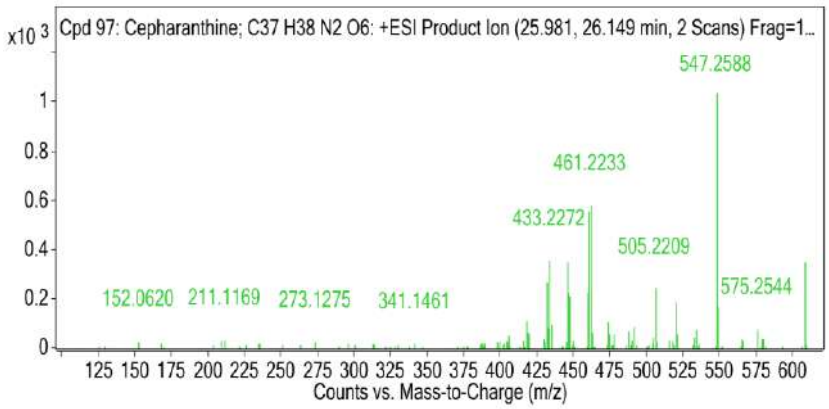
Irbesartan

$C_{25}H_{28}N_6O$
Mol. Wt.: 428.5



Cepharanthine

HR-LCMS data of the fresh *Psidium gaujava* L. leaves extract shows Cepharanthine has retention time of 25.98, 26.14 minutes in positive ESI mode which was represented as "E". Since 1950s, the Japanese have been using cepharanthine (CEP) to treat several kinds of acute as well as chronic illnesses including alopecia, xerostomia, leukopenia, and snake bites. Primarily CEP obtained from the plant Stephania cephalantha Hayata, this natural product has numerous of pharmacological qualities, comprising anti-oxidative, anti-inflammatory (Wang et al., 2023).



Preliminary phytochemical investigation highlights positive results against Alkaloids, Flavonoids, Tannin, Glycosides and polyphenol which reveals the plant sample as goldmine of phytochemical compound. The quantitative assessments of phytochemical constituents (TPC, TFC and TTC) shows that the plant extract contains substantial quantity of phytochemical constituents. This research result shows maceration extract show high TPC (316.0±7.9mg of GAE/g), TFC (257.9±4.4mg QE/g), TTC (563.6±4.5tannic acid equivalent (mg/g)) and antioxidant properties (IC_{50}=6.01mg/L and 125.0±2.1mM of ascorbic acid eqvt/g of dry wt.**)**. The antimicrobial assay showed good activity against five bacterial strains. The

following inhibition zones for *S. aureus*-19.33±0.57mm,*K. pneumonia*-21.33±2.08mm in soxhlet extract, *E. coli*-15.00±0.81mm, *S.aureus*- 18.67±1.15mm in sonication extract and *S. typhirium* -16.33±1.15mm maceration extraction. The qualitative HRLCMS analysis reveals the presence of substantial numbers of active molecules in the plant, which holds the significant role to cure different diseases. The numbers of molecules present in the extract have wide range of beneficial properties. Many of the molecules act as antioxidant, antibacterial, anti-cancer, anti-hypertensive agent, immunomodulatory, anti-inflammatory as well as anti-asthmatic, vasodilator, anti-viral (potential binder against SARS-COV-2) and antifungal. Besides this, some of the molecules present also used in food and beverage sweetener and preservatives.

CHAPTER FIVE: CONCLUSIONS

5.1 Conclusions

The findings revealed qualitative and quantitative differences in fresh *Psidium guajava* L. fresh leaves. Among the technique evaluated maceration extraction showed the highest TPC and TFC as well as the strongest antioxidant activity. The extract also demonstrated significant antimicrobial potential and antioxidant properties due to the presence of phenolic groups.

Phytochemical analysis of fresh *Psidium guajava* L. leaves identified several secondary metabolites, including phenolics, alkaloids, terpenoids, glycosides, and saponins. The antioxidant activity indicates the potential application of *Psidium guajava* L. leaves extract in the food industry. This work provides the first comprehensive analysis of secondary metabolites of fresh *Psidium guajava* L. leaves. As far as I know as compare with literature review Trichocarposide, Palmidin B, Patientoside A, Cinnzeylanine, Kenposide B, Azukisapogenol, Mammeisin and Trigofoenoside A secondary metabolites been reported for first time in *Psidium guajava* L. leaves.

This study discloses the pharmacokinetic applications of secondary metabolites as antioxidant, antimicrobial and anticancer properties. Additionally, it offers valuable insights into the quality evaluation of *Psidium guajava* L. leaves extracts and their broader application in health and industry.

5.2 Recommendations

The extraction of the molecules from the plant as in its natural form depends on the polarity of solvent used in the process. The complete information of the molecules present in the plant and efficacy of the extract has to be further evaluated for

- Toxicity and effective dose of the extract.
- Unidentified compound appeared in the HR-LCMS should be isolated and characterized.

Publication

"Khanal, B., Pokhrel, G., Khanal, R., Bhattarai, B. B., & Pokhrel, G. R. (2024). Bacterial Assay of Drinking Water Commercially Marketed in Bharatpur Metropolitan City, Chitwan, Nepal. *BMC Journal of Scientific Research*, *7*(1), 1–11. https://doi.org/10.3126/bmcjsr.v7i1.72937

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APPENDICES

APPENDIX 1: PHYTOCHEMICAL SCREENING PROTOCOL

1) Test for Alkaloids

About 500 mg extract was dissolved in 3 mL of 2 % (v/v) HCl. The solution was equally divided into two test tubes and the following tests were performed,

I. Meyer's Test

Few drops of Meyer's reagent were added to the first part. Formation of a pale-yellow precipitate indicates the presence of alkaloids.

II. Dragendorff's Test

Few drops of Dragendorff's reagent were added to the second part. Formation of orange-red precipitate indicates the presence of alkaloids.

2) Test for Terpenoids

To about 200 mg extract, 2 mL of chloroform ($CHCl_3$) and then 3 mL concentrated sulphuric acid (H_2SO_4) was added carefully. Formation of reddish-brown coloration at the interface indicates the presence of terpenoids.

3) Test for Coumarins

To about 1 mL of extract, 1 mL of 10 % sodium hydroxide (NaOH) solution was added. Formation of yellow color indicates the presence of coumarins.

4) Test for Flavonoids/Shinoda's Test

About 200 mg extract was dissolved in 2 mL methanol. To this solution, small piece of magnesium and 4-5 drops of concentrated hydrochloric acid (HCl) were added. Formation of orange color indicates the presence of flavonoids.

5) Test for Quinones

To about 2 mL extract, 1 mL freshly prepared ferrous sulphate ($FeSO_4$) solution and few crystals of ammonium thiocyanate (NH_4SCN) were added and the solution was treated with conc. sulphuric acid (H_2SO_4) drop by drop. The appearance of persistent deep red colouration indicates the presence of quinones.

6) Test for Polyphenols/ $FeCl_3$ Test

To about 1 mL extract, 1 mL distilled water was added followed by the addition of a few drops of 10 % (w/v) ferric chloride ($FeCl_3$) solution. The appearance of greenish blue coloration indicates the presence of polyphenols.

7) Test for Glycosides

About 500 mg extract was dissolved in 2 mL methanol and divided into two parts and the following tests were performed,

i. Molisch's Test

The first part was treated with 5 mL Molisch's reagent and conc. H2SO4 was added drop by drop from the side of the test tube without disturbing the solution. The appearance of a violet ring at the junction of two liquids which on shaking turns the solution into violet colour indicates the presence of glycosides.

ii. To the second part 2 mL of 25 % (v/v) NH_4OH solution was added and shaken vigorously. The appearance of cherry red colour indicates the presence of glycosides.

8) Test for Reducing Sugars

To about 1 mL extract, 1mL distilled water was added followed by addition of 1 mL Fehling's reagent (1,1 mixture of Fehling's solution A and B). Then the mixture was warmed over water bath for 30 minutes. The appearance of a brick red precipitate indicates the presence of reducing sugars.

9) Test for Saponins

About 500 mg extract was treated with hot water followed by shaking for 30 seconds. Formation of thick forth indicates the presence of saponins.

10) Test for Tannins

About 200 mg extract was boiled adding 10 mL distilled water. The mixture was cooled, filtered and few drops of $FeCl_3$ solution were added to the filtrate. The appearance of blue-black precipitate indicates the presence of tannins.

APPENDIX 2: PREPARATION OF REAGENTS.

i. Meyer's Reagent,

Mercuric chloride, $HgCl_2$ (0.679 g) was weighed in a 50 mL volumetric flask and dissolved in distilled water. To this solution, 2.5 g potassium iodide (KI) was added. The scarlet red precipitate was dissolved by shaking and volume was made up to the mark by adding distilled water.

ii. Dragendorff's Reagent,

Bismuth nitrate, $Bi(NO_3)_3$ (4.000 g) was dissolved in 5 N nitric acid (10 mL) to make solution A. Next, potassium iodide, KI (13.5 g) was dissolved in distilled water (20 mL) to make solution B. These two solutions were mixed together in a 50 mL volumetric flask.

Picric acid (0.25 g) was dissolved in 50 mL distilled water to make aqueous picric acid solution. The solution was neutralized with sodium bicarbonate ($NaHCO_3$). A strip of Whatmann no. 1 filter paper was dipped in the prepared solution. The paper was dried completely and protected from external contamination. Thus, prepared sodium picrate paper was used for Cyanogenic Glycoside detection.

3. Molisch's Reagent,

α-Naphthol (5.000 g) was dissolved in 50 mL methanol to prepare Molisch's reagent.

4. Neutral Ferric Chloride ($FeCl_3$) Solution,

Ferric chloride crystals (1.000 g) were dissolved in 100 mL distilled water. To this solution, sodium carbonate crystals were added little by little with stirring until the slight turbidity was persistent. Finally, the mixture was filtered and the colorless filtrate was used as neutral ferric chloride.

APPENDIX 3: STANDARD AND SAMPLE

Antioxidant Activity of Ascorbic Acid.

Calculation of % Radical Scavening and IC_{50} From DPPH Assay

Concentration(µg/ml)	Control	Absorbance of Ascorbic acid	%RSA
1	0.388	0.378	2.57
2	0.388	0.301	22.42
3	0.388	0.22	43.3
4	0.388	0.178	54.12
10	0.388	0.041	89.43
20	0.388	0.035	90.98

%RSA=(absorbance of control)-(absorbance of sample)/(absorbance of control)*100%

IC_{50}=(concentration of sample)-(intercept)/slope

IC_{50}= (50+13.273)/17.55

IC_{50}= 3.604ppm

Antioxidant Activity for Maceration extract of leaves.

Concentration(µg/ml)	Control	Abs. of sample(maceration)	%RSA
1	0.388	0.376	3.09
2	0.388	0.341	12.11
4	0.388	0.281	27.57
5	0.388	0.216	44.32
10	0.388	0.055	85.82
20	0.388	0.045	88.40

IC_{50} =(50+6.2509)/9.2509

IC_{50}=6.08ppm

Antioxidant Activity for Sonication extract of leaves.

Concentration(µg/ml)	Control	Abs. of sample(Sonication)	%RSA
2	0.388	0.378	2.57
3	0.388	0.323	16.75
4	0.388	0.25	35.56
10	0.388	0.1	74.22
20	0.388	0.049	87.37
30	0.388	0.047	87.88

IC_{50}= (50+7.760)/8.4285

IC_{50}= 6.852ppm

Antioxidant Activity for Soxhlet extract of leaves.

Concentration(µg/mL)	Control	Abs.of sample (soxhlet)	%RSA
1	0.388	0.362	6.70
2	0.388	0.349	10.05
3	0.388	0.322	17.01
4	0.388	0.25	35.56
5	0.388	0.229	40.97
10	0.388	0.061	84.27
20	0.388	0.051	86.85
30	0.388	0.049	87.37

IC_{50} = (50+5.0285)/8.989

IC_{50}=6.121ppm

Total Phenolic Content of Maceration extract of leaves

S.N.	Sample solution (µg/mL)	Wt. of dry extract per mL (g)	Absorbance	GAE conc. (µg/mL)	Mean ±S.D
1	100	0.001	0.323	30.77	
2	100	0.001	0.334	31.88	31.61±0.74
3	100	0.001	0.337	32.19	

Total Phenolic Content of Sonication extract of leaves

S.N.	Sample solution (µg/mL)	Wt. of dry extract per mL (g)	Absorbance	GAE conc. (µg/mL)	Mean ±S.D
1	100	0.001	0.254	23.80	
2	100	0.001	0.245	22.89	23.47±0.49
3	100	0.001	0.253	23.70	

Total Phenolic Content of Soxhlet extract of leaves.

S.N.	Sample solution (µg/mL)	Wt. of dry extract per mL (g)	Absorbance	GAE conc. (µg/mL)	Mean ±S.D
1	100	0.001	0.311	29.56	
2	100	0.001	0.299	28.35	28.08±1.63
3	100	0.001	0.279	26.33	

Total flavonoid content in Maceration extract of leaves.

S.N.	Sample solution (µg/mL)	Wt. of dry extract per mL (g)	Absorbance	QE conc. (µg/mL)	Mean ±S.D
1	100	0.001	0.042	26.30	
2	100	0.001	0.041	25.53	25.79±0.44
3	100	0.001	0.041	25.53	

Total flavonoid content in Sonication extract of leaves.

S.N.	Sample solution (µg/mL)	Wt. of dry extract per mL (g)	Absorbance	QE conc. (µg/mL)	Mean ±SD
1	100	0.001	0.043	27.07	
2	100	0.001	0.039	24.00	24.25±2.70
3	100	0.001	0.036	21.69	

Total flavonoid content in Soxhlet extract of leaves.

S.N.	Sample solution (µg/mL)	Wt. of dry extract per mL (g)	Absorbance	QE conc. C (µg/mL)	Mean ±SD
1	100	0.001	0.039	24.00	
2	100	0.001	0.038	23.23	22.47±2.03
3	100	0.001	0.034	20.15	

Total Phosphomolybdenum antioxidant Content of Maceration extract of leaves

S.N.	Sample solution (µg/mL)	Wt. of dry extract per mL (g)	Absorbance	GAE conc. (µg/mL)	Mean ±SD
1	200	0.001	0.190	25.30	
2	200	0.001	0.184	24.53	25+0.41
3	200	0.001	0.189	25.17	

Total Phosphomolybdenum antioxidant Content of Sonication extract of leaves

S.N.	Sample solution (µg/mL)	Wt. of dry extract per mL (g)	Absorbance	GAE conc.C (µg/mL)	Mean ±S.D
1	200	0.001	0.114	15.56	
2	200	0.001	0.114	15.56	15.47+0.14
3	200	0.001	0.112	15.30	

Total Phosphomolybdenum antioxidant Content of Soxhlet extract of leaves.

S.N.	Sample solution (µg/mL)	Wt. of dry extract per mL (g)	Absorbance	GAE conc. (µg/mL)	Mean ±S.D
1	200	0.001	0.119	16.20	
2	200	0.001	0.120	16.33	16.37±0.19
3	200	0.001	0.122	16.58	

Total Tannin content in Maceration extract of leaves.

S.N.	Sample solution (µg/mL)	Wt. of dry extract per mL m (g)	Absorbance	Tannin conc (µg/mL)	Mean ±S.D
1	200	0.001	0.1	80.00	
2	200	0.001	0.102	81.81	80.90±1.65
3	200	0.001	0.101	80.90	

Total Tannin content in Sonication extract of leaves.

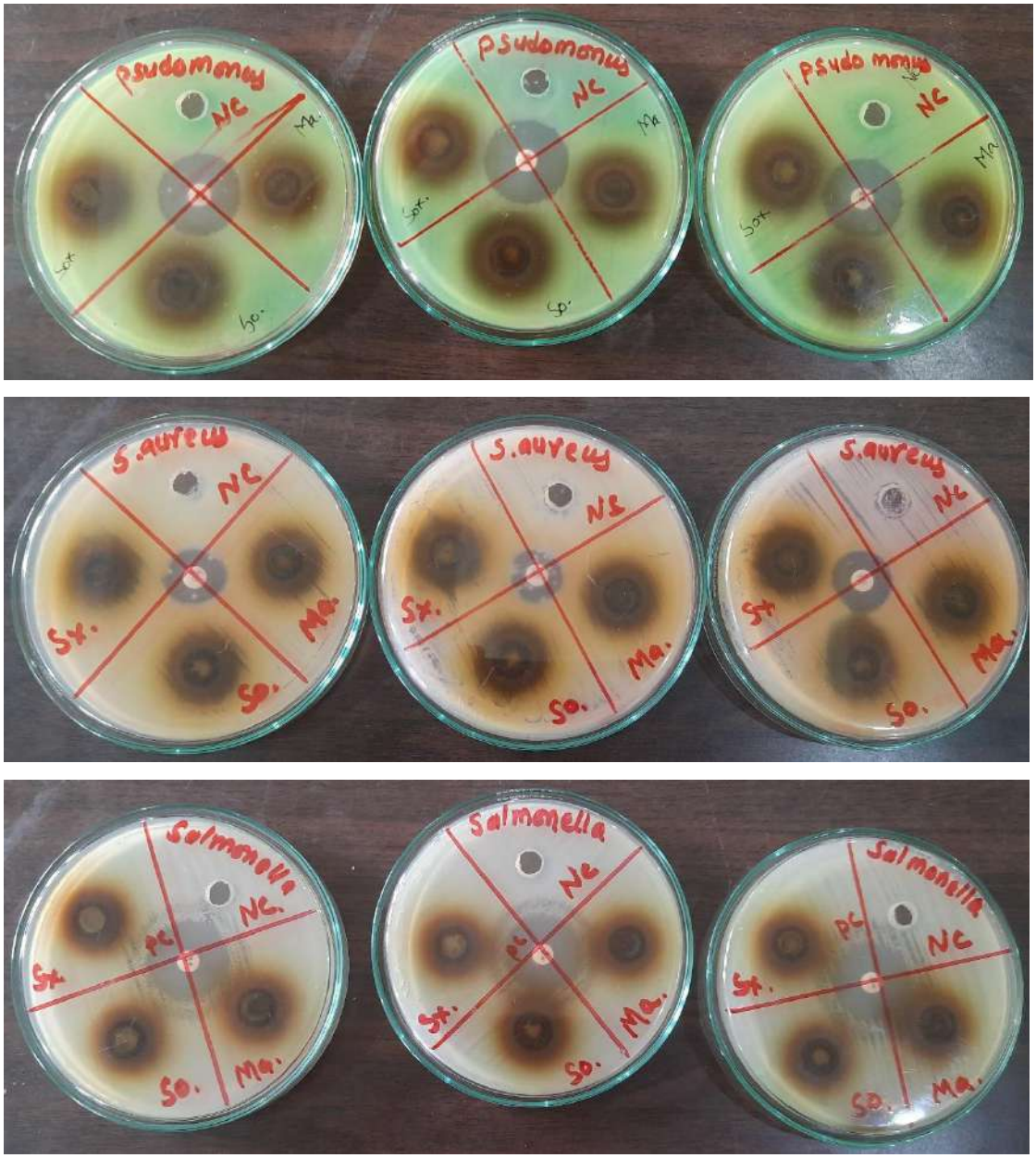
S.N.	Sample solution (µg/mL)	Wt. of dry extract per mL (g)	Absorbance	Tannin conc. C (µg/mL)	Mean ±S.D
1	200	0.001	0.115	93.63	
2	200	0.001	0.116	94.54	93.63±1.65
3	200	0.001	0.114	92.72	

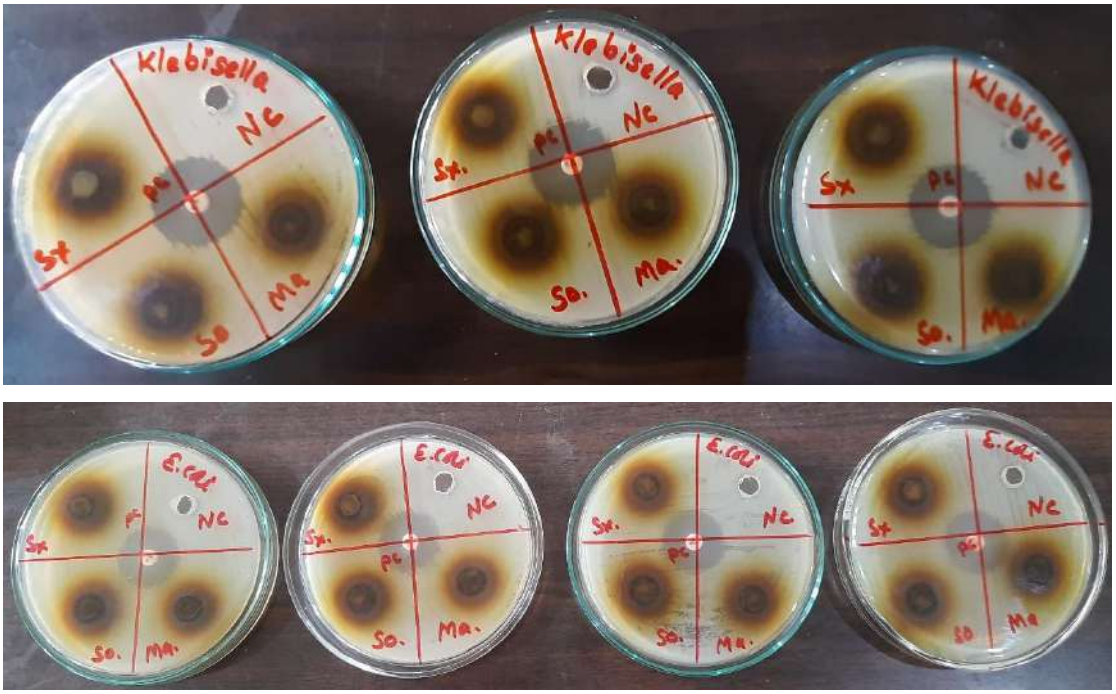
Total Tannin content in Soxhlet extract of leaves.

S.N.	Sample solution (µg/mL)	Wt. of dry extract per mL (g)	Absorbance	Tannin conc. (µg/mL)	Mean ±S.D
1	200	0.001	0.136	112.72	
2	200	0.001	0.137	113.63	112.72±0.90
3	200	0.001	0.135	111.81	

APPENDIX 4: PHOTO PLATES

APPENDIX 5: ANTIMICROBIAL PROPERTIES





Sx= Soxhlet extract Nc= Negative Control

So= Sonication extract Pc= Positive Control

Ma= Maceration extract

APPENDIX 6: ESI MODE

POSITIVE ESI MODE

Data File	TU-BMC-CD-17.d	**Sample Name**	TU-BMC-CD-17
Sample Type	Sample	**Position**	P1-A8
Instrument Name	QTOF	**User Name**	
Acq Method	metabolite_ESI_+VE_MSMS.m	**Acquired Time**	11/7/2024 12:11:30 AM
IRM Calibration Status	Success	**DA Method**	Default.m
Comment			

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

Compound Table

Compound Label	RT	Mass	Abund	Name	Formula	MFG Formula	DB Formula	DB Diff (ppm)	Hits (DB)
Cpd 1: 8-Hydroxy-2-chlorodibenzofuran; C12 H7 Cl O2	1.264	218.0151	13454	8-Hydroxy-2-chlorodibenzofuran	C12 H7 Cl O2	C12 H7 Cl O2	C12 H7 Cl O2	-7.38	2
Cpd 2: (-)-Annonaine; C17 H15 N O2	1.297	265.1104	92670	(-)-Annonaine	C17 H15 N O2	C17 H15 N O2	C17 H15 N O2	-0.44	6
Compound 3	1.373								
Cpd 4: Streptidine 6-phosphate; C8 H19 N6 O7 P	1.406	342.1085		Streptidine 6-phosphate	C8 H19 N6 O7 P	C8 H19 N6 O7 P	C8 H19 N6 O7 P	-9.28	10
Cpd 5: Trolamine; C6 H15 N O3	1.411	149.1042		Trolamine	C6 H15 N O3	C6 H15 N O3	C6 H15 N O3	6.54	1
Compound 6	1.428								
Compound 7	2.133		13025						
Compound 8	2.472								
Compound 9	2.856								
Compound 10	7.815								
Cpd 11: Patientoside A; C19 H21 Cl O8	8.178	412.0933		Patientoside A	C19 H21 Cl O8	C19 H21 Cl O8	C19 H21 Cl O8	-2.02	1
Cpd 12: O-Carbamoyl-deacetylcephalosporin C; C15 H20 N4 O8 S	8.491	416.1018		O-Carbamoyl-deacetylcephalosporin C	C15 H20 N4 O8 S	C15 H20 N4 O8 S	C15 H20 N4 O8 S	-3.89	2
Compound 13	12.239								
Compound 14	15.615								
Cpd 15: 1-Octen-3-yl primeveroside; C19 H34 O10	15.739	422.2131		1-Octen-3-yl primeveroside	C19 H34 O10	C19 H34 O10	C19 H34 O10	4.86	7
Compound 16	15.833								
Cpd 17: Naltrindole; C26 H26 N2 O3	15.857	414.1952		Naltrindole	C26 H26 N2 O3	C26 H26 N2 O3	C26 H26 N2 O3	-2.1	3
Compound 18	17.54								
Cpd 19: Isolimonic acid; C26 H34 O10	17.82	506.2095		Isolimonic acid	C26 H34 O10	C26 H34 O10	C26 H34 O10	11.13	5
Cpd 20: Plantaricin BN; C24 H36 O10	17.856	484.2278	20476	Plantaricin BN	C24 H36 O10	C24 H36 O10	C24 H36 O10	6.22	3
Cpd 21: Tobramycin; C18 H37 N5 O9	18.189	467.2594		Tobramycin	C18 H37 N5 O9	C18 H37 N5 O9	C18 H37 N5 O9	-0.55	1
Cpd 22: alpha,alpha'-Trehalose 6-palmitate; C28 H52 O12	18.547	580.3389	10010	alpha,alpha'-Trehalose 6-palmitate	C28 H52 O12	C28 H52 O12	C28 H52 O12	12.03	1
Compound 23	18.771		10300						
Cpd 24: 3-O-Protocatechuoylceanothic acid; C37 H50 O8	18.819	622.3486		3-O-Protocatechuoylceanothic acid	C37 H50 O8	C37 H50 O8	C37 H50 O8	3.2	4
Cpd 25: Trichocarposide; C22 H24 O9	18.828	432.1477		Trichocarposide	C22 H24 O9	C22 H24 O9	C22 H24 O9	-13.15	5
Cpd 26: L-Olivosyl-oleandolide; C26 H44 O10	18.918	516.2872		L-Olivosyl-oleandolide	C26 H44 O10	C26 H44 O10	C26 H44 O10	12.01	1
Compound 27	18.97								
Cpd 28: Perindopril; C19 H32 N2 O5	19.018	368.2285		Perindopril	C19 H32 N2 O5	C19 H32 N2 O5	C19 H32 N2 O5	7.11	8
Cpd 29: L-Olivosyl-oleandolide; C26 H44 O10	19.268	516.2867		L-Olivosyl-oleandolide	C26 H44 O10	C26 H44 O10	C26 H44 O10	13.04	1
Compound 30	19.322								
Cpd 31: Usambarine; C30 H34 N4	19.467	450.2809		Usambarine	C30 H34 N4	C30 H34 N4	C30 H34 N4	-5.76	2
Compound 32	19.591								
Cpd 33: Usambarine; C30 H34 N4	19.837	450.2805		Usambarine	C30 H34 N4	C30 H34 N4	C30 H34 N4	-4.89	2
Compound 34	20.082								
Cpd 35: L-Olivosyl-oleandolide; C26 H44 O10	20.085	516.2866		L-Olivosyl-oleandolide	C26 H44 O10	C26 H44 O10	C26 H44 O10	13.32	1
Cpd 36: Usambarine; C30 H34 N4	20.12	450.2807		Usambarine	C30 H34 N4	C30 H34 N4	C30 H34 N4	-5.21	2
Compound 37	20.274								
Compound 38	20.432								
Cpd 39: Glycosyl-4,4'-diaponeurosporenoate; C36 H50 O7	20.478	594.3548		Glycosyl-4,4'-diaponeurosporenoate	C36 H50 O7	C36 H50 O7	C36 H50 O7	1.37	2
Cpd 40: Tobramycin; C18 H37 N5 O9	20.727	467.2578		Tobramycin	C18 H37 N5 O9	C18 H37 N5 O9	C18 H37 N5 O9	2.83	1
Cpd 41: Usambarine; C30 H34 N4	20.747	450.2804	20272	Usambarine	C30 H34 N4	C30 H34 N4	C30 H34 N4	-4.52	2
Compound 42	20.795								
Compound 43	21.035								
Cpd 44: Usambarine; C30 H34 N4	21.055	450.2809		Usambarine	C30 H34 N4	C30 H34 N4	C30 H34 N4	-5.73	2
Cpd 45: Asparagoside B; C33 H56 O9	21.372	596.3968	11733	Asparagoside B	C33 H56 O9	C33 H56 O9	C33 H56 O9	-7.3	1
Compound 46	21.43								
Cpd 47: Inuline; C32 H46 N2 O8	21.486	586.329		Inuline	C32 H46 N2 O8	C32 H46 N2 O8	C32 H46 N2 O8	-6.18	1
Compound 48	21.66								
Compound 49	21.712								
Compound 50	21.737								

Cpd 51: Semilepidinoside A; C16 H20 N2 O6	21.806	336.1306		Semilepidinoside A	C16 H20 N2 O6	C16 H20 N2 O6	C16 H20 N2 O6	4.69	1
Compound 52	21.885								
Cpd 53: 23-trans-p-Coumaroyloxytormentic acid; C39 H54 O8	22.083	650.3825		23-trans-p-Coumaroyloxytormentic acid	C39 H54 O8	C39 H54 O8	C39 H54 O8	-0.9	2
Cpd 54: Inuline; C32 H46 N2 O8	22.083	586.3286		Inuline	C32 H46 N2 O8	C32 H46 N2 O8	C32 H46 N2 O8	-5.35	1
Cpd 55: 23-trans-p-Coumaroyloxytormentic acid; C39 H54 O8	22.412	650.3831		23-trans-p-Coumaroyloxytormentic acid	C39 H54 O8	C39 H54 O8	C39 H54 O8	-1.96	2
Cpd 56: Inuline; C32 H46 N2 O8	22.415	586.329		Inuline	C32 H46 N2 O8	C32 H46 N2 O8	C32 H46 N2 O8	-6.14	1
Compound 57	22.706								
Cpd 58: 23-trans-p-Coumaroyloxytormentic acid; C39 H54 O8	22.75	650.382		23-trans-p-Coumaroyloxytormentic acid	C39 H54 O8	C39 H54 O8	C39 H54 O8	-0.17	2
Cpd 59: 3-O-trans-Feruloyleuscaphic acid; C40 H56 O8	22.848	664.3976		3-O-trans-Feruloyleuscaphic acid	C40 H56 O8	C40 H56 O8	C40 H56 O8	-0.18	1
Cpd 60: Inuline; C32 H46 N2 O8	22.89	586.3288		Inuline	C32 H46 N2 O8	C32 H46 N2 O8	C32 H46 N2 O8	-5.71	1
Cpd 61: Aspartyl-Asparagine; C8 H13 N3 O6	23.121	247.0807		Aspartyl-Asparagine	C8 H13 N3 O6	C8 H13 N3 O6	C8 H13 N3 O6	-0.95	3
Cpd 62: Sulfoglycolithocholate; C26	23.178	513.2767		Sulfoglycolithocholate	C26 H43 N O7 S	C26 H43 N O7 S	C26 H43 N O7 S	-1.25	4
Cpd 63: 3-O-trans-Feruloyleuscaphic acid; C40 H56 O8	23.365	664.3976		3-O-trans-Feruloyleuscaphic acid	C40 H56 O8	C40 H56 O8	C40 H56 O8	-0.12	1
Cpd 64: 23-trans-p-Coumaroyloxytormentic acid; C39 H54 O8	23.516	650.3817		23-trans-p-Coumaroyloxytormentic acid	C39 H54 O8	C39 H54 O8	C39 H54 O8	0.29	2
Compound 65	23.572		11729						
Compound 66	23.589								

Cpd 67: Somniferine; C36 H36 N2 O7	23.777	608.2516		Somniferine	C36 H36 N2 O7	C36 H36 N2 O7	C36 H36 N2 O7	1.08	1
Cpd 68: Aspartyl-Asparagine; C8 H13 N3 O6	23.782	247.0806		Aspartyl-Asparagine	C8 H13 N3 O6	C8 H13 N3 O6	C8 H13 N3 O6	-0.67	3
Cpd 69: Aspartyl-Asparagine; C8 H13 N3 O6	24.036	247.0812		Aspartyl-Asparagine	C8 H13 N3 O6	C8 H13 N3 O6	C8 H13 N3 O6	-3.04	3
Cpd 70: 3-O-trans-Feruloyleuscaphic acid; C40 H56 O8	24.119	664.3984		3-O-trans-Feruloyleuscaphic acid	C40 H56 O8	C40 H56 O8	C40 H56 O8	-1.29	1
Cpd 71: Dipiperamide A; C34 H38 N2 O6	24.239	570.277	152242	Dipiperamide A	C34 H38 N2 O6	C34 H38 N2 O6	C34 H38 N2 O6	-7	4
Compound 72	24.244								
Cpd 73: Delphinine; C33 H45 N O9	24.42	599.3097	10521	Delphinine	C33 H45 N O9	C33 H45 N O9	C33 H45 N O9	-0.44	2
Cpd 74: Dipiperamide A; C34 H38 N2 O6	24.514	570.2754		Dipiperamide A	C34 H38 N2 O6	C34 H38 N2 O6	C34 H38 N2 O6	-4.3	4
Compound 75	24.548		11685						
Cpd 76: LysoPE(18:1(11Z)/0:0); C23	24.772	479.2955		LysoPE(18:1(11Z)/0:0)	C23 H46 N O7 P	C23 H46 N O7 P	C23 H46 N O7 P	11.83	3
Cpd 77: Delphinine; C33 H45 N O9	24.781	599.3147	11864	Delphinine	C33 H45 N O9	C33 H45 N O9	C33 H45 N O9	-8.72	2
Cpd 78: Arbekacin; C22 H44 N6 O10	24.783	552.3178	14445	Arbekacin	C22 H44 N6 O10	C22 H44 N6 O10	C22 H44 N6 O10	-10.67	2
Cpd 79: Dipiperamide A; C34 H38 N2 O6	24.871	570.2758	25415	Dipiperamide A	C34 H38 N2 O6	C34 H38 N2 O6	C34 H38 N2 O6	-4.65	4
Compound 80	24.94		10415						
Compound 81	24.963								
Cpd 82: Delcorine; C26 H41 N O7	25.041	479.295		Delcorine	C26 H41 N O7	C26 H41 N O7	C26 H41 N O7	-14.08	4
Compound 83	25.212		10199						
Cpd 84: Glycylseryiprolylmethionylphenylalanylvalinamide; C29 H45	25.345	635.3126		Glycylseryiprolylmethionylphenylalanylvalinamide	C29 H45 N7 O7 S	C29 H45 N7 O7 S	C29 H45 N7 O7 S	-3.9	1
Cpd 85: Aspartyl-Asparagine; C8 H13 N3 O6	25.393	247.0806		Aspartyl-Asparagine	C8 H13 N3 O6	C8 H13 N3 O6	C8 H13 N3 O6	-0.77	3

Cpd 86: LysoPE(18:1(11Z)/0:0); C23	25.429	479.2954		LysoPE(18:1(11Z)/0:0)	C23 H46 N O7 P	C23 H46 N O7 P	C23 H46 N O7 P	12.13	4
Compound 87	25.475								
Cpd 88: Isomigrestatin; C27 H39 N O7	25.529	489.2794		Isomigrestatin	C27 H39 N O7	C27 H39 N O7	C27 H39 N O7	-13.85	1
Cpd 89: Allosamidine; C25 H42 N4 O14	25.537	622.2703	12569	Allosamidine	C25 H42 N4 O14	C25 H42 N4 O14	C25 H42 N4 O14	-0.89	2
Cpd 90: Glycylseryiprolylmethionylphenylalanylvalinamide; C29 H45	25.61	635.3124		Glycylseryiprolylmethionylphenylalanylvalinamide	C29 H45 N7 O7 S	C29 H45 N7 O7 S	C29 H45 N7 O7 S	-3.57	1
Compound 91	25.659		17754						
Cpd 92: Isomigrestatin; C27 H39 N O7	25.773	489.2788		Isomigrestatin	C27 H39 N O7	C27 H39 N O7	C27 H39 N O7	-12.58	1
Cpd 93: LysoPE(18:1(11Z)/0:0); C23	25.779	479.2954		LysoPE(18:1(11Z)/0:0)	C23 H46 N O7 P	C23 H46 N O7 P	C23 H46 N O7 P	12.09	3
Compound 94	25.812								
Compound 95	25.907		12067						
Cpd 96: LysoPE(18:1(11Z)/0:0); C23	26.049	479.2951		LysoPE(18:1(11Z)/0:0)	C23 H46 N O7 P	C23 H46 N O7 P	C23 H46 N O7 P	12.7	4
Cpd 97: Cepharanthine; C37 H38 N2 O6	26.065	606.2743	173773	Cepharanthine	C37 H38 N2 O6	C37 H38 N2 O6	C37 H38 N2 O6	-2.2	3
Cpd 98: Americine; C31 H39 N5 O4	26.253	545.3031		Americine	C31 H39 N5 O4	C31 H39 N5 O4	C31 H39 N5 O4	-5.26	1
Cpd 99: Cepharanthine; C37 H38 N2 O6	26.438	606.273	19687	Cepharanthine	C37 H38 N2 O6	C37 H38 N2 O6	C37 H38 N2 O6	0.05	5
Cpd 100: Americine; C31 H39 N5 O4	26.627	545.3026	10123	Americine	C31 H39 N5 O4	C31 H39 N5 O4	C31 H39 N5 O4	-4.35	1

NEGATIVE ESI MODE

Qualitative Compound Report

Data File	TU-BMC-CD-17_.VE.d	**Sample Name**	TU-BMC-CD-17
Sample Type	Sample	**Position**	P1-A8
Instrument Name	QTOF	**User Name**	
Acq Method	metabolite_ESI_-VE_MSMS.m	**Acquired Time**	11/8/2024 4:27:15 AM
IRM Calibration Status	Success	**DA Method**	Default.m
Comment			

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

Compound Table

Compound Label	RT	Mass	Abund	Name	Formula	MFG Formula	DB Formula	DB Diff (ppm)	Hits (DB)
Cpd 16: Brompheniramine; C16 H19 Br N2	1.442	318.0731	340096	Brompheniramine	C16 H19 Br N2	C16 H19 Br N2	C16 H19 Br N2	0.33	10
Cpd 1: Quinic acid; C7 H12 O6	1.456	192.0633	333998	Quinic acid	C7 H12 O6	C7 H12 O6	C7 H12 O6	0.49	
Cpd 17: beta-D-Glc-(1->4)-alpha-L-Rha-(1->3)-beta-D-Glc; C18 H32 O15	1.486	488.1759		beta-D-Glc-(1->4)-alpha-L-Rha-(1->3)-beta-D-Glc	C18 H32 O15	C18 H32 O15	C18 H32 O15	-3.6	9
Cpd 18: Quinic acid; C7 H12 O6	1.533	192.0634		Quinic acid	C7 H12 O6	C7 H12 O6	C7 H12 O6	0.09	10
Cpd 19: 1-O-Caffeoyl-(b-D-glucose 6-O-sulfate); C15 H18 O12 S	1.538	422.0503		1-O-Caffeoyl-(b-D-glucose 6-O-sulfate)	C15 H18 O12 S	C15 H18 O12 S	C15 H18 O12 S	3.76	1
Cpd 20: Gallic acid; C7 H6 O5	4.4	170.0212	236998	Gallic acid	C7 H6 O5	C7 H6 O5	C7 H6 O5	2.07	4
Cpd 6: Gallic acid; C7 H6 O5	4.554	170.0212	340759	Gallic acid	C7 H6 O5	C7 H6 O5	C7 H6 O5	2.18	
Cpd 9: (-)-Catechin; C15 H14 O6	5.2	290.0786	742	(-)-Catechin	C15 H14 O6	C15 H14 O6	C15 H14 O6	1.37	
Cpd 21: 2-O-Galloylpunicalin; C41 H26 O26	5.684	934.073	14878	2-O-Galloylpunicalin	C41 H26 O26	C41 H26 O26	C41 H26 O26	-1.94	3
Cpd 22: Punicacortein B; C27 H22 O18	6.501	634.0826	39003	Punicacortein B	C27 H22 O18	C27 H22 O18	C27 H22 O18	-3.05	4
Cpd 23: 1-O-Galloylpedunculagin; C41 H28	6.624	936.0895		1-O-Galloylpedunculagin	C41 H28 O26	C41 H28 O26	C41 H28 O26	-2.82	3
Cpd 24: 1-O-Galloylpedunculagin; C41 H28	6.688	936.0887		1-O-Galloylpedunculagin	C41 H28 O26	C41 H28 O26	C41 H28 O26	-1.92	3
Cpd 8: Chlorogenic acid; C16 H18 O9	6.703	354.0941	1306	Chlorogenic acid	C16 H18 O9	C16 H18 O9	C16 H18 O9	2.82	
Cpd 7: Caffeic acid; C9 H8 O4	6.838	180.0418	2086	Caffeic acid	C9 H8 O4	C9 H8 O4	C9 H8 O4	2.32	
Cpd 11: (1R,6R)-6-Hydroxy-2-succinylcyclohexa-2,4-diene-1-carboxylate; C11 H12 O6	6.838	240.063	2384	(1R,6R)-6-Hydroxy-2-succinylcyclohexa-2,4-diene-1-carboxylate	C11 H12 O6	C11 H12 O6	C11 H12 O6	1.74	
Cpd 10: Ferulic acid; C10 H10 O4	6.838	194.0575	2384	Ferulic acid	C10 H10 O4	C10 H10 O4	C10 H10 O4	2.15	
Cpd 4: Genistein; C15 H10 O5	7.319	270.0529	10359	Genistein	C15 H10 O5	C15 H10 O5	C15 H10 O5	-0.14	
Cpd 14: Epigallocatechin gallate; C22 H18 O11	7.642	458.0851	736	Epigallocatechin gallate	C22 H18 O11	C22 H18 O11	C22 H18 O11	-0.33	
Cpd 25: Gossypetin 8-glucoside; C21 H20 O13	8.062	480.0911	19497	Gossypetin 8-glucoside	C21 H20 O13	C21 H20 O13	C21 H20 O13	-1.48	10
Cpd 26: 2-Protocatechoylphloroglucinoic arboxylate; C14 H10 O8	8.206	306.0375		2-Protocatechoylphloroglucinoic arboxylate	C14 H10 O8	C14 H10 O8	C14 H10 O8	0.14	10
Cpd 27: Myricetin 3-arabinoside; C20 H18 O12	8.335	450.0806	25963	Myricetin 3-arabinoside	C20 H18 O12	C20 H18 O12	C20 H18 O12	-1.65	3
Cpd 28: Quercetin 3-(2-galloylglucoside); C28 H24 O16	8.337	616.1081		Quercetin 3-(2-galloylglucoside)	C28 H24 O16	C28 H24 O16	C28 H24 O16	-2.66	2
Cpd 29: 3-(4-Chlorophenyl)-2H-1-benzopyran-2-one; C15 H9 Cl O2	8.613	256.0272	32665	3-(4-Chlorophenyl)-2H-1-benzopyran-2-one	C15 H9 Cl O2	C15 H9 Cl O2	C15 H9 Cl O2	7.31	1
Compound 30	8.667								
Cpd 31: Myricitrin; C21 H20 O12	8.681	464.0975		Myricitrin	C21 H20 O12	C21 H20 O12	C21 H20 O12	-4.31	10
Cpd 32: 8-Hydroxyluteolin 8-sulfate; C15 H10 O10 S	8.684	382.0002		8-Hydroxyluteolin 8-sulfate	C15 H10 O10 S	C15 H10 O10 S	C15 H10 O10 S	-1.93	2
Cpd 12: Ellagic acid; C14 H6 O8	8.693	302.0069	69080	Ellagic acid	C14 H6 O8	C14 H6 O8	C14 H6 O8	-1.96	
Cpd 33: Quercetin-4'-glucuronide; C21 H18 O13	8.71	478.0758		Quercetin-4'-glucuronide	C21 H18 O13	C21 H18 O13	C21 H18 O13	-2.13	8
Compound 34	8.748								
Cpd 35: Ellagic acid; C14 H6 O8	8.782	302.0073	182360	Ellagic acid	C14 H6 O8	C14 H6 O8	C14 H6 O8	-3.32	5
Cpd 36: Maclurin 3-C-(6''-p-hydroxybenzoyl-glucoside); C26 H24 O13	9	544.1235		Maclurin 3-C-(6''-p-hydroxybenzoyl-glucoside)	C26 H24 O13	C26 H24 O13	C26 H24 O13	-3.25	2
Cpd 37: 8-Hydroxyluteolin 8-sulfate; C15 H10 O10 S	9.021	381.9998		8-Hydroxyluteolin 8-sulfate	C15 H10 O10 S	C15 H10 O10 S	C15 H10 O10 S	-0.8	2
Cpd 38: Ceftibuten; C15 H14 N4 O6 S2	9.127	410.0405		Ceftibuten	C15 H14 N4 O6 S2	C15 H14 N4 O6 S2	C15 H14 N4 O6 S2	-12.19	1
Cpd 39: Ellagic acid; C14 H6 O8	9.147	302.0066	41647	Ellagic acid	C14 H6 O8	C14 H6 O8	C14 H6 O8	-1.04	4
Cpd 3: Luteolin 4'-O-glucoside; C21 H20 O11	9.353	448.1013	40237	Luteolin 4'-O-glucoside	C21 H20 O11	C21 H20 O11	C21 H20 O11	-1.57	
Cpd 2: Quercitrin; C21 H20 O11	9.353	448.1013	40237	Quercitrin	C21 H20 O11	C21 H20 O11	C21 H20 O11	-1.57	
Cpd 40: Luteolin 4'-O-glucoside; C21 H20 O11	9.382	448.1013		Luteolin 4'-O-glucoside	C21 H20 O11	C21 H20 O11	C21 H20 O11	-1.66	10

Cpd 41: (-)-Epigallocatechin 7-glucuronide; C21 H22 O13	9.507	482.1068		(-)-Epigallocatechin 7-glucuronide	C21 H22 O13	C21 H22 O13	C21 H22 O13	-1.68	10
Cpd 42: Bromadiolone; C30 H23 Br O4	9.797	526.078		Bromadiolone	C30 H23 Br O4	C30 H23 Br O4	C30 H23 Br O4	-0.01	1
Cpd 43: Glycyphyllin; C21 H24 O9	10.032	420.1428		Glycyphyllin	C21 H24 O9	C21 H24 O9	C21 H24 O9	-1.72	10
Cpd 44: Maclurin 3-C-(6"-p-hydroxybenzoyl-glucoside); C26 H24 O13	10.103	544.1228		Maclurin 3-C-(6"-p-hydroxybenzoyl-glucoside)	C26 H24 O13	C26 H24 O13	C26 H24 O13	-2.07	2
Cpd 45: 2",4",6"-Triacetylglycitin; C28 H28 O13	10.192	572.1538		2",4",6"-Triacetylglycitin	C28 H28 O13	C28 H28 O13	C28 H28 O13	-1.5	1
Cpd 46: Astringin; C20 H22 O9	10.421	406.1274		Astringin	C20 H22 O9	C20 H22 O9	C20 H22 O9	-2.5	10
Cpd 47: 2",4",6"-Triacetylglycitin; C28 H28 O13	10.654	572.1546		2",4",6"-Triacetylglycitin	C28 H28 O13	C28 H28 O13	C28 H28 O13	-2.86	1
Cpd 48: Piceatannol 4'-galloylglucoside; C27 H26 O13	10.765	558.1395		Piceatannol 4'-galloylglucoside	C27 H26 O13	C27 H26 O13	C27 H26 O13	-3.9	3
Cpd 49: Herbacetin; C15 H10 O7	11.167	302.0428	104623	Herbacetin	C15 H10 O7	C15 H10 O7	C15 H10 O7	-0.56	10
Cpd 50: 9,10-Dihydroxy-12,13-epoxyoctadecanoate; C18 H34 O5	12.573	330.2413	55475	9,10-Dihydroxy-12,13-epoxyoctadecanoate	C18 H34 O5	C18 H34 O5	C18 H34 O5	-2.08	8
Cpd 15: Garbanzol; C15 H12 O5	16.222	272.0681	5289	Garbanzol	C15 H12 O5	C15 H12 O5	C15 H12 O5	1.37	
Cpd 51: Flurogestone acetate; C23 H31 F O5	16.442	406.2158	84629	Flurogestone acetate	C23 H31 F O5	C23 H31 F O5	C23 H31 F O5	-0.61	4
Cpd 5: 9-HOTE; C18 H30 O3	17.392	294.2192	3895	9-HOTE	C18 H30 O3	C18 H30 O3	C18 H30 O3	1.13	
Cpd 52: Flurogestone acetate; C23 H31 F O5	17.537	406.2159	127695	Flurogestone acetate	C23 H31 F O5	C23 H31 F O5	C23 H31 F O5	-0.93	4
Cpd 53: Estradiol-17-phenylpropionate; C27 H32	17.757	404.2354	12536	Estradiol-17-phenylpropionate	C27 H32 O3	C27 H32 O3	C27 H32 O3	-0.7	4
Cpd 54: 2alpha-Hydroxypyracrenic acid; C39	17.989	634.3885	17037	2alpha-Hydroxypyracrenic acid	C39 H54 O7	C39 H54 O7	C39 H54 O7	-2.48	4
Cpd 55: 2alpha-Hydroxypyracrenic acid; C39	18.271	634.3887	35252	2alpha-Hydroxypyracrenic acid	C39 H54 O7	C39 H54 O7	C39 H54 O7	-2.83	4
Cpd 56: Azukisapogenol; C30 H48 O4	19.179	472.3563		Azukisapogenol	C30 H48 O4	C30 H48 O4	C30 H48 O4	-2.3	10
Cpd 57: Paramethasone acetate; C24 H31 F O6	19.244	434.2111		Paramethasone acetate	C24 H31 F O6	C24 H31 F O6	C24 H31 F O6	-1.55	8
Cpd 58: Descinolone acetonide; C24 H31 F O5	19.608	418.2147		Descinolone acetonide	C24 H31 F O5	C24 H31 F O5	C24 H31 F O5	2.02	6
Cpd 59: PIP(20:4(5Z,8Z,11Z,14Z)/16:0); C45 H80 O16 P2	20.022	938.4997		PIP(20:4(5Z,8Z,11Z,14Z)/16:0)	C45 H80 O16 P2	C45 H80 O16 P2	C45 H80 O16 P2	-8.06	7
Cpd 60: 2alpha-Hydroxypyracrenic acid; C39	20.116	634.3893		2alpha-Hydroxypyracrenic acid	C39 H54 O7	C39 H54 O7	C39 H54 O7	-3.71	4
Compound 61	20.172		12841						
Cpd 62: Caribenolide I; C33 H52 O11	20.202	624.3575		Caribenolide I	C33 H52 O11	C33 H52 O11	C33 H52 O11	-10.52	1
Cpd 63: 2alpha-Hydroxypyracrenic acid; C39	20.371	634.3898		2alpha-Hydroxypyracrenic acid	C39 H54 O7	C39 H54 O7	C39 H54 O7	-4.43	4
Cpd 64: PIP(20:4(5Z,8Z,11Z,14Z)/16:0); C45 H80 O16 P2	20.797	938.4997		PIP(20:4(5Z,8Z,11Z,14Z)/16:0)	C45 H80 O16 P2	C45 H80 O16 P2	C45 H80 O16 P2	-8	7
Cpd 65: 3-O-p-trans-Coumaroylalphitolic acid; C39 H54 O6	20.937	618.3942		3-O-p-trans-Coumaroylalphitolic acid	C39 H54 O6	C39 H54 O6	C39 H54 O6	-3.48	4
Cpd 66: 21-Fluoroprogesterone; C21 H29	21.018	332.2152		21-Fluoroprogesterone	C21 H29 F O2	C21 H29 F O2	C21 H29 F O2	-0.26	6
Cpd 67: PD 123319; C31 H32 N4 O3	21.209	508.2474	37561	PD 123319	C31 H32 N4 O3	C31 H32 N4 O3	C31 H32 N4 O3	0.07	9
Cpd 68: 3-O-p-trans-Coumaroylalphitolic acid; C39 H54 O6	21.293	618.395		3-O-p-trans-Coumaroylalphitolic acid	C39 H54 O6	C39 H54 O6	C39 H54 O6	-4.73	4
Compound 69	21.31								
Cpd 70: L-Urobilin; C33 H46 N4 O6	21.384	594.3474		L-Urobilin	C33 H46 N4 O6	C33 H46 N4 O6	C33 H46 N4 O6	-9.6	4
Cpd 71: LPA(18:2(9Z,12Z)/0:0); C21	21.486	434.2466		LPA(18:2(9Z,12Z)/0:0)	C21 H39 O7 P	C21 H39 O7 P	C21 H39 O7 P	-7.47	4
Cpd 13: Methoprene (S); C19 H34 O3	21.668	310.2506	1573	Methoprene (S)	C19 H34 O3	C19 H34 O3	C19 H34 O3	0.55	
Cpd 72: Palmidin B; C30 H22 O7	21.877	494.138		Palmidin B	C30 H22 O7	C30 H22 O7	C30 H22 O7	-2.99	9
Cpd 73: Palmidin B; C30 H22 O7	22.137	494.1383		Palmidin B	C30 H22 O7	C30 H22 O7	C30 H22 O7	-3.46	9
Cpd 74: 2-Dodecylbenzenesulfonic acid; C18 H30 O3 S	22.143	326.1919		2-Dodecylbenzenesulfonic acid	C18 H30 O3 S	C18 H30 O3 S	C18 H30 O3 S	-0.91	8
Compound 75	22.366								
Cpd 76: 2-Dodecylbenzenesulfonic acid; C18 H30 O3 S	22.505	326.1918		2-Dodecylbenzenesulfonic acid	C18 H30 O3 S	C18 H30 O3 S	C18 H30 O3 S	-0.82	8
Cpd 77: Anabaenopeptilide 90A; C46 H64 N8 O14	22.563	952.4424		Anabaenopeptilide 90A	C46 H64 N8 O14	C46 H64 N8 O14	C46 H64 N8 O14	12.43	1
Compound 78	22.738								
Cpd 79: MC-207,110; C25 H30 N6 O2	23.037	446.2447		MC-207,110	C25 H30 N6 O2	C25 H30 N6 O2	C25 H30 N6 O2	-3.76	2
Cpd 80: Mammeisin; C25 H26 O5	23.04	406.1795		Mammeisin	C25 H26 O5	C25 H26 O5	C25 H26 O5	-3.52	8
Cpd 81: Gradolide; C25 H34 O7	23.236	446.229	20644	Gradolide	C25 H34 O7	C25 H34 O7	C25 H34 O7	3.15	10
Cpd 82: Cincassiol B; C20 H32 O8	23.387	400.206	43668	Cincassiol B	C20 H32 O8	C20 H32 O8	C20 H32 O8	9.25	5
Cpd 83: Sufentanil; C22 H30 N2 O2 S	23.695	386.2011		Sufentanil	C22 H30 N2 O2 S	C22 H30 N2 O2 S	C22 H30 N2 O2 S	4.29	1
Cpd 84: MC-207,110; C25 H30 N6 O2	23.846	446.2322		MC-207,110	C25 H30 N6 O2	C25 H30 N6 O2	C25 H30 N6 O2	24.36	1

Cpd 85: MC-207,110; C25 H30 N6 O2	24.115	446.2468		MC-207,110	C25 H30 N6 O2	C25 H30 N6 O2	C25 H30 N6 O2	-8.55	2
Cpd 86: Blumenol C O-[apiosyl-(1->6)-glucoside]; C24 H40 O11	24.399	504.2557		Blumenol C O-[apiosyl-(1->6)-glucoside]	C24 H40 O11	C24 H40 O11	C24 H40 O11	2.65	6
Cpd 87: Kenposide B; C21 H36 O10	24.436	448.2272	432044	Kenposide B	C21 H36 O10	C21 H36 O10	C21 H36 O10	8.19	10
Cpd 88: Kenposide B; C21 H36 O10	24.79	448.2267	169189	Kenposide B	C21 H36 O10	C21 H36 O10	C21 H36 O10	9.22	10
Cpd 89: Sophoranone; C30 H36 O4	24.86	460.2622		Sophoranone	C30 H36 O4	C30 H36 O4	C30 H36 O4	-1.73	4
Cpd 90: Cinnzeylanine; C22 H34 O8	24.971	426.2205		Cinnzeylanine	C22 H34 O8	C22 H34 O8	C22 H34 O8	11.49	3
Cpd 91: Kenposide B; C21 H36 O10	25.138	448.227		Kenposide B	C21 H36 O10	C21 H36 O10	C21 H36 O10	8.48	10
Cpd 92: Flumethasone; C22 H28 F2 O5	25.19	410.1911		Flumethasone	C22 H28 F2 O5	C22 H28 F2 O5	C22 H28 F2 O5	-1.49	10
Cpd 93: Enterocin 900; C31 H33 N O2	25.453	451.2522		Enterocin 900	C31 H33 N O2	C31 H33 N O2	C31 H33 N O2	-2.29	2
Cpd 94: (4R,5S,7R,11x)-11,12 Dihydroxy-1(10)-spirovetiven-2-one 12-glucoside; C21 H34 O8	25.462	414.222		(4R,5S,7R,11x)-11,12-Dihydroxy-1(10)-spirovetiven-2-one 12-glucoside	C21 H34 O8	C21 H34 O8	C21 H34 O8	8.1	5
Cpd 95: Kenposide B; C21 H36 O10	25.501	448.227		Kenposide B	C21 H36 O10	C21 H36 O10	C21 H36 O10	8.66	10
Cpd 96: Cinnzeylanine; C22 H34 O8	25.556	426.2209		Cinnzeylanine	C22 H34 O8	C22 H34 O8	C22 H34 O8	10.48	3
Cpd 97: (4R,5S,7R,11x)-11,12 Dihydroxy-1(10)-spirovetiven-2-one 12-glucoside; C21 H34 O8	25.831	414.2218	694127	(4R,5S,7R,11x)-11,12-Dihydroxy-1(10)-spirovetiven-2-one 12-glucoside	C21 H34 O8	C21 H34 O8	C21 H34 O8	8.59	6
Cpd 98: Cinnzeylanine; C22 H34 O8	25.959	426.2208		Cinnzeylanine	C22 H34 O8	C22 H34 O8	C22 H34 O8	10.62	3

Cpd 99: Trigofoenoside A; C45 H74 O18	26.021	902.4784		Trigofoenoside A	C45 H74 O18	C45 H74 O18	C45 H74 O18	10.13	10
Cpd 100: Irbesartan; C25 H28 N6 O	26.206	428.237		Irbesartan	C25 H28 N6 O	C25 H28 N6 O	C25 H28 N6 O	-10.6	7

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राष्ट्रिय हर्बेरियम तथा वनस्पति प्रयोगशाला

गोदावरी, ललितपुर

पत्र संख्या: २०८१/०८२
च.नं. ७४

मिति २०८१/०५/२१
नेपाल सम्वत:११४४

विषय: नमूना पहिचान सम्वन्धमा।

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२०८१/५/२१

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. प्राप्त नमूनाको विवरण:- वनस्पतिको नमूना प्रजाति-१।

३. यस कार्यालयमा प्राप्त मिति:- २०८१/०५/२१

४. परिक्षणका आधारहरु:- (क) हर्बेरियममा भएका नमूनाहरु संगको तुलनात्मक अध्ययन
(ख) सन्दर्भ सामग्रीहरुको अध्ययन ।

५. पहिचान प्रतिवेदन:- प्राप्त नमूनाको Morphological अध्ययन तथा यस राष्ट्रिय हर्बेरियम तथा वनस्पति प्रयोगशालाको हर्बेरियममा राखिएका नमूनाहरु संगको तुलनात्मक अध्ययन गर्दा उक्त नमूना निम्नानुसार भएको पहिचान हुन गएको।

S.N	Scientific Name	Family	Remarks
1	*Psidium guajava* L.	Myrtaceae	

६. परिक्षण गर्ने अधिकारी:-

२०८१/५/२१
धन राज कँडेल
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भरतपुर, चितवन
Bharatpur, Chitwan

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Tribhuvan University

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