Chemical Investigation on Anti-inflammatory formulations used in Indigenous system of medicine

Thesis Submitted

to the University of Calicut in partial fulfilment of

the requirements for the award of the degree of

DOCTOR OF PHILOSOPHY IN CHEMISTRY

by

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DECLARATION

I, GIRIJA P.V hereby declare that the thesis entitled "Chemical Investigation on Anti-inflammatory formulations used in Indigenous system of Medicine "is an authentic record of the research work carried out by me under supervision and guidance of Dr. .K.K.VIJAYAN and Dr. RENUKA N.K., in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Chemistry of the University of the Calicut, and further that no part thereof has been presented before for any other degree of this or any other University.

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ACKNOWLEDGEMENT

This thesis would not have been possible without guidance and help of several individuals, who, in several possible ways contributed and extended their valuable assistance in completion of this study. It is a pleasure to thank all of them and though some of whom it is possible to give particular mention here.

First and foremost, my utmost gratitude to my supervisor Dr. K.K. Vijayan whose devotion to the work and continual efforts that made it a dream comes true. It cannot merely be expressed in words to thank him enough to pick me up as a Ph.D. student at the critical stage of my career and give right direction to it. I gained knowledge and skills whereas at the same time was bestowed by his excellent guidance and suggestions. I feel fortunate enough to work under his supervision. I offer my humble thanks and sincere gratitude to him for his valuable support, constructive criticism, patient hearing and motivational discussion during the course of my work. His enthusiasm, dedication, determination and integral view on research with an obligation for providing the best, has made a deep impression on me. He was always there to give emotional support, to remove my doubts, to encourage me, to motivate me whenever needed. I am indebted to him and consider it an honour to get an opportunity to work with him.

With due respect and immense gratitude, I acknowledge to my Co-supervisor, Dr Renuka N.K, for being as Co guide. I offer my humble thanks and sincere gratitude to her for her valuable support during the course of my work. She was always there to support me whenever needed.

I express my sincere thanks to former HOD of the chemistry department, Poovathinthodiyil Raveendran for allow me to re-registration

and enjoy the facility of the department. I thank present HOD of the chemistry department and all other teachers in the department of chemistry and nonteaching staff for their kind consideration and support. Many thanks to all research scholars and MSc students of the department of chemistry for their sincere support.

I remember all the teachers who taught me in my life and thanks to them. Their divine love has helped me. In this occasion I remember Dr. K.K. Krishnankutty, my M Phil guide, who has inspired me to join research, Dr Muhammed Shafi and all other teachers who taught me at chemistry department Calicut university.

I thank Dr Ramnath and Sri Sarath veitanary university Mannuthy for providing the facility to lyophilize the samples. I thank Care Keralam Koratty for providing the HPTLC result. I thank the principal KKTM Govt.College for allow me to do the anti-oxidant study at botany department.

I thank Dr Seena thomachan, former HOD of department of chemistry KKTM Govt.College,and all other teaching and nonteaching staff for various type of help provided to complete my work. I have enjoyed the facility of School of environmental sciences, MG university for the GC/MS, HPLC and LC MS/MS and QTOF MS result. I thank to, Smt. Shiny, Mr. Cyrus and Mr. Manu. Dr Pradnya A Jadhav helped me to get HRLCMS analysis from IIT Bombay. I thank her in this occasion with due respect.

I am blessed to have friends Dr. Dhanya, couldn't find words to express my feelings for her love, care and support they had devoted to me during my study. I also thank Dr. Nesy Department of Botany KKTM Government College Thrissur, for helping me to do the anti-oxidant study.

I also thank former and present colleagues at GWPTC Ernakulum for their support.

I would also like to take this opportunity to thank Director and section staff, DoR , Calicut University for reregistration of my PhD. I take this opportunity to thank Mr Rajesh for the effort taken to complete the binding work of my thesis in time.

I would like to thank my husband and children without their support I would have never ventured so far in academics and life.

My very special thanks to my father (Lt. Mr. Velayudhan), now who is not in this world but his divine blessings are always be there, creating a great force and faith in me. His blessings have always been a source of light and pillar of strength to me. I thank to my mother, sisters and brother for their divine blessings, affection and the Almighty who blessed me with strong will power, patience and confidence which helped me to complete the present work.

Girija P.V.

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LIST OF ABBREVIATIONS

WHO	:	World health organization
PHF	:	Poly herbal formulations
NSAIDs	:	Non-steroidal anti-inflammatory drugs
ROS	:	Reactive oxygen species
RNS	:	Reactive nitrogen species
HPTLC	:	High performance thin layer chromatography
MGP	:	Methanol extract of Gulguluthikthakam Kashayam residue
MGF	:	Methanol extract of Gulguluthikthakam Kashayam filtrate
MPP	:	Methanol extract of Prasarinyadi Kashayam residue
MPF	:	Methanol extract of Prasarinyadi Kashayam filtrate
TPC	:	Total phenolics content
TFC	:	Total flavonoids content
TAC	:	Total alkaloid content
TSC	:	Total steroid content
FRAP	:	Ferric ion reducing antioxidant power assay
DPPH	:	2, 2 diphenyl-1-picrylhydrazyl
STD	:	Standard
R_f	:	Retardation factor
MS	:	Mass spectroscopy
GCMS	:	Gas chromatography mass spectrometry
RT	:	Retention time
TIC	:	Total ion chromatogram
EI	:	Electron ionization
ESI	:	Electrospray ionization
HPLC	:	High performance liquid chromatography
LC-MS	:	Liquid chromatography mass spectrometry
HRMS	:	High resolution mass spectrometry
NP	:	Natural products
NPD	:	Natural product derivatives
APCI	:	Atmospheric pressure chemical ionization
CID	:	Collision-induced dissociation
QQQ	:	Triple quadrupole

TOF -MS	:	Time of flight mass spectrometry
UPLC	:	Ultra performance liquid chromatography
PLA2	:	Phospholipase A2
COX	:	Cyclooxygenase
PGs	:	Prostaglandins
UPLC-Q-ToF MS	:	Ultra performance liquid chromatography-quadrupole –time of flight mass spectrometry
CRF	:	Charge retention fragmentations
CMF	:	Charge migration fragmentations
RDA	:	Retro-Diels–Alder

Chapter 1
Introduction

1.1 Ayurveda

1.2 Polyherbal formulations (PHF)

1.3 Inflammation

1.4 Scope of the study

1.5 Aim of the study

Abstract

This chapter describes the popularity of polyherbal formulation in the treatment of inflammatory diseases compared to single herbal formulation.

1.1 Ayurveda

Ayurveda, is the indigenous therapeutic system of India and supposed to be of more than 5000 years heritage. This name is coined from two words, i.e., *Ayur* meaning *Life* and *Veda* meaning *Science,* the Science of Life [1]. According to the *Moola Sidhant*, Ayurveda defines three dynamic pathophysiological entities (Doshas), as the basis for all body function. The three Doshas are termed as Vata, Pitta, and Kapha. Kapha Dosha governs the nervous and musculo-skeletal systems [1,2,3]. At the cellular level, Vata Dosha can be associated with signaling pathways regulating cell growth, differentiation, and cell death. Vata Dosha also governs movements of cells, molecules, nutrients, and wastes [4,5]. The Pitta Dosha is responsible for transformative processes such as digestion, metabolism, energy production, and maintenance of immunity [1,2,3]. At the cellular level, Pitta Dosha can be associated with actions of enzymes, growth factors, hormones, and the reactions required for energy

homeostasis and maintenance of basal metabolism [4,5]. Kapha Dosha acts to form and maintain body mass, shape, and flexibility [1,2,3]. At the cellular level, anabolic processes (such as biosynthesis of macromolecules) and coordination of gene and protein function may be associated with Kapha Dosha [4,5,6].

In Ayurveda, one's basic "body constitution" is termed as "Prakriti." Prakriti arises due to a unique combination of fixed amounts of the three Doshas at the time of conception. Although one's Prakriti (genotype) is fixed, one's Doshas are in dynamic equilibrium, and optimal function of each Dosha and normal interactions between Doshas are essential for good health. Accordingly, individuals with "balanced" Doshas (Sama Prakriti) are less susceptible to disease than individuals with abnormal Doshas. For example, inflammatory diseases are associated with vitiation of Pitta Dosha [7]. A specific illness manifests when the vitiated Dosha(s) interact with weaknesses in specific organs (Dhatus). Conversely, pathogenic factors can also trigger abnormality of the Doshas and weaken the Dhatus [7].

Ayurveda involves the use of natural elements to eliminate the root cause of diseases by restoring balance. Herbal medicines have existed worldwide with a long-recorded history and they were used in ancient Chinese, Greek, Egyptian, and Indian systems of medicines for various therapeutic purposes. WHO has estimated that about 80% of the world's inhabitants still rely mainly on traditional medicines for their health care [8].

In India more than 15000 medicinal plants have been recorded, and about 50-55% of these are used for curing various ailments in the different indigenous system and folk medicine. When combining multiple

herbs, these preparations were found to give a better therapeutic effect and reduce toxicity [9].

Approximately 90% Ayurvedic preparations are based on poly herbal formulations [10]. Ayurvedic plants have a stronger action on the body than either food or spices. Such actions enable the plants to reverse patho-physiological processes and stabilize the doshas. Ayurvedic medicines prepared from the herbs are said to have no side effects when prepared as per the established Ayurveda Texts and used appropriately [11]. Herb possesses infinite potencies (saktis) and works wonders. Public interest for the treatment with complementary and alternative medicine is mainly due to increased side effects in synthetic drugs, lack of curative treatment for several chronic diseases, high cost of new drugs, etc. Ayurvedic medicines exist in different formats, including decoctions, powders, pastes, fermented products, tablets, and medicinal butters [12].

The realization that most chronic diseases are multi -genic and hence, a multi- targeted approach is required to ameliorate/cure the condition. Holistic treatment is the hallmark of Ayurveda therapy. Hence, it demands that one herb or one drug alone would not cure the imbalance of Dosha. So, in most of the cases, a combination of medicinal plants is recommended for treatment, a type of multitargeted or combinatorial approach. Unlike the Allopathic system which uses mainly synthetic chemicals designed for specific target receptors that primarily give symptomatic relief, Ayurveda involves the use of natural products to eliminate the root cause of diseases by restoring balance.

Recent investigations utilizing modern scientific protocols, have identified many pharmacologically active ingredients of the Ayurvedic medicines as well as their usefulness in drug therapy [13]. Basically, it is the phytochemical constituent in the medicinal plants which lead to the

desired healing effect. These may be any one of the following constituents or a combination of them such as alkaloids, flavonoids, terpenoids, steroids, saponins and terpenoids, lactones and phenolics. A single herb may even contain more than one of the aforementioned phytochemical constituents, which works synergistically with each other in producing pharmacological action. Even though the active phytochemical constituents of individual plants have been well established, they usually present in minute amounts, and hence are insufficient to achieve the desirable therapeutic effects. However, scientific studies have revealed that these plants of varying potency when combined may theoretically produce a greater result, as compared to individual use of the plant and also the sum of their individual effect. This phenomenon of positive herb-herb interaction may be considered as synergism. Certain pharmacological actions of active constituents of herbals are significant only when potentiated by that of other plants. These formulations have plant-based pharmacological agents which may exert synergistic, potentiate, agonistic and /or antagonistic actions by virtue of its associated diverse active principles. This pharmacological principle works together in a dynamic way to produce maximum therapeutic efficacy with minimum side effect [14]. Based on the nature of the interaction, there are two mechanisms on how synergism acts [15]. In one, the pharmacokinetic synergism, the ability of herbs to facilitate the absorption, distribution, metabolism and elimination of the other herbs is focused. In the second, Pharmacodynamic synergism the effect of the active constituents with similar therapeutic activity targeted to a similar receptor or physiological system. Apart from this, it is considered that multiple factors and interactions cause diseases in most of the cases, leading to both visible and invisible symptoms. Here, a combination of herbs may act on multiple targets at the same time to provide a thorough relief [16].

When synergism is considered, poly herbal formulations offer some benefits not available with single herb preparations. It is evident that better therapeutic effect can be reached with multi-constituent formulation. For this, a lower dose of the herbal preparation may be needed to achieve desirable pharmacological action, thus reducing the risk of deleterious side-effects. These benefits have resulted in the popularity of Poly herbal formulations (PHF) in the market when compared to single herbal formulations.

1.2 Polyherbal formulations

In Ayurveda, most of the classical preparations are polyherbal, with a combination of 3 to 30 different plants. Ayurvedic medical books appeared by the 8th century BCE include procedural instructions for surgeries and herbal remedies as well as the history of how Ayurveda evolved. The *Charak Samhita*, *Sushurta Samhita* and *Ashtanga Hridaya* form the triad of classical texts called the *Brhattrayi*. These books detail the basic theories of Ayurveda. The Ayurvedic literature "Sarangdhar Samhita " dated centuries ago in 1300 A.D. has highlighted the concept of poly herbalism in this ancient medicinal system and poly herbalism confers some benefits not available in single herbal formulation [17]. Therapeutic effect of herbal medicines is exerted due to the presence of different phytoconstituents and the effects are further potentiated when compatible herbals are formulated together in PHFs. This claim is based on reports of research that have been done on PHF to evaluate their effectiveness and these are published in international journals [18,19.20].

1.3 Inflammation

Inflammation is a defense mechanism in the body. It is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or irritants, and is a protective response

involving immune cells, blood vessels, and molecular mediators. The function of inflammation is to eliminate the initial cause of cell injury, clear out necrotic cells and tissues damaged from the original insult and the inflammatory process, and initiate tissue repair [21].

Inflammation can be classified as either acute or chronic [22]. Acute inflammation is the initial response of the body to harmful stimuli and is achieved by the increased movement of plasma and leukocytes (especially granulocytes) from the blood into the injured tissues. A series of biochemical events propagates and matures the inflammatory response, involving the local vascular system, the immune system, and various cells within the injured tissue. Acute inflammation is a short-term process, usually appearing within a few minutes or hours and begins to cease upon the removal of the injurious stimulus. Acute inflammation occurs after a cut on the knee, a sprained ankle or a sore throat. It's a short-term response with localized effects, meaning it works at the precise place where a problem exists. In the case of acute inflammation, blood vessels dilate, blood flow increases and white blood cells swarm the injured area to promote healing, said Dr. Scott Walker, a family practice physician at Gunnison Valley Hospital in Utah. This response is what causes the injured area to turn red and become swollen. Prolonged inflammation, known as chronic inflammation, leads to a progressive shift in the type of cells present at the site of inflammation, such as mononuclear cells, and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process.

Unlike acute inflammation, chronic inflammation or silent inflammation can have long-term and whole-body effects. Chronic inflammation is also called persistent, low-grade inflammation because it produces a steady, low-level of inflammation throughout the body. In

many ways, this condition can be considered "silent pain" in that the immune system continues to attack at the cellular level but without the perception of pain. Like free radicals in the human system, the existence of silent inflammation is not a disease, but only an indication that a potential medical problem lingers. Silent inflammation has been linked to depression, cancer, heart disease, diabetes, psoriasis, allergy, atherosclerosis, obesity, asthma, neurodegenerative diseases, such as Alzheimer's disease and stroke. Currently, there are no prescription drugs that specifically target chronic inflammation, according to an article published in the Johns Hopkins Health Review.

So, inflammation needs to be well managed. Nowadays it is managed by non-steroidal anti-inflammatory drugs (NSAIDs), Steroids and Supplements. But long-term use of steroids can lead to vision problems, high blood pressure, and osteoporosis. Long-term use of NSAIDs increased risk of several conditions, including peptic ulcer disease and kidney disease [23]. Hence, for the proper management of inflammatory conditions that form the root causes of many chronic disease's drugs free of such side effects are required. Many recent investigations have established the complex interactions between various immunomodulatory cytokines, and different signaling pathways in chronic inflammatory conditions. Monocytes and macrophages contribute to chronic inflammatory conditions by producing higher amounts of prostaglandins, superoxide anions, and lysosomal enzymes leading to tissue damage. Hence, in the management of chronic inflammatory conditions a multi molecular formulation that may produce multi targeted therapeutic intervention. This idea forms the basis for the present study to investigate the two polyherbal Ayurveda formulations that have been used to treat many inflammatory diseases. Free radicals are generated inside

our body during metabolic activity. Our body produces anti - oxidants to nullify the free radicals. But in certain circumstances there occurs an imbalance in the free radicals and antioxidants ratio. This leads to inflammatory diseases such cardiovascular diseases, cancers [24], neurodegenerative diseases, Alzheimer's disease [25] and etc.

1.4 Scope of the study

- Unpleasant side effects associated with prolonged use of current anti-inflammatory drugs have necessitated a need for new drugs with no side effects.
- Natural antioxidants in medicinal herbs have received attention over time, since they do not possess the side effects associated with their synthetic counterparts.
- Ayurvedic formulations/preparations have been prescribed as anti-inflammatory medicines since immemorial.
- Analysis and identification of molecular entities responsible for the prescribed action of Ayurveda formulations is not fully studied.

1.5 AIM of the study

- Phytochemical evaluation of Anti-inflammatory Ayurveda formulations, Prasarinyadi kashayam and Gulguluthikthakam kashayam.
- Qualitative Characterization of molecules by employing modern scientific tools.
- Characterization will be carried out employing HPTLC, LC/MS, HRLCMS and UPLC-QTOF- MS.

Gulguluthikthakam Kashayam is mainly used for inflammatory conditions so it is good for osteoarthritis, skin diseases and it is an excellent medicine for all types of pain.

Gulguluthikthakam Kashayam is prepared from 29 plants as per Ashtanga Hridayam. "Ashtanga Hridayam", a compendium of the Ayurvedic System by Vagbhata considered as “Heart or Essence of all the Eight Branches of Ayurveda” is one of the primary ancient root texts of Ayurveda. The Ashtanga Hridayam continues to serve as a root source for Ayurvedic philosophy and protocol, providing clear guidelines in all aspects of health.

The plants used for the Gulguluthikthakam Kashayam are *Holarrhena antidysenterica* (Roth)DC. (Seed), *Piper longum* L. (Root), *Piper nigrum* L., *Piper brachystachyum* Wall.ex Hook.f. (Root), *Picrorhiza kurroa* Royle ex Benth. (Root), *Plumbago zeylanica* L. (Rhizome), *Rubia cordifolia* L. (Stem), *Curcuma longa* L.(Rhizome), *Cyperus rotundus* L. (Rhizome), *Cyclea peltata* (Lam.) Hook.f.&Thoms (Tube root), *Commiphora mukul* (Hook.ex Stocks) Engl. (Resin), *Celastrus paniculatus* Willd (Seed), *Cuminum cyminum* L. (Fruit), *Cedrus deodara* (Roxb.ex D.Don)G.Don (Wood), *Embelia ribes* Burm.f.(Fruit), *Adhatoda vasica* Nees (Root), *Azadirachta indica* (L.)A.Juss.(Stem), *Alpinia calcarata* Roscoe (Rhizome), *Acorus calamus* L.(Rhizome), *Aconitum heterophyllum* Wall.ex Royle(Tube root), *Anethum graveolens* L.(Fruit), *Scindapsus officinalis*(Roxb.)Schott (fruit), *Saussurea lappa* (Decne)Sch-Bip. (Root), *Semecarpus anacardium* L.f. (Seed), *Solanum indicum* L. (Root), *Zingiber officinale* Rosc. (Rhizome), *Trichosanthes dioica* Roxb. (Whole Plant), *Tinospora cordifolia* (Willd) Miers ex Hook.f.&Thoms (Stem), *Trachyspermum roxburghianum* (DC.) Wolff (Fruit).

Prasarinyadi Kashayam is mainly used for shoulder related problems. This formulation is prepared as per Sahasrayogam, the Ayurveda Text, using 6 medicinal plants. Sahasrayogam, is an important classic compiled by Kerala tradition of Ayurveda practitioners. This is a compendium of more than thousand Ayurveda medicinal preparations involving all Ayurveda systems procedures. *Allium sativum* L. (Bulb), *Alpinia calcarata* Roscoe (Rhizome), *Merremia tridentata* (L.) Hallier f. (Whole plant), *Sida rhombifolia* L. (Root), *Vigna mungo* (L.) Hepper (Seed) and *Zingiber officinale* Rosc. (Rhizome) are the plants used in the preparation of Prasarinyadi Kashayam.

Gulguluthikthakam Kashayam and Prasarinyadi Kashayam have been procured from the market prepared by Ayurveda Institute, The Arya Vaidya pharmacy, Coimbatore.

The present study on Ayurvedic formulation consisted of seven chapters.

Chapter 1 describes the importance of Ayurveda in the management of inflammatory diseases. Scope and aim of the study also included in this chapter.

Chapter 2 includes the list of work carried out in the Ayurvedic formulations. Reported pharmacology and chemical constituents of the plants taken for the preparation of the Prasarinyadi kashayam and Gulguluthikthakam kashayam also given in this chapter.

Chapter 3 includes anti-oxidant study and HPTLC study of methanol extract of Gulguluthikthakam kashayam and Prasarinyadi kashayam.

Chapter 4 is based on the GCMS study of volatile principles separated from Prasarinyadi Kashayam and Gulguluthikthakam Kashayam.

Chapter 5 and 6 are based on the LCMS study of Prasarinyadi Kashayam and Gulguluthikthakam kashayam respectively.

Chapter 7 gives a brief overview of the present study, important findings and scope for the research in future.

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Chapter 2
Review of Literature

2.1. Reported studies on Ayurveda formulations.

2.2. Pharmacology of the plants used in the preparation of Prasarinyadi kashayam and Gulguluthikthakam kashayam.

2.3 Reported chemical constituents of the plants used in the preparation of Prasarinyadi kashayam and Gulguluthikthakam kashayam.

Abstract

Analysis and identification of molecular entities responsible for the prescribed treatment of the Ayurveda formulations is not fully studied. There are few studies available. They are enlisted in this chapter. Pharmacology and the chemical constituents of the plants used in the preparation of kashayam also included

Analysis and identification of molecular entities responsible for the prescribed treatment of the Ayurveda formulations is not fully studied. There are few studies available. They are enlisted in table 2.1.

2.1 Reported studies on Ayurvedic formulations

Table 2.1. Studies on Ayurveda formulations

SI No.	*Title*	*Reference*
1.	The gas chromatography-mass spectrometry study of one Ayurvedic formulation avipathi churnam.	Kotteswari.M et al 2020 [1]
2.	The GC–MS study of one ayurvedic formulation tiktaka Ghrita.	Prabhu K et al 2020 [2]
3.	Validation and quantification of major biomarkers in Mhasudarshan Churna-an ayurvedic polyherbal formulation through high performance thin layer chromatography.	Prabhjot Kaur 2020 [3]
4.	Chromatographic Analysis of Kutajarista—an Ayurvedic Polyherbal Formulation.	Shivali Garg and Bhutani K.K 2008 [4]
5.	Chemical profiling of a polyherbal formulation by tandem mass spectroscopic analysis with multiple ionization techniques	Sulaiman C. T et al 2020 [5]

6.	Chromatographic and Tandem mass spectroscopic characterization of Ayush Kwath Churnam-an Ayurvedic immunomodulatory drug during COVID-19.	Madhavankutty M et al 2020 [6]
7.	Phytochemical analysis and cellular uptake study of an Ayurvedic formulation, Vyaghryadi Kashayam used in the clinical management of COVID-19.	Nandakumar G et al 2021[7]
8.	Chemical Profiling of an Indian Herbal Formula Using Liquid Chromatography Coupled with ElectroSpray Ionization Mass Spectrometry.	Sulaiman C. T. and Indira Balachandran 2014 [8]
9.	Liquid chromatography mass spectrometry analysis and phytochemical screening of trimad: an ayurvedic polyherbal formulation.	Sureh P Khadke and Aniket A Kuvalekar 2018[9]
10.	Evaluation of polyherbal Ayurvedic formulation 'Peedantak Vati' for anti-inflammatory and analgesic properties.	Acharya Balkrishna 2019[10]
11.	Phytochemical standardization of panchavalkala: An ayurvedic formulation and evaluation of its anticancer activity in cervical cancer cell lines.	Aphale S et al 2018 [11]
12.	Standardization of Hinguvacadi Churna - An Ayurvedic Formulation and Identification of Piperine as a marker compound	Gayathri Devi et al 2010 [12]
13.	Validated high performance thin layer chromatography method for the quantification of bioactive marker compounds in Draksharishta, an ayurvedic polyherbal formulation.	Divya Pillai et al 2016 [13]
14.	Standardization of Ayurvedic Formulation (Marichyadi Vati) Using HPLC and HPTLC Methods	Pathan Imran Khan et al 2014[14]
15.	Coronil, a Tri- Herbal Formulation, Attenuates Spike-Protein-Mediated SARS-CoV-2 Viral Entry into Human Alveolar Epithelial Cells and ProInflammatory Cytokines Production by Inhibiting Spike Protein-ACE-2 Interaction	Acharya Balkrishna 2021[15]
16.	HPTLC method for routine quality control of Ayurvedic formulation Drakshadi gutika.	Jain V et al 2013 [16]
17.	GC MS analysis of one Ayurvedic formulation "Nasika churnam".	Sharmila D et al 2021 [17]
18.	The GC MS analysis of one ayurvedic medicine Aragwadharishtam	Hassan Mohammad et al 2019 [18]
19.	The GC MS study of one Ayurvedic medicine. Vasakadyaristam	Kumar et al 2019 [19]
20.	Anti-oxidant Studies of One Ayurvedic Medicine Aswagandharishtam.	Kotteswari M et al 2018 [20]
21.	GC-MS Analysis of an Ayurvedic medicine "Modified Arjunarishta".	Santhosh kumar B et al 2017 [21]
22.	Estimation of total alkaloid in Chitrakadivati by UV-Spectrophotometer	Manjunath Ajanal et al 2012 [22]

23.	Gas chromatography – Mass spectrometric analysis of Haritakyadi eye drops: A poly herbal compound for ophthalmia neonatorum.	Srikantha K V et al 2019 [23]
24.	GC-MS Analysis and Anti-Oxidant Activity of Manasamitra Vatakam: A Herbomineral Formulation.	Srikalyani V et al 2020 [24]
25.	Chromatographic Fingerprinting of Sarasvata Churna–an Ayurvedic Polyherbal Formulation for Epilepsy.	Rahul Kaushik et al 2018 [25]
26.	GC MS Analysis of One Ayurvedic Medicine Sahacharadi Kashayam.	Praveen Kumar et al 2018 [26]
27.	The GC-MS Study of One Ayurvedic Preparation Katakakhadiradi Kashayam.	Angeline Jessica et al 2016 [27]
28.	GC MS analysis of one Ayurvedic polyherbal formulation "Patoladi kwatha churnam" for psoriasis.	Senthamil Kavitha M et al 2021 [28]
29.	Solid phase extraction of Ayurvedic lipid based formulation gritas and analysis of its contents by high performance thin layer chromatography and gas chromatography mass spectrometry.	Selvakumar D& Vijaya S 2015[29]
30.	Analegesic Effects of Dashmula, an Ayurvedic Preparation, versus Diclofenac Sodium in Animal models	Ravi Shekar Singh et al 2012 [30]
31.	Antioxidant Properties of the Ayurvedic Formulation Thriphala and its constituents	Vani T et al 1997 [31]
32.	Pharmacognostical and physico-chemical analysis of Pathyadi Varti-A polyherbal Ayurvedic formulation	Varun B Gupta et al 2012 [32]

2.2. Pharmacology of the plants used in the preparation of Prasarinyadi kashayam and Gulguluthikthakam kashayam.

Plants taken for the preparation of both the kashayam have different pharmacology. Reported pharmacology of each plant is given below.

The pharmacological studies revealed that *Cuminum cyminum* L. exerted antimicrobial, insecticidal, anti-inflammatory, analgesic, antioxidant, antidiabetic, antiplatelet aggregation, hypotensive, bronchodilatory, immunological, contraceptive, anti-amyloidogenic, anti-

osteoporotic, aldose reductase, alpha-glucosidase and tyrosinase inhibitory effects, protective and central nervous effects [33,34].

Celastrus paniculatus Willd seed have emollient, stimulant, laxative, emetic and expectorant properties. They have also been used as an anti-inflammatory, anti-asthmatic, antileprotic and useful in abdominal disorders, beri-beri and sores [35].

Celastrus paniculatus Willd reported activities are antiviral, anti-bacterial, insecticidal, anti-inflammatory, anti-spermatogenic, sedative, anti-fatigue and analgesic, hypolipidemic. It is arthrogenic, anti-rheumatic, aphrodisiac, emetic, laxative, nervine tonic [36].

Scindapsus officinalis (Roxb.) Schott fruit is reported to have anti-diarrheal, hypoglycemic, aphrodisiac, expectorant, antirheumatic, diaphoretic, carminative, anthelmintic, anti-inflammatory and analgesic activity and has significant application in treating bronchitis and helminthiasis [37].

Picrorhiza kurroa Royle ex Benth. root is anti-inflammatory. It is used in traditional and modern medicines for liver disorders, fever, asthma, and jaundice. The plant is reported to possess hepatoprotective principles such as iridoid glycosides and antioxidants [38,39].

The roots of *Saussurea lappa* (Decne)Sch-Bip. have been found to exhibit anti-inflammatory, hepatoprotective, anti-ulcer, anti-cancer, immunomodulatory and pesticidal activities [40].

Piper nigrum L.is reported to have biological properties such as anti-hyper -tensive, anti-asthmatic, anti-microbial, anti-oxidant, anti-cancer, antidiarrheal, anti-depressant, anticonvulsant, analgesic, immunomodulatory, hepatoprotective and digestive activity [41].

Rubia cordifolia L. stem exhibit different biological properties such as antimicrobial, anticancer, hypoglycemic, haemostatic, antipyretic, analgesic, anthelmintic, anti-inflammatory. It is also used in purifying blood, diuretic, in liver complaints, in pains of joints and uterine pains [42].

Reported biological activity of *Cyperus rotundus* L. are anti-inflammatory, antipyretic, hypotensive and used for the treatment of dysmenorrhea, stomach disorder and menstrual irregularities [43].

Curcuma longa L. has been attributed numerous pharmacological activities including antioxidant, anti-cancer, antimicrobial, anti-inflammatory antibiotic antibacterial, antifungal [44].

Adhatoda vasica Nees is a well-known plant in Ayurveda and Unani medicine. It has been used for the treatment of various disease particularly for the treatment of inflammatory and cardiovascular diseases. It also shows anti-oxidant, anti-ulcer, antimicrobial, antithrombotic, anti-mutagenic, antifungal, antibacterial, hypoglycaemic and immunomodulatory activity [45,46].

Tinospora cordifolia (Willd) Miers ex Hook.f.&Thoms is an important medicinal plant which is used for its anti-inflammatory, immunomodulatory, antidiabetic and antiallergic properties [47].

Solanum indicum L.has many medicinal values, in the treatment of hypertension, urinary tract infection, as an immune booster, diabetes mellitus, enhancement of kidney function [48].

All the parts of *Azadirachta indica* (L.)A.Juss. have been used traditionally for the treatment of inflammation, infection, fever, skin diseases and dental disorders [49].

Reported biological properties of *Semecarpus anacardium* L.f. are anti-inflammatory, anti-cancer, antiarthritic, anti-oxidant, hypolipidemic, hypoglycemic, anti-atherogenic, antifungal, antifertility and neuroprotective [50,51].

Commiphora mukul (Hook.ex Stocks) Engl .is extensively used for the treatment of different ailments including dysmenorrhea, endometritis, hypercholesterolemia, hypertension, bronchitis, inflammation, arthritis, cancer and cardiovascular disorders [52].

Plumbago zeylanica L.has antiplasmodial, antimicrobial, antifungal, anti-inflammatory anti-hyperglycemic, antiatherosclerotic, antidiarrheal, antiallergic, antidiabitic, hepatoprotective and hypoli pidaemic property [53].

Piper brachystachyum Wall.ex Hook.f. roots are useful in asthma, bronchitis, dyspepsia, anorexia vitiated conditions kapha and pitta [54].

Embelia ribes Burm.f. is a highly valuable medicinal plant with anthelmintic, carminative, antibacterial, antibiotic, hypoglycaemic and antifertility properties [55].

Acorus calamus L .has several medicinal properties, which is used in the treatment of insomnia, melancholia, neurosis, remittent fevers, delirium and hysteria. It is the important ingredient of various Unani and Ayurvedic preparations prescribed for headache, migraine, body ache and severe inflammatory pain [56,57].

Trichosanthes dioica Roxb. exhibit hepatoprotective activity, wound healing activity, antioxidant activity, antibacterial activity, anti-inflammatory activity, cholesterol-lowering activity and antidiabetic activity [58].

Zingiber officinale Rosc. rhizome is used traditionally to treat the common cold, headache, muscular and rheumatic disorders. Reported pharmacological activities on ginger include antimicrobial, antioxidant, anti-inflammatory, hepatoprotective and antinociceptive [59].

Alpinia calcarata Roscoe is the major part of indigenous medicinal formulation for the treatment of respiratory ailments, arthritis, diabetics, indigestion, impurities of the blood, throat inflammation, voice improvement and to marinate youthful vigor. Research reveals its antibacterial, antioxidant, anti-inflammatory, gastro protective, antifungal, aphrodisiac and antinociceptive [60].

Aconitum heterophyllum Wall.ex Royle is used in curing hysteria, throat infection, dyspepsia, abdominal pain, diabetes and is considered as a valuable febrifuge, nervine tonic. Pharmacological evaluations on the plant include antipyretic, analgesic, antifungal, antimicrobial, insecticidal, brime shrimp cytotoxic, antiviral, hypolipidemic, ant-diabetic and immunostimulant activities and is used to treat diseases of nervous system, digestive system, rheumatism and fever [61].

Holarrhena antidysenterica (Roth)DC is antidiabetic, anti-plasmodial, antidiarrheal, antibacterial, anti-urolithic, antioxidant, anti-hyperglycemic and anti-hyperlipidemic [62].

Anethum graveolens L. is antimicrobial, antifungal, antioxidant, insecticidal, anti –inflammatory, antispasmodic, anti-diabetic, anti-cancer and anti-hyperchole- sterolaemic [63].

Cyclea peltata (Lam.) Hook.f.&Thoms is anti-inflammatory, analgesic, anti-hemorrhagic, gastro protective, antioxidant and cardio protective. It is used in the diseases like fever, diarrhea,

pruritus, dermatoses, worms, asthma, tumors, heart diseases and wounds [64,65].

Trachyspermum roxburghianum (DC.) Wolff is antioxidant, antibacterial, anti-mutagenic and antimicrobial, Insecticidal, Antifungal activity, hepato-protective activity, analgesic effects, anti-inflammatory effects [66,67].

Piper longum L. is insecticidal and acaricidal, anti-inflammatory, Immunomodulatory, anti-cancer, antifungal, antiamoebic activity, antiasthmatic, anti-microbial, antidiabetic, hypercholesterolaemia, antioxidant and analgesic [68].

Merremia tridentata (L.) Hallier f. have strong antioxidant, anti-inflammatory, gastro protective and anti-arthritic effects. In the traditional system of medicine, it is used for various ailments such as piles, swelling, ulcers, affections, and stiffness of the joins, hemiplegia, and urinary infection [69,70,71].

Allium sativum L., number of biological activities are reported for the plant some of them are, anticancer, antioxidant, anti-inflammation, lipid lowering, antithrombotic, antihyper cholesterolemia and reduce cardiovascular diseases [72][73].

Vigna mungo (L.) Hepper seeds are used in inflammatory, rheumatism, affections of the nervous system, diseases of the liver [74].

Sida rhombifolia L. is anti-rheumatic, antipyretic, analgesic, anti-asthmatic, laxative, demulcent, diuretic, appetite stimulant, anti-inflammatory, anti-oxidant, antibacterial, hepatoprotective, vasorelaxant and hypotensive [75].

The wood of the *Cedrus deodara*(Roxb.ex D.Don)G.Don is anti-inflammatory, analgesic, immunomodulatory, antispasmodic, anticancer antihyperglycemic, anti-apoptotic, antibacterial, anti -sarcoptic, anticonvulsant, insecticidal, anxiolytic and molluscicide [76].

2.3. Reported chemical constituents of the plants used in the preparation of Prasarinyadi kashayam and Gulguluthikthakam kashayam.

All the plants used in the preparation of both the kashayam and compounds identified. They are given below.

Anethum graveolens L. (Fruit)

Major compounds isolated from the plants are Dihydrobezofuran, 2, 3-dihydro-7- methoxy-2-methyl-5, 6-methylene dioxybenzofuran, d-caravone, isodillapiole from oil, anethofuran, α and β-phellandrene from flower and seed, dillether, 4-viyl-2-methoxyphenol, 4-hydroxy-3-methyl-6-(1-methylethyl) cyclohex-2-en-1-one and nonacosa-10-one from dill flower. Piperitone, 8-dehydro-p-cymene and linalylacetate, limonene (33%), α-phellandrene (20.61%), including pinene, diterpene, dihydrocarvone, carvacrol, methylbenzoate, 1, 5- cineole, p-cymene, safrole, α-terpinene, α-pinene, cineole, myrcene, para -myrcene, dillapiole (26%), isomyristicin, myristicin, myristin, apiol and dillapiol. carvone- $C_{10}H_{14}O$ (30 – 60%) [77], Dillanoside $C_{24}H_{22}O_{10}$. Graveolone $C_{14}H_{12}O_4$**,** *Dillapional*- $C_{12}H_{12}O_5$[2]. Furanocoumarin: - 5-(4"-hydroxy-3" methyl-2"- butenyloxy)-6, 7-furanocoumarin, oxypeucedanin, oxypeucedanin hydrate and falcarindiol. Alkaloid: -Piperine, Sterols: -β-sitosterol and its glycosides from seeds [78].

Aconitum heterophyllum Wall. ex Royle. (Tubroot)

Alkaloid: - atisine, $C_{22}H_{33}NO_2$ (0.4%) and hetisine $C_{20}H_{27}O_{35}N$, benzoyl –heteratisine, heterophyllidine $C_{21}H_{31}NO_5$, heterophylline

($C_{21}H_{31}NO_4$), atidine, histisine, hetidine, heterophyllisine $C_{22}H_{33}NO_4$ 178-l 79^0, heteratisine $C_{22}H_{33}NO_5$, isoatisine $C_{22}H_{33}NO_2$, dihydroatisine, hetisinone, diterpenoid .lactone: -Atisenol isolated from the root $C_{20}H_{28}O_3$ mp-161^0C [79].

***Acorus calamus* L. (Rhizome)**

The plant has been extensively investigated and a number of chemical constituents from the rhizomes, leaves and roots of plants have reported which includes α, β and γ-asarone $C_{12}H_{16}O_3$(Mwt-208) (50.09%), (*E*)-methylisoeugenol (14.01%) $C_{11}H_{14}O_2$(Mwt-178), methyl eugenol (8.59%) ($C_{11}H_{14}O_2$), β-asarone (3.51%), α-cedrene (3.09%) and camphor (2.42%), asaryl-aldehyde (Chief constituent of the oil), ascarone, pinene, eugenol. α and β-pinenes, myrcen, camphene, p-cymene, car-3-ene, sabinene, α-terpinene, β-phellandrene, γ-terpinene, cis-ocimene, limonene, terpinolene and trans- β- ocimene. Epoxyisoacoragermacrone, acolamone, acoramone along with isoeugenol methylether (Z, Z)4, 7-decadienal, Calarene, 9-aristolene(25%) and β-gurjunene(10aristolene) (75%). Flavone glycoside: -Luteolin, 6, 8, C-diglucoside, Selinane type sesquiterpenes: -acolamone, isoacolamone, glycoside: -acorin, Sesquiterpenes and sesquiterpenic alcohols: - arylindane, calamen, calamenone, calamendiol and isocalamendiol, α-cedrene, acoragermacrone, calamusenone, iso calamusenone, preisocalamendiol, shyobunone, epishyobunone, isoshyobunone, sesquiterpene hydrocarbon: (-)cadala-1, 4, 9-triene, alkaloid: -choline.Acoradin 2, 4, 5-trimethoxy benzadehyde, 2, 5-dimethoxybezoquinon, flavanoids: -galangin .sterol: -sitosterol.Acids: Myristic(1.3), palmitic(18.2), stearic(7.3) oleic(29.1) linleic (24.5) and arachidic(3.2)acids detected y GC in rhizome .Carbohydrates: maltose(0.2), glucose (20.7), fructose(79.1%)detected in rhizome, (Z)3-(2, 4, 5-trimethoxyphenyl)-2-propenal and 2, 3-dihydro-4,

5, 7-trimethoxy-1-ethyl-2-methyl-3-(2, 4, 5- trimethoxyphenyl)indene isolated from rhizome.phenylpropane derivative: - 1-(p-hydroxyphenyl)1-(O-acetyl)-prop-2-ene [80].

***Adhatoda vasica* Nees. (Root)**

Glycerides of acids: - arachidic 3.1%, behenic 11.2%, lignoceric 10.7%, cerotic 5%, oleic 49.9% and linoleic acids 12.3% and β-sitosterol (2: 6%) .protein (8.5 %), vitamine C (5.2%), fats (2.5%) .Alkaloid from root*:* -Vasicine(7.5%), Vasicinone(mp-209^{0}C), 9-acetamido-3, 4-dihydropyrido-(3, 4-b)-indole, vasicol, vasicinolone, vasicinol, Adhatodine, deoxyvasicine, deoxyvasicinone, l-vasicinone, maiontone, Anisotine.Glucoside from root: -O-ethyl-α-D-galactoside, β-glucoside, galactose .sitosterol-β-D-glucoside. Sterol from root;-Daucosterol, sitosterol.Glucose from root: -D-glucoside, D-galactose.Chalcone from root: -2'-glucosyl-4-hydroxyl – oxychalcone.Galactoside from root: -O-ethyl-β-D –galactoside. Hydrocaron from the root: - Tritriacontane. Lignins: -Guaiacyl-, syringil-and p-hydroxyphenyl propane building units yielded vanillin, syringaldehyde p-hydroxybenzaldehyde.Aliphatic hydroxyl ketones: -37-hydroxyhexatetracont-1-en -15-one and 37-hydroxyhexatetracontan-19-one and 29-methyltriacontan-1ol [45].

***Allium sativum* L. (Bulb)**

Sulphur containing amino acids, alliin, methyl alliin and pentylalliin. Diallyltetra, penta-, hexa- and heptasulphides. protein (6.3%), fat (0.1 %), carbohydrates (29.0%), vitamin C (13 mg/100 g). Sulphur containing compound, allylpropyl disulfide, $C_6H_{12}S_2$ (6%), diallyl disulfide$C_6H_{10}S_2$ (6%), and two more sulfur-containing compounds. Ajoene [(E & Z)4, 5, 9-trithiadodeca -1, 6, 11-triene-9-oxide], diallyl trisulphide, 3-vinyl-4H [1, 2]-dithiin, 2-vinyl –4H [1, 3]-dithiin and proteoerubosideB isolated from the bulb. Isoutyl iso

thiocyanate, Diallyl sulphide, Allylmethyl sulphide, Allylmethyltrisulphide, Methyl-2-propenyl thioether, di(2-propenyl) thioether and 1, 2-dithio cyclo pent-3-ene. Gibberellins A3 and A7. Insulin (2->1) and levan (2->6) glycoside. Steroid, saponin 1 and2.Isolation of furostanol glycoside-sativoside B1 from bulb, sativoside R1 and R2 from roots. Allixin. Protodesgalactotigonin, desgalactotigonin, F –gitonin. Pectic substance composed of glucose (78.63%), galacturonic acid (38.4) mannose(7.46) arabinose(2.4%).Se-containing poly saccharides(SeGPS)isolated. Acids, Palmitic, stearic, oleic, linoleic and linolenic acids lysolecithin, lecithin, phosphatidylserin, phosphatidylinositol and phosphatidylethanolamine. Furostanol glycoside protodeoeruboside B [81].

***Alpinia calcarata* Roscoe (Rhizome)**

Isolation of 1'-acetoxychavicol acetate, 1'-acetoxyeugenol acetate and 1'-hydroxychavicol acetate and p-hydroxycinnamaldehyde. Essential oil contain methyl cinnamate (20 to 30%), d-pinene. 1, 8-cineole (17.72%), camphor (11.71%), α-myrcene(10.38%) and Borneol (6.29%).α-pinene (22.5%), β-pinene (36.7%) and limonene(13.8%), cineol (69%), terpinen-4-ol, α-terpineol(6.9%), Camphor, α-myrcene, Linalool, cedrol and eugenol .It contan gallic acid .It contain Calcaratarin D.It also contain tannin, phlobaphenes, starch, flavonoids, quercetin, tectochrysin -5-glucoside, kaempferol, quercetin-3-methyl ether isorhamnetin, kaempferide ($C_{16}H_{12}O_6$), galangin and its 3-methyl ether.Isolation of di(p-hydroxy-cis-styryl) methane along with p-hydroxycinnamaldehyde[82].

***Azadirachta indica* (L.) A. Juss.(Stem)**

Isolation and characterization of β-sitosterol (nimbosterol), nonacosane. flavonoids kemferol, quercetin, Nimbidinin, odoratne,

Azadirone, limocinone, nombandiol, myricetin, nimbin (0.04%) nimbinin $C_{28}H_{34}O_6$ (0.001 %),Epoxyazadiradione, nimbosterol (0.03%), nimbidin(.4%) essential oil (0.02%), Phenolic compounds, tannin (6.0%), sulfur-containing product $C_{13}H_{27}0\ _7S_2$, Carbohydrates containing L-arabinose, L-fucose, o-galactose, and D-glucuronic acid. Acids, aldobiuronic acid (4-0(D-glucopyranosyluronic acid)-D-galactopyranose). Nimatone, nimbin Desacetyl nimbin, Tetranortriterpenoids-isonimolide and isolimolide. Seco-tetranortriterpenoids-isonimbinolide. Diterpenoids-nimbionone, nimbione, Isonimolide, limocine B, melianin A, nimbinone, nimosone, nibosone, methylnimbionone, methylnimbiol, sugiol, margolone, margolonone [83], isomargolonone, nimbonone, nimbonolone and nimbionol. Water soluble polysaccharides-GIII DO'_2 Ia and GIII DO'_2 IIa, CSP1, 2, 3 isolated from bark. Isolation of 24-methylenelophenol, Azadiradione, diterpene-nimbilicin and nimocidin. Tetranorterpenoids-limbocinin and limbocidin, 1α-methoxy-1, 2-dihydro epoxy azadiradione, 1β, 2β: 14β, 15β diepoxyazadiradione, nimbinol, Azadirachtin from heartwood Sulphur containing compound Dipropyl di sulphide (major volatile) also presents in the plant. Polyacetate derivative margosine, margaosinone and margaosinolone, nimbosodione, nimbisonol and demethylnimbionol, diterpenoids-margosone, margosolone, nimbinene, 6-deacetyl nimbinene, arabinofucoglucans Gla and Glb. Tricyclic diterpenoids-margocin, margocinin, margocilin, nimbilin, nimolinin and 28-deoxo nimbolide are reported in the plant. Mahamoodin, epoxy azadirone, nimbin, gedunin, 7-deacetylgedunin, azadiradione, deacetylnimbin, 17-hydroxy azadiradione, nimbinol, Cis and trans -3, 5-diethyl-1, 2, 4-trithiolanes. Pentanortriterpenoids-nimbinene, 6-deacetyl nimbinene, nimbandiol, 6-O-acetyl nimbandiol. Trinortriterpinoid-limbonin, epoxyazadiradione, azadiradione, C7-bezoates of tetranor triterpenoids(I, II, III)isolated from kernel .Fruits

contain 1-cinnamoyal melianolone, 1- cinnamoyal -3, 11-dihydroxymeliacarpin, nimbo chalcin, nimbocetin, 5-hydroxy methylfurfural.Presence of Azadirol, kulactone, tetranortriterpene alcohol-17-epinimbocinol, nimbocinol in the seed coat.Limbonin along with epoxyazadira dione and azadiradione are reported from kernel.From leaves the following compounds are identified Tetranortriterpenoids-6-deacetyl nimbinal and 28-deoxonimbolide, hyperoside, quercetin, rutin, meldenindiol, meldenindiol, meldenin, isomeldenin, 4α, 6α-dihydroxyA-homo azadirone, isorhamnetin, cyclic tri and tetra sulphides, limonoid group**:** -Azadirachtin, Deacetylnimbin, 6-Deacetylnimbinor. Desacetyl Nimbin $C_{28}H_{34}O_{8,}$ Solannolide, Carbohydrates: -Mannose, glucosamine, arabinose, galactose, fructose, xylose, glucose [84].

***Cedrus deodara* (Roxb.ex D. Don) G. Don (wood)**

Isolation of ethyl 23-methylpentacosanoate from stem bark. 8-C methyl taxifoline, dihydroquercetin, 8-C methylquercetin, quercetin, sitosterol, tannins 8.25%, non-tannins 6.95%. sesquiterpenoids—alpha and beta-himchalenes. Acids- butyric and caproic acids, isopimaricacid, borneol and its esters. p-methylacetophenone, p-methylΔ3 tetrahydroacetophenone Dihydroflavanol-cedeodarin, cedrin, dihydromyricetin, cedrinoside, taxifolin, hemasecolone. Lignans-meso secioisolariciresinol, Matairesinol, dibenzylbutyrolactone, cedrusinin, cedrusin and its 4-glucoside, dihydro dihydro coniferyl alcohol, lariciresinol, isolarici –resinol, taxifolin-3-glucoside [85].

***Commiphora mukul* (Hook.ex Stocks) Engl. (Resin)**

Flavanoids: -quercetin, quercetin-3-O-α-arabinoside, quercetin-3-O-β-D-galactoside ,quercetin - 3-O-β-L-rhamnoside, quercetin-3-O-β-D-glucuronide, ellagic acid, pelargonidin-3, 5-di-O-glucoside from flowers. Guggulipid is hypocholesteremic. steroids—gugglsterones Z and E,

gugglusterone VI, guggulsterols I–V, .VI, Z. sesamin, cholesterol, and a few other steroids. Terpenes: -6.4% myrcene, 11% dimyrcene, and some polymyrcene, acids, diterpenoid: - cembrene A and mukulol. 20α-hydroxy-4-pregnen-3-one, 20β-hydroxy-4-pregnen-3-one, 16β-hydroxy-4, 17(20z)-pregnadien-3-one, 20 Hydroxy prognene-3, 10-dione, 16 α-hydroxy progesterone, Commipherol and 16α-hydroxy-4-pregnen-3-one. phenylpropanoids-methylchavicol [52].

***Celastrus paniculatus* Willd. (Seed)**

Normal triglycerides (20.2%), polar-triglycerides (44.4%), polarnonglyceridic esters (11.0%). acid: -palmitic acid, stearic acid, oleicacid, linoleic acid, linolenic acid. Sesquiterpenoid: -1β, 6α-diacetoxy-9β-benzoyloxy-8β-cinnamoyloxy-β-dihydroagarofuran. 1β, 6α, 8β-triiacetoxy-9α-(β-furancarbonyloxy)-β-dihydroagarofuran, β-dihydroagarofura sesquiterpenoids, 4, 4-dimethyl-1, 3-dioxan-5-ol. Alkaloids: -magnoflorin, celastrine and paniculatine, sterol: - celastrol [86]

***Cuminum cyminum* L. (Fruit)**

Phenylpropanoids: -methylchavicol (30ppm) .terpenes: 1, 8-cineole (0.2-0.4%)cuminaldehyde (20–40% of the oil) and *p*-cymene, pinene, dipentene, cumene, cuminic alcohol, phellandrene, alpha-terpenol, Cumene, α-terpinol, α-phellandrene, limonene, α-terpinene, α- pinene, apigenin-7-O- glucopyranoside, gallic acid, apigenin-5-O-glucopyranoside, luteolin-7-O- glucopyranoside, β- pinene (11.49%), camphene, myrcene, γ-terpinene, terpinolene, linalool, pulegone, myrtenal, 2, 3-dihydrocuminal, 1, 4-p-menthadien-7-al, 1, 3-p-menthadien-7-al, γ-terpinene (12.17%), p-menthadienal(26.24%). 14.5%lipids. flavonoid glycosides; apigenin, luteolin protein (18.7%),

ether extract (15.0%), carbohydrates (36.6%), fibre (12.0%), carotene, vitamin A (870 IU/100 g), and vitamin C (3 mg/100 g) [33].

***Curcuma longa* L. (Rhizome)**

Terpenes: - 1, 8-cineole, camphor. Curcumin and monodesmethoxycurcumin;60% of turmerones which are sesquiterpene ketones, alpha. -phellandrene (1%), sabinene (0.6%), cineol (I%), borneol (0.5%), zingiberene (25%), tolylmethyl carbinol, α-pinene, β-pinene, camphene, limonene, terpinene, caryophyllene, linalool, curcumene, carotene, diferuloyl methane of the formula $C_{21}H_{20}0_6$. A glycan-ukonan-A composed of arabinose, xylose, galactose, glucose, rhamnose, galacturonic acid, ukonan-B and C- composed of arabinose, xylose, galactose, glucose, rhamnose, galacturonic acid, ukonan-D- composed of arabinose, galactose, glucose, mannose .curcuminoid pigments-;1-(4-hydroxy-3-methoxyphenyl)-7-(3, 4-dihydroxyphenyl)-1, 6-heptadien-3, 5-dione, 1, 7-bis(4-hydroxyphenyl) -1, 4, 6-heptatrien-3-one.Two phenolics-1, 5-bis(4-hydroxy-3-methoxyphenyl)-penta-(1E, 4E)-1, 4-dien-3-one, 1-(4-hydroxy-3-methoxy phenyl) -5-(4-hydroxyphenyl -penta-(1E, 4E)-1, 4-dien-3-one.Sesquiterpines-germacrone -13-al, 4-hydroxybisabola-2, 10-dien-9-one, 4-methoxy-5- hydroxyl bisabola -2, 10-dien-9-one, 2, 5-dihydroxybisabola-3, 10-diene, procurcumadiol.

Sterols: -Campesterol, Stigmasterol, terpinolene (28.9%), Beta-sitosterol, curcumenone, dehydrocurcurdione, (4S, 5S)germacrone-4, 5-epoxide. Cholesterol, bisabola-3, 10dien-2-one, α-turmerones (59.60%) major constituent in the oil (1.3%), Bisacumol, bisacurone, β-turmerones, fattyacids, Isoborneol, curcumenol, isoprocurcumenol, procurcumenol, zedoaronediol, epi pro curcumenol, Camphor, α-atlantone (2.4%), turmerol (20%), Eugenol, 4, 5-dihydroxy bisabola-2, 10-diene, Peptide: -turmerin, Curdione, Curzerenone, monode methoxy curcumin identified

in rhizome. Curlone, Feruloyl-p-coumaroyalmethane, di-p-coumaroyalmethane, 4-Hydroxycinnamoyal (feruloyl)methane, bis-4-hydroxycinnamoyal (feruloyl)methane, 2-(hydroxymethyl anthraquinone, Hexahydrocurcumin, demethoxycurcumin [91].

***Cyclea peltata* (Lam.) Hook. F &Thoms. (Tubroot)**

Alkaloid: tetrandrine, (+) curine, (+) tetrandrine, homoaromaline, (+) isochondrodendrine, magnoflorine, isotetrandrine, berbamine, fangchinoline, cycleanine and cycleanorine. Phaeanthine and its(1S, 1'S) enantiomer (tentrandrine), dicentrine(major alkaloid), (-)limacine, (+)thalrugosin, (+) homoaromolin, cycleapeltine, (-)2'-norlimacin (+)cycleabarbatine, (+)tetrandrine-2'-β-N-oxide, (+)cycleanorine, (-)repandine, (+)daphnandrine, (-)curine, (+)cocclaurine and (-)N-methylcoclaurine [88].

***Cyperus rotundus* L. (Rhizome)**

Sesquiterpene pyridine alkaloids: rotundines A-C (0.21%-0.24%); bufadienolide glycosides (0.62%-0, 74%) β-caryophyllene and their oxides constitute 70% of the oil. δ-cadinene and α-copaene isolated from the tubers. Steroid alkaloids: conessine, isoconessimine, kurchessine. Beta-sitosterol. sesquiterpenes. eudalne: - sesquiterpene alcohol, isocyperol, fat, sugar, gum, carbohydrates: - Glucose, sucrose, maltose, fructose, essential oil, albuminous matter, starch, fiber, and ash. Sesquiterpene hydrocarbons, sesquiterpene epoxides, sesquiterpene ketones, monoterpene and aliphatic alcohols, Saponin-oleanolic acid -3-o-neohesperidoside isolated from tubers. Rhamnetin-3-O-rhamnosyl (1-4) rhamnopyranoside, caryophyllene from root detected in free sugar fraction, protein (4%). Humulene [43].

***Embelia ribes* Burm. f. (Seed)**

Compounds isolated from Embelia ribes are embelin, embelinol, embeliol, rapanone, homoembelin, homorapnone, vilangine, an alkaloid-christembine, a resinoid, tannins, homo embelin, embelinrapanone, quercitol, quarvital, palmitic acid, oleic acid, sitosterol, daucosterol, vidangin and volatile oil [55].

***Holarrhena antidysenterica* (Roth) DC. (Seed)**

Alkaloids, regholarrhenine-A, -B, -C, -D, -E and-F isolated from stem bark along with pubescine, norholadiene, pubescimine, kurchinin, kurchinine, kurchinidine, holarrifine, holadiene, kurchilidine, kurchamide, kurcholessine, kurchessine, conessine, conessimine and isoconessimine, and steroids ;-kurchinicin and holadyson, 3a-Aminoconan-5-ene, Dihydroconcuressine, Dihydroisoconessine, Dihydroisoconessimine, Concuressine, Conessimine, Kurcholessine, N_2 –methylholarrhimine, N_3-methylholarrhimine, N20 –methylholarrhimine, N .N'-Tetramethylholarrhimine, Holarrhidine, Holarrimin, Holonamine, α-Hydroxyconessine, Kurchamine, Kurchessine, holacine, holacimine . Conessine isolated from bark and seed. From the leaf Holadysamine, Holadysine, Isoconessimine, Kurchimine, Kurchiphyllamine Kurchiphylline and Kurchaline are isolated. Aspartic acid and arginine are found as major amino acids. Holarricine isolated from seed[89].

***Picrorhiza kurroa* Royle ex Benth. (Root)**

Kutkin (mixture of picroside-1and kutkoside) isolated from roots. Apocynin(4-hydroxy-3-methoxyacetophenone). Kutkoside (glucoside). Sterols, D-mannitol, kutkiol, kutkisterol and a ketone (identical with apocynin). Picroliv is a mixture of picroside I(6'-O-trans-cinnamoyal-catalpol) and kutkoside .Curcurbitacin glycoside: - picroside I, II(6-vanilloylcatalpol) [94]and III(6'-(4-hydroxy-3-metoxycinnamoyal)catalpol,

isolated from root.Iridoid glycoside-6-feruloyl catalpol isolated with veronicoside, picein, androsin and minecoside.Beta-D-6-cinnamoyl glucopyranose, 1-[2-(3-hydroxy-4-methoxyphenyl)ethyl]β-laminaribose, (1R, 5R, 6S, 7R, 8S, 11S)6, 7, 8-trihydroxy 2, 10-dioxatricyclo [6, 2, 1, 0]undecane, Catalpol, 20(R)3β, 16α, 20, 25-tetrahydroxy-2β-(β-D-glucopyranosyloxy)-10α-cucurbit-5-ene-22-one.Three cucurbitacine-16α-hydroxy-22, 23, 24, 25, 26, 27-hexanor-2β-(β-D- gluco pyranosyloxy)10α-cucurbit-5-en-3, 11, 20-trione, 20(R) 16α, 20-dihydroxy-24, 25, 26, 27-tetranor-2β-(β-D- gluco pyranosyloxy)10α-cucurbit-5-en-3, 11, 22-trione, 20(R)3β, 16α, 20-trihydroxy-24, 25, 26, 27-tetranor-2β-(β-D- gluco pyranosyloxy)10α-cucurbit-5-en-11, 22-dione. Cucurbitacine glycosides-2β-(glucosyloxy) 3, 16, 20, 25-tetrahydroxy-9-methyl-19-norlanosta-5, 23-dien-22-one, 25-(acetoxy) -2β-(glucosyloxy)-3, 16, 20-trihydroxy-9-methyl-19-norlanosta-5, 23-dien-11, 22-dione, 2β-(glucosyloxy)-16, 20-dihydroxy-9-methyl-19-norlanosta-5, 24-dien-3, 11, 22-trione,

2β- (glucosyloxy)-16, 20, 22-trihydroxy-9-methyl-19-norlanosta-5, 24-dien-3, 11, -dione, 2-O-glucosides of cucurbitacinB, 23, 24-didehydrocucurbitacinB and arvenin III.Six cucurbitacins;- 25-(acetoxy)-2β-(β-D-glucopyranosyloxy)-3, 16, -dihydroxy-9-methyl-19-norlanosta-5, 23-dien-22-one, 25-(acetoxy)-2β-(β-D-glucopyranosyloxy)-3, 16, 20-trihydroxy-9-methyl-19-norlanosta-5, 23(Z)-dien-22-one, 2-(β-D-glucopyranosyloxy)-3, 16, 20-trihydroxy-9-methyl-19-norlanosta-5, 24-dien-22-one, 2β-(β-D-glucopyranosyloxy)-3, 16, -dihydroxy-4, 4, 9, 14-tetramethyl-19-norpregn-5-en-20-one, 2, 3, 16, 20, 25-pentahydroxy-9-methyl-19-norlanost-5-en-22-one, 2β-(6-O-cinnamoyl-β-D-glucopyranosyloxy)-3, 16, 20, 25-tetrahydroxy-9, -methyl-19-norlanost-5-en-20-one, (2β, 9β, 10α, 20ξ24 ,ξ)20, 24-epoxy-2β-(β-D-

glucopyranosyloxy)-16, 25-dihydroxy-9-methyl-19-norlanost-5-en-3, 11-dione, (2β, 3β, 9β, 10α, 16α, 20ξ24 ,ξ)20, 24-epoxy-2β-(β-D-glucopyranosyloxy)-3, 16, 25-trihydroxy-9-methyl-19-norlanost-5-en-11-one, (2β, 9β, 10α, 16α, 20ξ24 ,ξ)20, 24-epoxy-2β-(β-D-glucopyranosyloxy)-16, 25, 26-trihydroxy-9-methyl-19-norlanost-5-en-3, 11-dione, (2β, 9β, 10α, 16α, 20ξ24 ,ξ) 20, 24-epoxy-2β-(β-D-glucopyranosyloxy)-3, 16, 25, 26-tetrahydroxy-9-methyl-19-norlanost-5-en-11-one, (2β, 9β, 10α, 16α, 20β, 20Z)2β-(β-D-glucopyranosyloxy)-16, 20, 26-trihydroxy-9-methyl-19-norlanost-5, 24-dien-3, 11-dione, (2β, 9β, 10α, 16α, 20β, 20Z) 2β-(β-D-glucopyranosyloxy)-3, 16, 20, 25-tetrahydroxy-9-methyl-19-norlanost-5, 24-dien-11-one [90].

***Piper longum* L. (Root)**

Piperolactam A and B, piperadione. aristolactam A, cepharadione A and B, cepharanoneB, nor cepharadioneB2-hydroxy-1-methoxy-4H-dibenzo [de, g] quinolie-4, 5-(6H)-dione isoutylamide longamide, guineensine, pluviatilol and methyl pluviatilol. Alkaloid-piperinonaline and piperundecalidine piperine and piperlongumine and also methyl-3, 4, 5-trimethoxy cinnamate. volatile oil, carbohydrates, protein, saponins, amygdalin, isobutylamide of an unsaturated acid. Pipernonaline and piper undecalidine. piper lactam. The petroleum ether extract yielded N-isobutyl-decatrans-2-trans-4-dienamide, piperine. Essential oil contains n-hexadecane, n-heptadecane, n-octadecane, n-nonadecane, n-cicosane, n-hencosane, α-thujene, terpinolene, zingiberene, p-cymene, p-methoxy acetophenone, dehydrocarveol, phenylethyl alcohol, and two new – monocyclic sesquiterpenes. L-tyrosine, L-cysteine hydrochloride, free acids -DL-serine, and L-aspartic acid. palmitic, hexadecenoic, stearic, linoleic, oleic, linolenic, higher saturated acids, arachidic, and behenic acids, sylvatine, sesamin, dieudesmin, hentriacontane, hentriacontane-16-

one, triacontanol, and β-sitosterol. Aristolactams and dioxoaporphines, long chain isobutyl amide, longamide, guineensine, lignans, pluviatilol, methyl pluviatilol (fargesin), sesamin and asarinine [68].

***Piper nigrum* L. (Fruit)**

Alkaloid- piperine (5 to 9%), piperidine (5%), N -isobutyl eicosa-trans-2-trans-4-dienamide, piperetine, piperlin, piperolein A, and piperolein, essential oil (1 to 2%), fat (7%), starch, lignin, gum, fat (1%); proteins (7%) and ash containing organic matter (5%). Chavacine is a soluble pungent concrete resin, hentria contanone, hentriacontane, hentriacontanol, β-sitosterol, palmitic, linoleic, oleic, and linolenic acids, amides, piperyline, piperoleins A and B, and N-isobutyl-cicosa-trans-2-trans-4-dienamide. (E)β-ocimen, guaiene, (ZE)farnesole, δ-cardinol and guaiacol. N-trans-feruloyltyramine, N-5-(4-hydroxyphenyl) 2E, 4E penta dienoyl piperidine, guineensine, N-isobutyl-2E, 4E, 8Z-eieosa trienamide, N-isobutyl-2E, 4E-octadeca dienamide and pellitorine.Phenolc amides-Coumaperine, N-trans-feruloylpiperidine, ferupiperine and dihydro ferupiperine.(E, E)-N-(2-methylpropyl)-2, 4, -decadienamide, (E, E, E)-13-(1, 3-benzodioxaol-5yl)-N-(2-methylpropyl)-2, 4, 12, tridecatrienamide and, (E, E, E)-11-(1, 3-benzodioxaol-5yl)-N-(2-methylpropyl)2, 4, 10, undecatrienamide, piperonal, piperine, piperoleineB, (2E, 4E)-N-isobutyl-2, 4-decadienamide [91].

***Piper brachystachyum* Wall ex Hook.f.(Root)**

Unsaturated amides-brachystamides A and B. Pyrrolidides-brachyamide A and B, brachystine along with asarinin, guineensine, pipataline, pluviatilol methylpluviatilol, sesamine, sitosterol, retrofractamide A and pipercide (retrofractamide B)[56].

***Plumbago zeylanica* L. (Rhizome)**

Binaphthaquinone-chitanone and lupeol, α and β amyrins, taraxasterol and Ψtaraxasterol, Plumbagin, 3-chloroplumbagin, 3, 3-biplumbagin, binaphtho – quinone: - 3', 6' –bi plumbagin, isozeylinone, zeylinone, elliptinone, and droserone. Plumbagozeylanone. Dihydro naphthaquinone -dihydroserone. Amino Acids-Aspartic acid (9.9), tryptophan (9.7), tyrosine (4.5), threonine (4.1), alanine (3.5), histidine (3.4), glycine (1.7), methionine (0.5) and hydroxyprline, (0.2%) from aerial parts. Naphthaquinone-methylene-3, 3'-diplumbagin, chitranone, maritinone, 2-methylaphthazarin, pluamgozeylanone and zeylanone from root. Zeylanone, isozeylanone, plumagic acid, plumagin, β-sitosterol, vanilic acid, catechol tannin, glucose, steroidal glycoside, isoshinanolone and 1, 2(3)-tetrahydro-3, 3'-bi plumbagin [92].

***Rubia cordifolia* L. (Stem)**

1, 3-Dihydroxy-2-hydroxymethyl-9, 10-anthraquinone: lucidin. Anthraquinones and their glycosides - purpurin (trihydroxyanthraquinone), munjistin (xanthopurpurin -2-carboxylic acid); besides xanthopurpurin, peudopurpurin (purpurin-3-carboxylic acid), free alizarin as well as its glucoside, pentacylic triterpenic acids—rubicoumaric and rubifolic acids. From roots Hexapeptides-RA-1, II, III, IV, V, VII-as minor constituent.1-acetoxy-6- hydroxy-2-methyl anthraquinone-3-O-α-rhamnosyl(1->4) α-glucoside, Anthraquinones-1-hydroxy-2-carboxy-3-methoxyanthraquinone, 1, 4-dihydroxy-2-carbo methoxyanthra quinone, 1, 3-dihydroxy-2-methoxymethylanthraquinone, 1, 4-dihydroxy-2-hydroxyl methylanthraquinone, 1-methoxy-2-methoxymethyl-3-hydroxyanthra quinone, 4-hydroxy-2-carboxyanthraquinone, 1-hydroxy-2-hydroxy methyl anthraquinone, 1, 4-dihydroxy-6-methylanthraquinone, 1-hydroxy -2-methyl anthraquinone,

1, 4-dihydroxy-2-methylanthrax quinone, 1, 5-dihydroxy-2-methylanthraquinone, 3-prenyl-5-methoxy-1, 4-aphthaquinone. Nordamnacanthal, physcion, rubicoumaric acid, rubifolic acid, 1-hydroxy-2 methoxy anthrax quinone, 1, 4-dihydroxy-2-methyl-5-methoxyanthraquinone(1, 4-dihydroxy -2-methyl-8-methoxyanthraxquinone), 1, 3-dimethoxy -2-carboxy anthraquinone, Rubiadin, Fernane derivative-rubiatriol, Alizarin, 2-methyl-1, 3, 6-trihydroxyanthraquinone, β –sitisterol, scopoletol, oleanolic acid acetate, mollugin, 1-hydroxy- 2-methyl-9, 10-anthraquinone, 1, 3-dihydroxy-2-ethoxy methyl-9, 10-anthraquinone, Lucidin, Primeveroside, Ruberythric acid [93].

***Saussurea lappa* (Decne)Sch-Bip. (Root)**

Camphene (0.04%), phellandrene (0.4%), terpene alcohol (0.2%), d-costen (6.0%), P-costen (6.0%), aplotaxene (20.0%), costol (7 .0%), di-hydrocostus lactone (15.0%), costos lactone (10.0%), costic acid (14.0%), a glucoside, resin (6.0%), tannin, inulin (about 18.0%), a fixed oil, sugar. Lactone- saussurea lactone, in 20 to 25% yield. Alkaloid: - saussurine (%.05), costunolide, dehydro costuslactone, costic acid, palmitic and linoleic acids, betasitosterol and alpha-cyclocostunolide. alantolactone, beta-cyclocostunolide and *iso*-alantolactone, Diosgenin, Dihydrocostuslactone isolated as an active principle. Sesquiterpenoid – saussureal, Costunolide, dihyro costunolide, 4β-methoxydehydrocostus lactone, 15-hydroxydehydro costus lactone. Aminoacid-sesquiterpene adduct-aussureamines A, B, C, D, E. Alpha and beta Peltatin.Lignin glycoside-(-) massoniresinol-4”-O-β-D-lucoside. Isodehydrocostus lactone and isozaluzainin, Guaianolide -12-methoxy-dihydro costuslactone [94].

***Scindapsus officinalis* (Roxb.) Schott. (Fruit)**

Glycosidic substances—scindapsin A and B. Carbohydrates-sugars, rhamnose, fructose, glucose and xylose together with some di-and tri-saccharides. Fatty acid -11-hydroxy-cis, cis-5, 8-tetracosadienoic acid, cyclopropenoid fatty acids[37].

***Semecarpus anacardium* L. f. (Seed)**

Semecarpol, biflavonoids- Semicarpetin, jeediflavanone, galluflavanone, semicarpuflavanone tetrahydrobustaflavone, tetrahydroamentoflavone and anacarduflavanone, nallaflavone, semicarpetin, anacardic acid, aromatic amines and bhilawanol. Dimeric Flavonoid-nalla flavanone [50,51].

***Sida rhombifolia* L.(Root)**

Three types of alkaloidal constituents, beta-phenethylamine, quinazolin and carboxylated triptamines, methyl beta-phenethylamine, S-(+) Nb-methyltrypto phanmethyl ester, Vasicinol, Vasicinone, Vasicine, Cholin, Hypaphorine methyl ester Hypaphorin and betaine have been isolated. Four ecdyson-20-hydroxy ecdyson, 2-deoxyecdyson, 2-deoxy hydroxy ecdyson-3-O-β-D-glucopyranoside and 20- hydroxyecdysone-3-O-β-D-glucopyranoside were reported. Isolation of scopoletin, scoporone, ethoxy-ferulate, kaempferol-3-O-β-D-glucosyl-600-α-D-rhamnose, quindolinone, 11-methoxy- quindoline, harmine, quindoline and cryptolepine salt [95].

***Solanum indicum* L. (Root)**

Diosgenin, beta-sitosterol, lanosterol, solamargine, solasodine and tomatidenol. carpestrol. Alkaloids: - solanine and solanidine. Fruits are reported to contain enzymes Dioscin, methylprotoprosapogenin A of dioscin, methylprotodioscin, protodioscin, steroidal glycoside-indioside

A, carpesterol, 3β-(p-hydroxy) bezoyloxy-22α-hydroxy-4α-methyl-5α-stigmast-7-ene-6-one. Isoanguivine, solasonin and indiosides A-E, ascorbic acid, acaffeic acid, caffeoyl quinic acid and naringenin also have been reported[96].

***Tinospora cordifolia* (Willd.) Miers ex Hook.f.&Thoms (Stem)**

Giloin, $C_{23}H_{32}0_{10}$ a glycoside, mp 226 to 228°C, gilenin, $C_{17}H_{18}0_5$, a nonglycoside bitter, mp 210 to 212°C, gilosterol, $C_{28}H_{48}O$ mp 192 to 193°C. Tinosporine-the furanoid bitter principle. Clerodane furano-diterpene with the molecular formula $C_{20}H_{22}0_8$ cordifolia, alkaloid – berberine, bitter principles, including columbin, chasmanthin, palmarin and tinosporon, tinosporic acid and tinosporol. Tinosporide, furanoid diterpene glucoside. Diterpenoid furanolactone. Phenolics lignin-3-(α, 4-dihydrxy-3-methoxybenzyl)4-(4-hydroxy-3-methoxybenyl) tetrahydrofuran, noacosan-15-one, octacosanol and β-sitosterol, Magnoflorine (.07%), clerodane diterpenoid-18-norclerodane glucoside-tinosporaside [97].

***Trachyspermum roxburghianum* (DC.) Wolff (Fruit)**

Coumarins—Seselin, bergaptene, 7-methoxy-6-methyl coumarin, 7-hydroxy-6- methyl coumarin, angelicin, psoralen, bergapten, umbelliferone. Beta-sitosterol, d-limonene (35.1), alpha-terpinene (19.4), *d*-linalool (4.7), *dl*-terpineol (5.7) and *dl*-piperitone (13.6%). Thymol content is 1.7% and β- cyclo-lavandulic acid $C_{10}H_{16}O_2$ [66,67].

***Trichosanthes dioica* Roxb. (Whole plant)**

The seed extract of *T. Dioica* contains 7-oxidihydrokarounidol-3-benzoate as the most predominant component. Nicotinic acid, riboflavin, vitamin C, thiamine, 5-hydroxytryptamine, cucurbita-5, 24-dienol. Colocynthin, trichosanthin, hentriacontane, elaeostearic, linoleic, oleic and saturated acids. From the fruits of Trichosanthes 14 cucurbitane

glycosides (khekadaengosides A-J, M-N, cucurbitacin J 2-O-b-glucopyranoside and cucurbitacin K 2-O-b-glucopyranoside), a hexanorcucurbitane glycosides (khekadaengoside K) and octanorcucurbitane (khekadaengoside L) were isolated along with two known cucurbitane glucosides (cucurbitacin2-Obd-glucopyranoside and 25-O-acetyl-cucurbitacin 2-O-b-glucopyranoside). charantin and cucurbitacin have been reported and isolated; Galactose-specific lectins have been purified from seeds and roots of Trichosanthes. The roots also contain an abortifacien t protein, trichosanthin, which is a ribosome-inactivating protein (RIP), a similar RIP, trichokirin, was also found in the seeds of Trichosanthes [98,99].

***Zingiber officinale* Rosc. (Rhizome)**

Zingiberene, B-sesquiphellandrene, Ar-curcumene, γ-cardinene, γbesabolene, sesquisabinene hydrate, zingiberinol, citronellol, Sesquiphellandrene, Geranyl acetate, Terpinolene, copaene, Zerumbone, Camphene, 1, 8-cineol, Geranial, Neral, Phellandrene, Linlool, camphene, 1, 8-cineole, phellandrene, geranial, neral, zingiberine, borneol, gingerol, beta bisabolene, alpha zingiberene, gingerols, shogaols, Citral, Diarylheptenones, Diterpenes, gingesulphonic acid and Mono acyl di galactosyl glycerols.α-farnesene, resin, starch, Homologous Phenols: -[6] gingerol (i.e., where n = 4). Gingerols with 7, 8, 9, and possibly 16 carbon atoms in the side chain have been reported. Gingediols, methylgingediols, gingediacetates, methyl gingedi acetates.6-Gingediol or [6] Gingerdiol, 10-Gingediol $C_{21}H_{36}O$.8- Gingediol $C_{19}H_{32}O_4$, gingerol [100].Zingerone, dihydrogingerol, hexahydrocurcumin, (6)-dehydrogingerdione, (10)-dehydrogin gerdione, (6)-gingerdione, (10)- gingerdione, hexahydrocurcumin, des-methylhexahydrocurcumin [70]. Gingerols 1, II, III.Aminoacids: -aspartic acid, Threonine, serine, Glycine, cysteine, Valine, isoleucine, leucine, argentine. Undecan-2-one, Zerumbodienone.Humulene epoxide 1 and 2, (8) gingerols,

Galanolactone, (E)8β, 17-epoxylabd-12-en-15, 16-dial, Diarylheptenones-gingerinones A, B, C, iso gingerinonesB, meso-3, 5-diacetoxy-1, 7-bis (4-hydroxy-3-methoxy phenyl) heptanes, 3, 5-diacetoxy-1-(4-hydroxy-3, 5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl) heptanes. Dehydroxytetrahydrocurcumin, Hexahydrocurcumin, 5-hydroxy-7- (4-hydroxyphenyl)-1-(4-hydroxy-3-methoxyphenyl)-3-heptanone, 3, 5-diacetoxy-7-(3, 4-dihydroxyphenyl)-1-(4-hydroxy-3-methoxy phenyl)heptanes, 5-hydroxy-1-(4-hydroxy-3, 5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-3-heptanone, 5-hydroxy-7-(4-hydroxy-3, 5-dimethoxyphenyl) -1-(4-hydroxy-3-methoxyphenyl)-3-heptanone, (3S, 5S)3, 5-diacetoxy 1, 7-bis(3, 4-dihydroxy-phenyl)heptanes, (3R, 5S)3, 5-dihydroxy1, 7-bis(4-hydroxy-3-methoxy phenyl)heptanes, 7-(3, 4-dihydroxyphenyl)1-(4-hydroxy-3-methoxyphenyl)hept-4-en-3-one,

(3S, 5S)3, 5-dihydroxy1, 7-bis(4-hydroxy-3-methoxyphenyl) heptanes, 6-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-4-decen-3-one, (3R, 5S)3acetoxy-5-hydroxy-1-(4-hydroxy-3-methoxyphenyl) decane, (3R, 5S)5-acetoxy-3-hydroxy-1-(4-hydroxy-3-methoxyphenyl) decan, 3, 5-diacet oxy-1-(4-hydroxy-3-methoxyphenyl) decane, (3R, 5S)3, 5-diacetoxy-1-(4-hydroxy-3-methoxyphenyl) decan (3R, 5S)3, 5-diacetoxy-1-(3, 4-dimethoxyphenyl) decan, 6-ginger sulphonic acid

Monoacyl Galactosyl Glycerol-ginger glycolipids A, B and C. (+) angelicoidenol-2-O-β-D-glucoside. Heptanes-2-ol6-methylhept-5- ene-2-one.6, 4-dichloro flavan [101].

***Merremia tridentata* (L.) Hallier f. (Whole plant)**

The aerial parts of M. tridentata contain flavonoids, diometin, luteolin, and their 7-O-β-D glucosides [70]. Do decanoic acid and Nonanoic acid Methyl ester have. been reported from root using GC [103].

***Vigna mungo* (L.) Hepper (Seed)**

24-epiclerosterol, 24α and 24β-methyl-5 α-cholest-8(14) en-3 β-ols, 24 α -ethyl-5 α-cholest-8(14) en-3 β-ols, 14α, 24α and 14α, 24β-di methyl-5 α-cholest-9(11) en-3 β-ols, 14α, methyl 24 α -ethyl-5 α-cholest-9(11) en-3 β-ols, 14α, methyl 24 β -ethyl-5 α-cholest-9(11), 25-dien-3 β-ol, 24 α -ethyl-5 α-cholest-9(11) en-3 β-ol, 24-methylenepollinastanol, β-sitosterol. It contains various isoflavones such as genistein, 2'-hydroxy-genistein, 2'-hydroxydaidzein, Kievitone, Dalbergioidin, Cyclokievitone, 5-hydroxykievitone, 2'-hydroxy dihydrodaizein, Isoferririn, Eurenol, Glycinol, Demethylvesititol, Kievitonehydrate, 4'-O-methyl kievitone, Cyclokievitonehydrate, 5-deoxy kievitone hydrate, hemicellulose A and hemicelluloses [74].

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Chapter 3

Antioxidant study and HPTLC study

3.1. Introduction

3.2. Result and Discussion

3.2.1. Methanol extract preparation

3.2.2. Phytochemical Analysis

3.2.3. Quantitative analysis of phytochemicals

3.2.4. Anti-oxidant study

3.2.5. HPTLC study of phenolics

3.2.6. HPTLC study of flavanoids

3.2.7. HPTLC study of alkaloids

3.3. Experimental

3.4. Conclusion

References

Abstract

Several types of reactive species are generated in the body as a result of metabolic reactions in the form of free radicals. These species may be either oxygen derived or nitrogen derived and called pro-oxidants. They attack macromolecules including protein, DNA and lipid etc. causing cellular/tissue damage. To counter their effect, the body is endowed with another category of compounds called antioxidants. In a healthy body, pro-oxidants and antioxidants maintain a ratio and a shift in this ratio towards pro-oxidants gives rise to oxidative stress. Continued oxidative stress due to free radicals can lead to chronic inflammation which in turn could mediate the most chronic diseases including cancer, diabetes, cardiovascular, neurological, and pulmonary diseases. Radical scavenging activity is associated with the quantity of secondary metabolites namely poly phenols, alkaloids and terpenoids present in the preparation. This chapter describes the identification and quantification of phytochemicals and antioxidant activity of Gulguluthikthakam Kashayam and Prasarinyadi Kashayam.

3.1. INTRODUCTION

Free radicals and reactive oxygen species (ROS) and its role in human diseases became an important aspect of health and disease management. Antioxidant is a general term for any compound that can counteract unstable molecules called free radicals which are the products of normal cellular metabolism. Free radicals damage DNA, cell membranes, and other parts of cells. Free radicals are unstable, short-lived and highly reactive because of their odd number of electron(s). Because free radicals lack a full complement of electrons, they abstract electrons from other cellular biomolecules and damage those molecules in the process. Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) can be generated in the human body by various endogenous or exogenous sources, exposure to different pathological states or physiochemical conditions, such as industrial solvents and radiation [1]. Free radicals at low or moderate levels are vital to human health, involving their physiological roles in the function of many cellular signaling systems and acting as weapons for the host defense system. However, free radicals have a pivotal role in a diverse range of degenerative diseases like atherosclerosis, cancer, inflammatory disease, asthma, diabetes, kidney diseases [2, 3].

Antioxidants are chemicals that bind with free radicals and nullify their effect from causing damage to biological molecules. Antioxidants bind with free radicals and effects the termination of oxidative chain reactions, and the free radicals are no longer able to attack the cell. Antioxidant defense in the organisms against reactive oxygen species (pro- oxidants and free radicals) produced during normal metabolic processes may be endogenous or exogenous.

Endogenous (enzymatic and non-enzymatic) antioxidants are produced by our body which are used to combat various free radicals.

A balance between free radicals and antioxidants is necessary for proper physiological function in the body. Cellular damage by free radicals contributes to the etiology of many chronic health problems. Antioxidants prevent free radical induced tissue damage by preventing the formation of radicals, scavenging them, or by promoting their decomposition. Oxygen concentration plays an important role in determining which free radicals are generated. The detrimental effect of free radicals causing health damages is termed oxidative stress [2]. Oxidative stress is a harmful process and can damage cell structures which ultimately can develop inflammatory diseases. Continued oxidative stress can lead to chronic inflammation. Chronic inflammation is a pathological condition characterized by continued active inflammation response and tissue destruction. Many of the immune cells including macrophages, neutrophils and eosinophils are involved directly or by production of inflammatory cytokine in the pathology of chronic inflammation. Moreover, many studies suggest that chronic inflammation could have a serious role in the most chronic diseases including cancer, diabetes, cardiovascular, neurological, and pulmonary diseases [4].

Various plant-derived or synthetic antioxidant and anti-inflammatory compounds have been used to treat oxidative insult and inflammation, as these substances are capable in scavenging of ROS and inflammatory mediators [5]. However, the synthetic compounds have been claimed to cause mild to severe adverse effects. On the other hand, usage of herbal medication has increased over the past three decades, and ~80% of the world's population living in the developing nations rely on plant-based medicinal products as the primary source of healthcare.

Although several herbal compounds have been widely used as antioxidant or anti-inflammatory substances, many of them are yet to be investigated to elucidate their molecular mechanism of antioxidant activity. Therefore, identification of novel and effective therapeutic substances from medicinal plants with low or no side effects became prioritized research. The phenolic compounds and flavonoids are a family of bioactive constituents that have been demonstrated as promising sources for new medications to treat inflammation and related chronic painful diseases [6].

Many Ayurvedic formulations and modern medicines are available for the treatment of oxidative stress related diseases. The studies conducted by WHO revealed that Ayurvedic formulations are highly effective in some of the diseases, e g; rheumatoid arthritis, caused by oxidative stress where the modern medicines failed [2]. Radical scavenging activity of Ayurvedic drugs is associated with the quantity of secondary metabolites namely poly phenols, alkaloids and terpenoids. Plant phenolics are widely distributed in the plant tissues, and play a vital role as highly effective free radical scavengers [4]. Natural antioxidants have attracted much interest because of their ability to scavenge free radicals [5].

In the present study two polyherbal formulations, Gulguluthikthakam Kashayam and Prasarinyadi Kashayam that are widely used in Ayurveda therapeutics for the treatment of various chronic inflammatory ailments are investigated for their antioxidant potential by standard analytical chemical protocol. A number of methods have been developed to measure the efficiency of pure compounds or the plant extracts in vitro [7, 8]. These standard protocols are followed with minor modifications.

High Performance Thin Layer Chromatography

High Performance Thin Layer Chromatography (HPTLC) technique is a sophisticated and automated form of the thin-layer chromatography (TLC) with better and advanced separation efficiency and detection limits [9]. It is useful in qualitative and quantitative analysis of natural products, pharmaceuticals, botanicals, forensics, foods and chemicals. HPTLC is a quicker analytical technique, extremely reproducible and has superior detection sensitivity than TLC. The in-situ derivatization is unique to HPTLC [10].

Principle of separation is adsorption, desorption and differential migration between the stationary and mobile phase, the same as that of TLC. The uniform particle size of the stationary phase (5-6µm) provides higher efficiency than TLC because adsorbents are small and uniform in size. Small particle size reduces sample diffusion resulting in compact bands or spots. Furthermore, the smaller particle size and uniform thinner layer significantly increase detection sensitivity and analysis speed.

Steps involved in the HPTLC analysis are [11]

1. Selection of chromatographic layer
2. Sample and standard preparation
3. Activation of pre coated plate
4. Application of sample and standard
5. Selection of mobile phase
6. Chamber saturation
7. Chromatographic development and drying
8. Derivatization
9. Detection and documentation.

3.2. RESULT AND DISCUSSION

3.2.1. Methanol extract

Gulguluthikthakam Kashayam after centrifuge, lyophilization and dried at 50 degree celsius produced Gulguluthikthakam Kashayam residue and filtrate weighed 48 and 90 gm respectively and that of Prasarinyadi Kashayam are 76 and 29 gm respectively. The % yield of methanol extract from Gulguluthikthakam Kashayam residue and filtrate are 57.88 and 5.56 and that of Prasarinyadi Kashayam are 6.84 and 22.10. The values are given in Table 3.1.

Table 3.1. Methanol Extracts obtained

Quantity of extracts from 1500mL Kashayam			
Item weighed	**Quantity(gm)**	**Quantity of methanol extract(gm)**	**% Yield of methanol extract**
Gulguluthikthakam Kashayam residue	48	27.78	57.88
Gulguluthikthakam Kashayam filtrate	90	5	5.56
Prasarinyadi kashayam residue	76	5.2	6.84
Prasarinyadi kashayam filtrate	29	6.41	22.10

3.2.2. Phytochemical analysis of methanol extract of Gulguluthikthakam Kashayam and Prasarinyadi Kashayam:

The results of the phytochemical analysis of methanol extracts of kashayam revealed the presence of different biochemical constituents, such as alkaloids, flavonoids, phenolics, tannins, steroids, amino acids, carbohydrates, terpenoids, saponins and glycosides in different concentration. The result is depicted in Table 3.2. Methanol extract of Gulguluthikthakam kashayam residue showed the maximum number of

phyto constituents except proteins and amino acids. Least number of phytochemical tests was answered by methanol extract of Prasarinyadi kashayam residue. Secondary metabolites like alkaloids, terpenes, phenolic and flavonoids are present in all extracts in high concentration.

Table 3.2. Preliminary phytochemical profile of methanol extract of kashayam

SI No.	Phytochemical test	Name of the test	MGP	MGF	MPP	MPF
1.	Tannins	Lead acetate, $FeCl_3$	+	+	+	+
2.	Steroids	Salkowski test	+++	+++	+++	+++
3.	Flavonoids phenolics	Ferric chloride test	+++	+++	+++	+++
		Lead acetate solution test	+++	+++	+++	+++
		Alkaline reagent test	+++	+++	+++	+++
4.	Saponins	Frothing test	+	-	-	-
5.	Amino acids & Proteins	Ninhydrin test Biuret test	-	+	+	+
6.	Alkaloids	Wagner's Hager's &Mayer's test	+++	+++	+++	+++
7.	Carbohydrates	Molisch's test	+	+	+	+
8.	Cardiac glycosides	Keller Killiani test	+	+	-	+
9.	Terpenoids	Salkowski test	++	++	++	++

+Present, ++ Moderately present, +++ Highly present, - Absent (Quantify +; ++; +++ etc.)

MGP-Methanol extract of Gulguluthikthakam Kashayam residue

MGF- Methanol extract of Gulguluthikthakam Kashayam filtrate

MPP- Methanol extract of Prasarinyadi Kashayam residue

MPF- Methanol extract of Prasarinyadi Kashayam filtrate

3.2.3. Quantitative analysis of phytochemicals

Quantitative analysis of phytochemicals has been done using the standard procedure.

3.2.3.1. Total phenolics content (TPC)

Total phenolics content determined on the basis of the hydroxyl (-OH) group of phenolics compounds reduces the phosphomolybdic acid to molybdenum blue in the presence of an alkaline medium. The blue color complex was then spectrophotometrically measured at λ 760nm.

Total quantity of phenolics obtained in the extract is shown in Table 3.3. Total phenolics were calculated in terms of gallic acid equivalent per mL. Maximum phenolics content was estimated by methanol extract of Gulguluthikthakam kashayam residue (57.9764 µg EGA/mL) followed by Prasarinyadi kashayam filtrate (42.5294 µg EGA/mL). The lower value of TPC was observed in Prasarinyadi kashayam residue (35.8106 µg EGA/mL) followed by Gulguluthikthakam kashayam filtrate (39.05 µg EGA/mL). As the free radical scavenging activity of phenolic compounds is facilitated by their hydroxyl group [12] total phenolic concentration observed for Gulguluthikthakam kashayam and Prasarinyadi kashayam had confirmed its scavenging property.

Table 3.3. Total Phenolics content

Sample	Quantity of phenolics equivalent to gallic acid per mL
Gulguluthikthakam kashayam residue	57.9764 µg
Gulguluthikthakam kashayam filtrate	39.05 µg
Prasarinyadi kashayam residue	35.8106 µg
Prasarinyadi kashayam filtrate	42.5294 µg

3.2.3.2. Total flavonoids content (TFC)

Flavonoids present in the extract formed a charge transfer complex with Al^{3+} present in the Aluminium chloride. This complex is highly stable in the aqueous medium, which then interacts with the sodium nitrite in the alkaline medium to form a yellow color complex that is spectroscopically measured at λ 415nm.

All the extracts were evaluated for the quantitative determination of total flavonoids, the results obtained are entered in Table 3.4. Total flavonoids were calculated in terms of rutin equivalent per mL The perusal of the data reveals that TFC was higher in the methanol extract of Gulguluthikthakam kashayam residue (80.75 µg E rutin/mL) followed Prasarinyadi kashayam filtrate by (53.22 µg E rutin/mL) while lower flavonoid quantity was observed in methanol extract of Prasarinyadi kashayam residue (39.56 µg E rutin/mL). Antioxidant property of flavonoids depends on the presence of a free OH group. Plant flavonoids have antioxidant properties in *vivo* and in *vitro* and act as antioxidants [13]. High quantity of flavonoids observed in both the kashayam reinforce its antioxidant property and the anti-inflammatory activity.

Table 3.4. Total Flavonoids content

Sample	Quantity of Flavonoids equivalent to rutin per mL
Gulguluthikthakam kashayam residue	80.75 µg
Gulguluthikthakam kashayam filtrate	51.93 µg
Prasarinyadi kashayam residue	39.56 µg
Prasarinyadi kashayam filtrate	53.22 µg

3.2.3.3. Total alkaloid content (TAC)

Higher quantity of alkaloid found in methanol extract of Gulguluthikthakam kashayam residue (45.66%) followed by methanol extract of Gulguluthikthakam kashayam filtrate. The lower quantity of TAC observed in methanol extract of Prasarinyadi kashayam filtrate (3.0833%) followed by extract of Prasarinyadi kashayam residue (13. 875%). The values obtained are given in table 3.5.

Alkaloids are very important as these possess multiple physiological and pharmacological activities, such as, analgesic, anti-inflammatory, antioxidant, anti-tumor, and antibacterial. Alkaloids containing OH and NH groups which can donate hydrogen ions to free radicals can act as antioxidants [14]. The quantity of alkaloids observed in both the kashayam supports its anti- radical activity.

Table 3.5. Total Alkaloids content

Sample	Quantity of Alkaloid (%)
Gulguluthikthakam kashayam residue	45.66
Gulguluthikthakam kashayam filtrate	31.916
Prasarinyadi kashayam residue	13.875
Prasarinyadi kashayam filtrate	3.0833

3.2.3.4. Total steroid content (TSC)

Higher quantity of total steroids found in methanol extract of Gulguluthikthakam kashayam residue (8.76%) and lower quantity found in Prasarinyadi kashayam filtrate (0.1%) and medium values are found in methanol extract of Gulguluthikthakam kashayam filtrate (5.977%) and Prasarinyadi kashayam residue (7.6696%). The values are given in table 3.6. The free hydroxyl group on the aromatic ring is responsible for the antioxidant properties of steroids. The hydrogen from this group is donated to the free radical. Both the kashayam contain steroids in the range 5.9 -8.76%. This strengthens its antioxidant property and anti-inflammatory activity.

Table 3.6. Total Steroids content

Sample	Quantity of Steroids (%)
Gulguluthikthakam kashayam residue	8.76
Gulguluthikthakam kashayam filtrate	5.977
Prasarinyadi kashayam residue	7.669
Prasarinyadi kashayam filtrate	0.1

3.2.4. Antioxidant Property of Gulguluthikthakam Kashayam and Prasarinyadi Kashayam:

Antioxidants are vital substances that possess the ability to protect the body from the damage caused by free radical induced oxidative stress [15]. Natural antioxidants have benefits over synthetic antioxidants because the later has side effects [16] [17,18].

The methanol extract of Gulguluthikthakam kashayam and Prasarinyadi kashayam were used for antioxidant study. Antioxidant property of extracts determined by FRAP, DPPH and modified hydroxyl radical scavenging assays.

3.2.4.1. Ferric ion reducing antioxidant power assay: Antioxidant property of extracts was determined using FRAP assay. In this method, the antioxidants reduce the $Fe^{3+}$to Fe^{2+}. This ion then conjugates with the ferricyanide ion to form a Prussian blue color product, which is spectrophotometrically measured at λ 700 nm. Higher absorbance indicates higher free radical scavenging potential. The free radical scavenging potential of the compound can be attributed to its antioxidant potency. The free radical scavenging values of the plant extracts tested in this study are illustrated in Figure 3.1. Absorbance values are given in Table 3.7. Antioxidant study by FRAP assay shows MGP and MPF has higher radical scavenging activity than ascorbic acid (positive control) and MPP and MGF have approximately the same radical scavenging activity. Total phenolic and flavonoids are approximately the same quantity in MPP and MGF. Radical scavenging activity of MGP is in correlation with the secondary metabolites calculated for MGP. From the quantitative analysis it is found that all the secondary metabolites are high in MGP. In the case of MPF, secondary metabolites are in the order TFC >TPC >TAC>TSC. It is one of the most rapid tests and very useful for routine analysis. All the extracts showed an increasing trend in radical scavenging activity with increasing extract concentration. It ascertains its anti-inflammatory activity.

Table 3.7. Antioxidant study by FRAP Assay

Extracts	Concentration (μgmL^{-1})	Absorbance
Ascorbic acid (STD)	100	0.2253±0.0031
	150	0.2567±0.0062
	200	0.2817±0.0043
	250	0.3045±0.002
	300	0.3225±0.0016
	350	0.3425±0.0017
MGP	100	0.157±0.002
	150	0.2145±0.0567
	200	0.264±0.004
	250	0.3356±0.0607
	300	0.4092±0.0593
	350	0.519±0.0076
MGF	100	0.1456±0.0423
	150	0.2052±0.0165
	200	0.2464±0.0126
	250	0.2691±0.0119
	300	0.3189±0.0123
MPP	100	0.1789±0.0136
	150	0.1931±0.007
	200	0.2317±0.007
	250	0.2778±0.0108
	300	0.2969±0.0112
MPF	100	0.1982±0.003
	150	0.2738±0.012
	200	0.325±0.0035
	250	0.3789±0.0196
	300	0.4556±0.0069

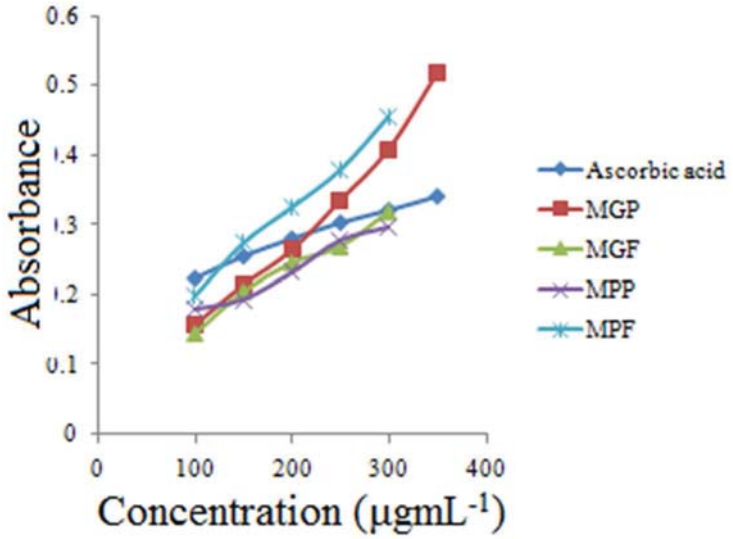

Figure 3.1. Antioxidant study by FRAP Assay

3.2.4.2. Modified hydroxyl radical scavenging assay: The result of hydroxyl radical scavenging assay was presented, in the Figure (3.2 and 3.3) and Table (3.8 and 3.9). The extracts inhibit the generation of hydroxyl radicals in a concentration-dependent manner and a maximum inhibition in •OH generation was observed by MGP (at 525.196 µgmL^{-1}) with the equation y = 0.102x - 3.570, R^2 = 0.997 and minimum for MGF (1421.40 µgmL^{-1}) with the equation y=0.032x+4.515, R^2=0.978. Positive control Mannitol has an IC50 value 481.639 µgmL^{-1} with the equation y=0.0978x+3.281, R^2=0. 991.The IC_{50} value calculated in the case of MPP and MPF are found to be 754.935 µgmL^{-1} with the equation y=0.062x+3.194, R^2=0.986 and 1359.2 µgmL^{-1} with the equation y=0.030x+9.224, R^2=0.951 respectively. Moderate inhibition in •OH generation was observed by MPP and MPF. Hydroxyl radical is considered as the major contributor for the oxidative stress related diseases including inflammation. All extracts inhibited •OH generation and hence, contribute to anti-inflammatory properties.

Table 3.8. Antioxidant study by Modified hydroxyl radical scavenging Assay

Extracts	Concentration(µg/mL)	Absorbance (415 nm)	Hydroxyl radicals scavenged (%)
MGP	100	0.4282±0.0003	6.0067±0.0552
	200	0.4082±0.0002	10.4111±0.0335
	300	0.3764±0.0002	17.3763±0.0335
	500	0.3548±0.0002	22.1173±0.0335
	700	0.3337±0.0002	26.7632±0.0456
	900	0.3175±0.0002	30.3189±0.0335
MGF	100	0.4358±0.0003	5.3532±0.0552
	200	0.424±0.0001	6.9285±0.0335
	300	0.3925±0.0005	13.8498±0.1097
	500	0.3773±0.0003	17.1934±0.0552
	700	0.3628±0.0003	20.376±0.0552
	900	0.3423±0.0003	24.8756±0.0552
MPP	100	0.4221±0.002	7.3456±0.4483
	200	0.4172±0.0003	8.4211±0.067
	300	0.3896±0.0007	14.4863±0.1536
	500	0.3787±0.0001	16.8715±0.0335
	700	0.3474±0.0005	23.7489±0.1005
	900	0.3353±0.0003	26.412±0.0552
MPF	100	0.4124±0.0002	9.4746±0.0456
	200	0.4053±0.0004	11.03307±0.077
	300	0.3926±0.0002	13.828±0.0438
	500	0.3793±0.0004	16.7398±0.083
	700	0.3582±0.0004	21.3784±0.0956
	900	0.3393±0.0003	25.5194±0.067
Mannitol (STD)	100	0.3853±0.0003	15.4375±0.0552
	200	0.3582±0.0004	21.3784±0.0956
	300	0.3175±0.0002	30.319±0.0336
	400	0.2616±0.0051	42.5812±1.1098
	500	0.2169±0.0086	52.3924±1.878
	600	0.1696±0.0089	62.7745±1.9500

Table 3.9. IC50 value of extracts in Modified hydroxyl radical scavenging Assay

Sample	IC50 value(µg/mL)
STD	481.639
MPF	1359.2
MGP	525.196
MPP	754.935
MGF	1421

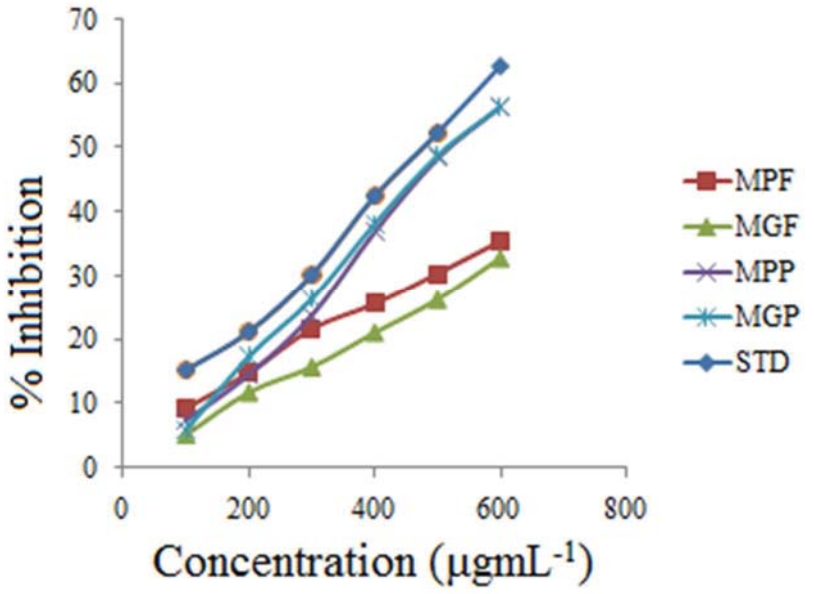

Figure 3.2. Inhibition (%) of hydroxyl radical by Methanol extract of Gulguluthikthakam Kashayam residue (MGP), Gulguluthikthakam Kashayam filtrate (MGF), Prasarinyadi Kashayam residue (MPP), Prasarinyadi Kashayam filtrate (MPF) and Mannitol as STD.

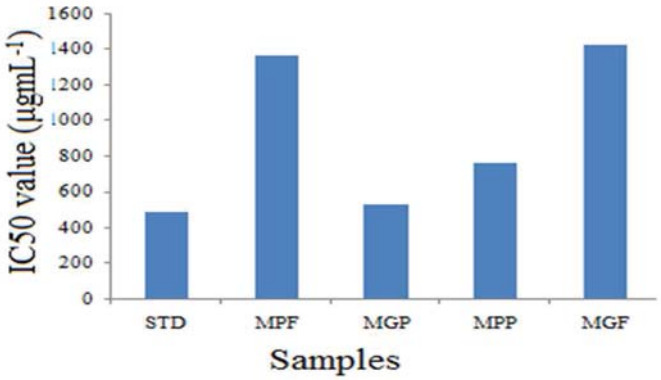

Figure 3.3 IC 50 value of Methanol extract of Gulguluthikthakam Kashayam residue (MGP), Gulguluthikthakam Kashayam filtrate (MGF), Prasarinyadi Kashayam residue (MPP), Prasarinyadi Kashayam filtrate (MPF) and Mannitol as STD in hydroxyl radical scavenging assay.

3.2.4.3. DPPH scavenging assay: DPPH assay method is very simple and is also quick for manual analysis for the total radical scavenging property. This method is not only specific to any particular antioxidant but also applies to the overall radical scavenging capacity of the sample. The DPPH is reacted with absolute ethanol to yield a purple color DPPH radical. The presence of antioxidants which include polyphenols and flavonoids will scavenge the formed DPPH radical and thereby a decreased color will be observed which is spectrophotometrically measured at 517 nm. In this study Figure 3.4 and Table 3.10, showed that all samples had significant levels of radical scavenging activity in a dose dependent manner. All samples inhibited DPPH radicals formed to different extent. IC50 value also calculated, Figure 3.5 and Table 3.11, Higher radical scavenging activity found to be for MGP (317.33 μgmL^{-1}) calculated from the equation y=0.075x+26.20, R^2=0.874 and lower for MPF(652.5925 μgmL^{-1}) calculated from the equation y=0.054x+14.76, R^2=0.990 .While that for L-Ascorbic acid(standard) was found to be 216.5137 μgmL^{-1} and calculated from the equation y=0.092x+26.4, R^2=0.961.MGF and MPP got intermediate inhibition values as 483.0769 μgmL^{-1} (y=0.052x+24.88, R^2=0.862) and 495.3406 μgmL^{-1} (y=0.091x+4.924, R^2=0.956 respectively .Low value of IC50 indicates high radical scavenging nature and high anti-inflammatory activity .

Table 3.10. Antioxidant study by DPPH radical scavenging Assay

Extracts	Concentration (µg/mL)	Absorbance (517 nm)	DPPH radicals scavenged (%)
MGP	100	0.3747±0.0009	26.6588 ± 0.186
	200	0.2882±0.0014	43.5897 ± 0.274
	300	0.2364±0.0011	53.7222 ± 0.2165
	400	0.1962±0.0011	61.6167 ± 0.2055
	500	0.1863±0.0012	63.5349 ± 0.2257
	600	0.1744±0.001	65.8576 ± 0.196
MGF	100	0.3976±0.0009	22.1831 ± 0.1664
	200	0.309±0.0005	39.5185 ± 0.1036
	300	0.2829±0.0002	44.6206 ± 0.0452
	500	0.2255±.0005	55.9535 ± 0.0882
	800	0.1898±.001	62..8498 ± 0.188
MPP	100	0.4578±0.0008	10.3934 ± 0.1494
	200	0.3965±0.0007	22.3918 ± 0.1356
	300	0.3178±0.002	37.7960 ±0 .3625
	400	0.2879±.0011	43.6484 ± 0.2074
	500	0.2466±.0008	51.7518 ± 0.1569
	600	0.2267±0.0002	55.6273 ± 0.0452
MPF	100	0.4179±0.001	18.2031 ± 0.1975
	300	0.3449±0.0006	32.4916 ± 0.1222
	500	0.2877±0.0007	43.6941 ± 0 .1374
	700	0.2364±0.0005	53.7352 ± 0.0883
	800	0.2095±0.0003	58.9939 ± 0.0492
	900	0.1943±0.0003	61.9625 ± 0.0597
Ascorbic acid (STD)	100	0.3559±0.0073	30.3386±1.4248
	200	0.2470±.0104	51.6604±2.0366
	300	0.1873±.0111	63.3392±2.175
	400	0.135±.0058	73.5825±1.1306
	500	0.0882±0.01	82.7363±1.964
	600	0.0554±0.0087	89.1629±1.6943

Table 3.11. IC 50 value of extracts in DPPH radical scavenging Assay

Sample	IC50 value(µg/mL)
STD	216.514
MGP	317.333
MGF	483.077
MPP	495.341
MPF	652.593

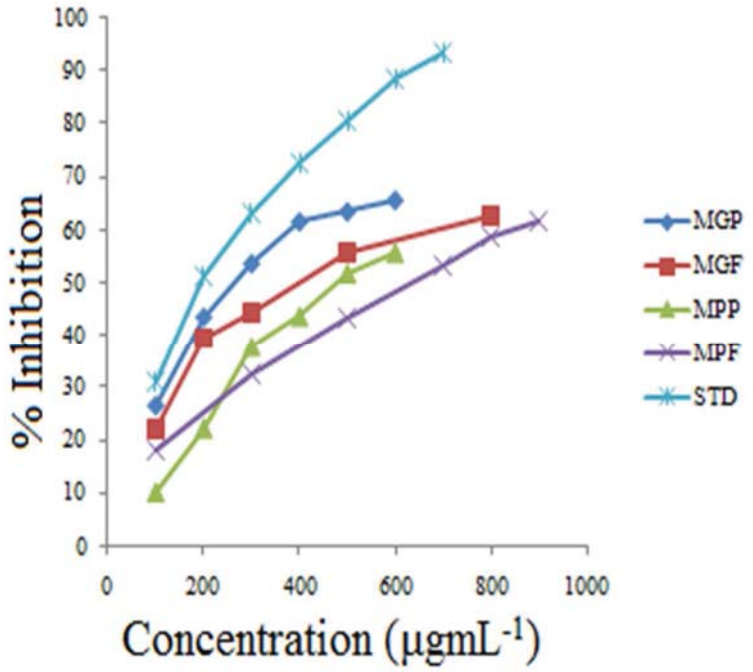

Figure 3.4. Graph of inhibition (%) of DPPH free radical by Methanol extract of Gulguluthikthakam Kashayam residue (MGP), Gulguluthikthakam Kashayam filtrate (MGF), Prasarinyadi Kashayam residue (MPP), Prasarinyadi Kashayam filtrate (MPF) and Ascorbic acid as STD

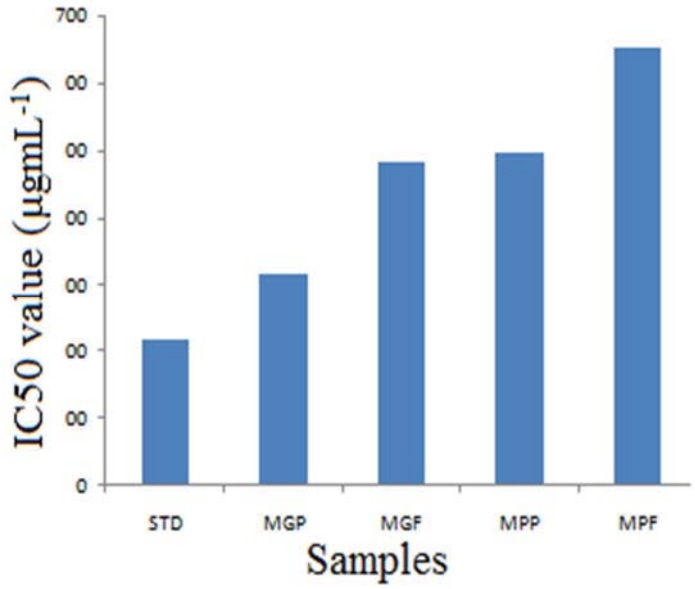

Figure 3.5. IC 50 value of Methanol extract of Gulguluthikthakam Kashayam residue (MGP), Gulguluthikthakam Kashayam filtrate (MGF), Prasarinyadi Kashayam residue (MPP), Prasarinyadi Kashayam filtrate (MPF) and Ascorbic acid as STD in DPPH assay**.**

3.2.5. HPTLC study of phenolics

HPTLC studies of phenolics are presented in Table 3.12. Some of the spots are common to all the extracts with the R_f values-0.89, 0.76 and 0.66. The presence of more phenolic compounds in the methanol extract of Gulguluthikthakam kashayam residue confirmed the spots in HPTLC. Prasarinyadi kashayam filtrate and Prasarinyadi kashayam residue have the same number of spots with approximately the same R_f values. Standard, Gallic acid, gave a spot with R_f value 0.48. All the extracts gave a spot with R_f value near to that of gallic acid that means gallic acid present in both kashayam. Since the spray reagent used was specific to phenolics all the spots shown in Figure 3.6 are due to the phenolics.

Table 3.12. HPTLC study of phenolics

Phenolics determination by HPTLC		
extract	**R_f**	**Standard**
Gulguluthikthakam Kashayam residue	0.89, 0.76, 0.66, 0.62, 0.58, 0.48, 0.31	0.48
Gulguluthikthakam Kashayam filtrate	0.89, 0.76, 0.66, 0.62	
Prasarinyadi kashayam residue	0.89, 0.76, 0.66, 0.63	
Prasarinyadi kashayam filtrate	0.89, 0.76, 0.66, 0.64	

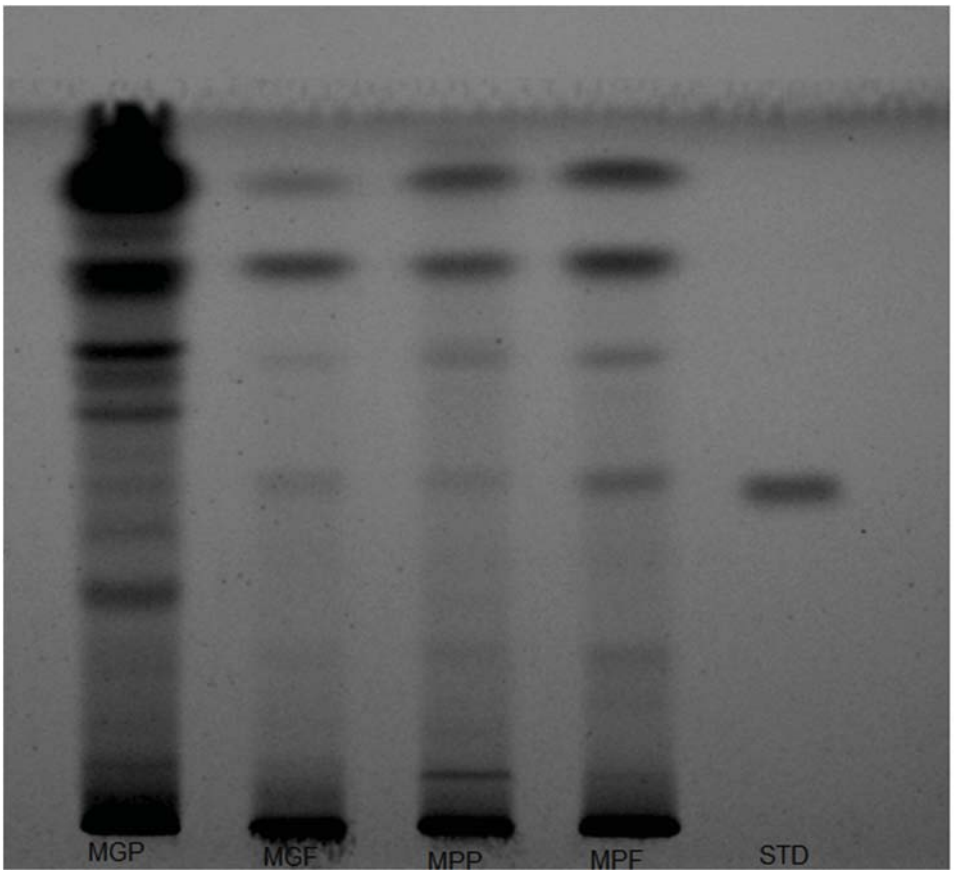

Figure 3.6. HPTLC of Phenolics

3.2.6. HPTLC study of flavonoids

HPTLC study of flavonoids are presented in Table 3.13. The spots with R_f values 0.3, 0.47, 0.59 are the same in all extracts, meaning the same compounds are present in all extracts. Number of spots higher in the Gulguluthikthakam kashayam residue compared to others indicate that more flavonoids are present in Gulguluthikthakam kashayam residue. When comparing the spots observed in the Gulguluthikthakam kashayam residue and Gulguluthikthakam kashayam filtrate, Gulguluthikthakam kashayam residue contains a larger number of flavonoids. Consider the Prasarinyadi kashayam filtrate and Prasarinyadi kashayam residue both have the same number of flavonoids with approximately the same R_f values. Spots revealed in the Figure 3.7 are due to flavonoids only because the plate was sprayed with the, aluminium chloride, reagents specified for flavonoids. Standard used for the flavonoids was quercetin and it got a R_f value 0.51. A feeble band approximately the same as that of quercetin have been seen in all extracts indicating the presence of quercetin.

Table 3.13. HPTLC study of flavonoids

Flavonoids determination by HPTLC		
Extract	**R_f**	**Standard**
Gulguluthikthakam Kashayam residue	0.87, 0.75, 0.68, 0.65, 0.6, 0.59, 0.5, 0.47, 0.3	0.513
Gulguluthikthakam Kashayam filtrate	0.3, 0.47, 0.59, 0.75	
Prasarinyadi kashayam residue	0.3, 0.47, 0.59, 0.76	
Prasarinyadi kashayam filtrate	0.3, 0.47, 0.59, 0.77	

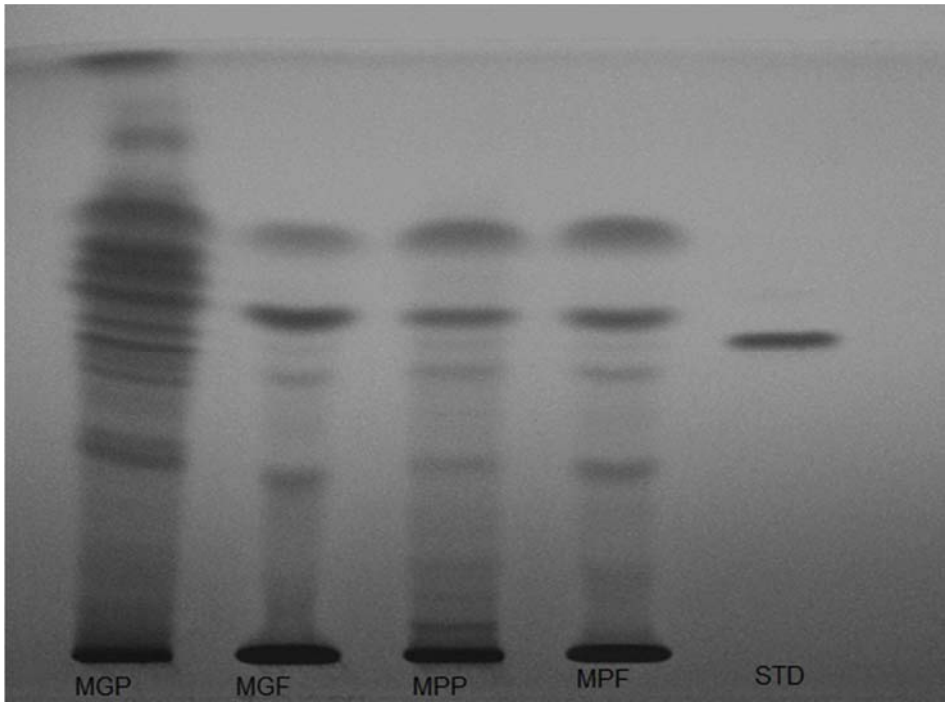

Figure 3.7. HPTLC of flavonoids

3.2.7. HPTLC study of alkaloids

HPTLC study of alkaloids are presented in Table 3.14. The spots with R_f values, 0.04, 0.09, 0.21, 0.38, are common to all extracts meaning that the same alkaloids are found in all extracts. Gulguluthikthakam Kashayam residue got more spots with R_f values 0.04, 0.09, 0.15, 0.21, 0.24, 0.29, 0.38, 0.4, 0.49, 0.61, 0.68, 0.74, and 0.86. Gulguluthikthakam Kashayam filtrate, Prasarinyadi kashayam filtrate and Prasarinyadi kashayam residue have the same number of alkaloids with approximately the same R_f values. Standard used was vasicine and gave a spot with R_f value 0.04 was present in all extracts and confirmed its presence in all extracts Figure 3.8.

Table 3.14. HPTLC study of alkaloids

Alkaloid identification by HPTLC		
Extract	**R_f**	**Standard**
Gulguluthikthakam Kashayam residue	0.04, 0.09, 0.15, 0.21, 0.24, 0.29, 0.38, 0.4, 0.49, 0.61, 0.68, 0.74, 0.86	0.04
Gulguluthikthakam Kashayam filtrate	0.04, 0.09, 0.21, 0.38	
Prasarinyadi kashayam residue	0.04, 0.09, 0.21, 0.39	
Prasarinyadi kashayam filtrate	0.04, 0.09, 0.21, 0.40	

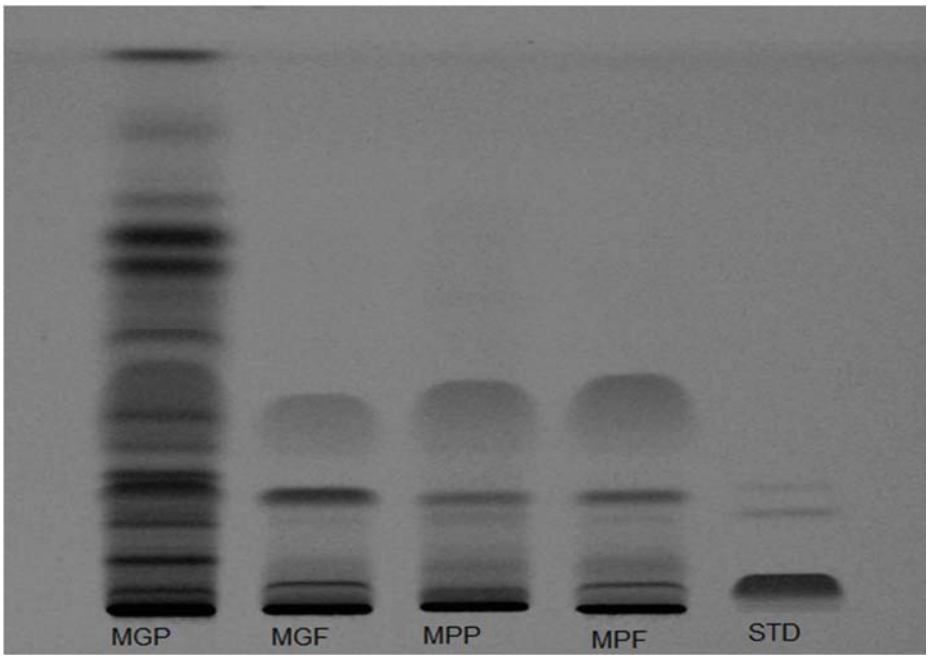

Figure 3.8. HPTLC of alkaloids

3.3. Experimental

Chemicals: DPPH purchased from Sigma-Adrich Germany.2 -deoxy-D-ribose extra pure, ascorbic acid and mannitol were obtained from Loba chemie Pvt Ltd, ethanol from Heyman group Ltd UK, Methanol from Spectrochem Mumbai India. All other chemicals of analytical grade were purchased from Merck.

Instruments: Centrifuge machine used to separate the kashayam into filtrate and residue. Lyophilizer was used for the removal of water from the filtrate. Hot air oven used to dry the residue at 50^0C.Soxhlet apparatus used for the extraction and Elico UV-Vis spectrophotometer used for measuring the absorbance in antioxidant study.

Preparation of Methanol extract: Gulguluthikthakam Kashayam and Prasarinyadi Kashayam are procured from an Ayurveda Institute the Arya Vaidya pharmacy, Coimbatore. The kashayam is a water extract and comes as a suspension. This suspension is administered to the patients and advised them to take 30 mL aliquots two or three times daily. The water dissolved compounds in the suspension will get absorbed to the system easily whereas the undissolved residue may /may not. To ascertain whether the residue also gives some amount of antioxidant activity to facilitate the therapeutic value, the decoction is separated into the soluble portion and the residue and studied separately.

About 1500mL kashayam was centrifuged and the residue obtained dried in the oven at 50^0C. The filtrate was lyophilized to get the semi solid dark brown colored substance. The residue and the lyophilized filtrate were extracted with methanol using Soxhlet extractor. The methanol extracts were shaken with petroleum ether to remove petroleum ether soluble fractions (fatty substances). Methanol extract of residue and

the filtrate of both the kashayam were analyzed for phytochemical contents and antioxidant activity using standard procedure.

Phytochemical analysis of methanol extract of Gulguluthikthakam Kashayam and Prasarinyadi Kashayam:

Identification of phytochemical constituents is carried out using standard procedure as described by Rahman et al [23] and Shanmugam et al [24].

Test for Carbohydrate

Molisch's Test: 2 mL of the extract taken in a test tube and 2 mL of the Molisch's reagent is added and shaken carefully, then about 1 mL. of conc. H_2SO_4 is poured through the side of the test tube and allowed to stand for one minute. A violet color ring appears at the junction of the two layers indicating the presence of carbohydrates.

Fehling's test: Equal volume of Fehling's A (Copper sulfate in distilled water) and Fehling's B (Potassium tartrate and Sodium hydroxide in distilled water) reagents are mixed and few drops of sample is added and boiled, a brick red precipitate of cuprous oxide formed.

Test for Alkaloids

The following color tests are used to detect the presence of an Alkaloid in the sample.

(1) Wagner's test: Two drops of Wagner's reagent are added to 2mL of extract and mixed well. Alkaloids give a reddish-brown precipitate with Wagner's reagent [Solution of iodine in potassium iodide].

(2) Hager's test: 2mL of extract is mixed with a few drops of Hager's reagent [saturated solution of Picric acid]. It produces yellow color precipitate indicating the presence of alkaloids.

(3) Mayer's test: 1mL of extract is mixed with a few drops of Mayer's reagent (potassium mercuric iodide solution). Resulting cream color precipitate indicates the presence of alkaloids.

Test for amino acids and proteins

(1) Ninhydrin test: To 2 mL aqueous extract 2 mL alcoholic Ninhydrin is added and then heated. Formation of blue to Violet color indicates the presence of amino acids.

(2) Biuret test: 0.5mg extract mixed with 40 % NaOH solution and a few drops of 1% $CuSO_4$ solution is added. The solution turns violet in color for proteins.

Test for Tannin

(1) Ferric chloride test: In a test tube 1 mL extract and 2 mL ferric chloride reagent are mixed and shaken well. An intense green or blue color developed indicates the presence of tannins.

(2) Lead acetate test: 1 mL extract and few drops of 10% lead acetate are mixed and shaken well. Reddish brown precipitate is formed indicate the presence of tannins

Test for Glycoside

Killer – Killiani test: To an extract in Glacial Acetic Acid, a few drops of $FeCl_3$ and conc. H_2SO_4 is added. Formation of reddish-brown color at the junction of two layers and changing of the upper layer into bluish green indicates presence of Glycoside.

Test for Saponins

About 1 mL of extract is diluted to 10 mL by distilled water and shaken in a graduated cylinder for 15 minutes. Formation of 1cm layer of froth indicates presence of Saponins.

Test for Phenols

In a test tube 2 mL of extract mixed with 2 mL of $FeCl_3$ solution. Blue or deep green color of the solution is suggestive to the presence of phenols.

Test for Flavonoids

(1) Ferric chloride test: In a test tube 1mL extract is treated with a few drops of ferric chloride solution. Then the formation of green color indicates the presence of flavonoids.

(2) Alkaline reagent test: When the extract is treated with sodium hydroxide solution, increase the intensity of yellow color and decolorize by the addition of dilute hydrochloric acid.

(3) Lead acetate solution test. When the test solution is treated with 10% lead acetate solution yellow precipitate formed indicates the presence of flavonoids.

Test for Sterols and Triterpenoids

Salkowskis' test: Take the extract in chloroform with few drops of concentrated sulfuric acid, shaken well and allow to stand for some time, red color appears in the lower layer indicate the presence of sterols and the formation of yellow color indicate the presence of triterpenoids.

Quantification of phytochemicals

Estimation of Total Phenolics by modified Folin –Ciocalteau method

The amount of total phenol contents was determined by the standard spectroscopic methods with slight modification [18]. To 1 mL extract (100-1000µg/mL) distilled water is added to make up to 3.5mL. Then 0.125 µL Folin's reagent is added and incubated at room temperature for 6 minutes. Then 1.25 mL of 7% sodium carbonate is added and incubated at room temperature for 90 minutes. The absorbance is measured at 760nm.Total phenolics content was determined from extrapolation of a calibration graph which was made from Gallic acid.

Estimation of Flavonoids by Aluminium Chloride method

The amount of total flavonoids contents was determined by the spectroscopic methods with slight modification [25]. To 1mL of the extract (concentration varying from 50 to 250 µg) distilled water is added to make up to 2.5mL.Then 75 µL of 5% $NaNO_2$ is added and incubated at room temperature for 5 minutes. Then 150 µL of 10% $AlCl_3$ and incubated at room temperature for 6 minutes. Then 300 µL of 1M NaOH is added and mixed well and the resulting pink color solution is spectrophotometrically measured at 415 nm. The calibration graph prepared by preparing rutin solution at concentrations 50-250 µg/mL in alcohol.

Estimation of Total Alkaloids by gravimetric method

The amount of total alkaloid contents was determined by the gravimetric methods with slight modification [24]. 1gm of extract dissolved in the minimum amount of alcohol and then pH of the solution is set to 2 with HCl. Then the pH of the solution is raised to 12 with NaOH and extracted with ethyl acetate. The extract was collected and

evaporated to dryness by rotary evaporation under reduced pressure and weigh the obtained residue and calculate the % alkaloid as per the equation

%Alkaloid = (W2-W1/Weight of sample) x100

W1-weight of filter paper

W2-weight of filter paper + alkaloid precipitate

Estimation of Total Steroids by Harborne (1973)

The amount of total steroid contents was determined by the gravimetric methods with slight modification [24]. 1gm of extract is hydrolyzed by boiling in 50 mL dilute hydrochloric acid solution for about 30 minutes. It is filtered using weighed Whatman filter paper (42). Then the filtrate is transferred to a separating funnel. Equal volume of ethyl acetate is added to it, mixed well and allowed to separate into two layers. The ethyl acetate layer recovered, while the aqueous layer was discarded. The extract is dried at 100^0C for 5 minutes in a steam bath. It is then heated with amyl alcohol (equal amount) to extract the steroid. Precipitate is filtered through reweighed Whatman filter paper (42), allowed to dry and the dried extract is cooled in a desiccator and reweighed. The process is repeated two more times and an average is obtained.

The concentration of steroid was determined and expressed as a percentage as in the total alkaloid determination.

Antioxidant Property of Gulguluthikthakam Kashayam and Prasarinyadi Kashayam

Ferric ion reducing antioxidant power: Ferric reducing property or antioxidant power is determined as per the standard procedure of Qyaizu M with slight modification [26]. To 1mL of the extract (100-350µg/ mL) 2.5mL phosphate buffer and 2.5mL of 1% potassium ferricyanide are added. Then the mixture heated in a water bath at 50^0C for 20 minutes, then rapidly cooled and mixed with 2.5mL of 2.8% trichloroacetic acid. From this solution 2.5 mL is pipetted out and mixed with 2.5mL distilled water and 0.5mL of 1% ferric chloride. The resulting solution mixed well and kept aside for 10 minutes. Absorbance of the resulting solution measured using UV-Vis spectrometer at λ 700 nm. Ascorbic acid used as a positive control.

Modified hydroxyl radical scavenging assay: The hydroxyl radical scavenging activity of the methanol extract of Gulguluthikthakam Kashayam and Prasarinyadi Kashayam was determined by the procedure described by Shanmugam et al with slight modification[24] .In a test tube, 1.5mL of phosphate buffer mixed with 1mL of 10mM 2-deoxy-D-ribose, .2.5mL of 20mM Na_2-EDTA, .2.5mL of 20mM $FeCl_2$ solution, 0.1mL of sample solution or standard (100-900µg/mL), 1.9mL distilled water and 0.5mL of 10mM H_2O_2 rapidly in this order. The resulting mixture was incubated for 1Hr at 37^0C in a water bath. After that the reaction was arrested by adding 2.5mL of 2.8% tri chloro acetic acid then added 2.5mL of 1% thio barbituricacid and kept the reaction mixture at 100^0C in a boiling water bath for 10 minutes. Cool the mixture under running tap water and recorded the absorbance at 415 nm. Mannitol was the positive control used. Hydroxyl radical scavenging activity expressed in

percentage. The percent antioxidant or radical scavenging activity was calculated using the following equation.

$$\% \text{ Hydroxyl radical scavenging activity } = \left(\frac{\text{Test sample absorbance}}{\text{Blank sample absorbance}} \right) \text{ x } 100$$

DPPH scavenging assay: In a test tube 0.250mL of 0.5mM DPPH radical solution mixed with 2mL of the extract or the standard solution and the reaction mixture was vortexed for 10 s and incubated in the dark at room temperature for 30 minutes. The absorbance is recorded at 517 nm by UV-Vis spectrometer. Ascorbic acid was used as the standard antioxidant compound [29]. The free radical scavenging activity of each fraction was determined by comparing its absorbance with that of a blank solution (no sample). The ability to scavenge the DPPH radical was expressed in percentage. The percentage antioxidant activity was calculated using the equation given

% Antioxidant activity= (A0-A1)/A0 x 100.

Where A0 –absorbance of blank solution and A1-absorbance of sample.

Statistical analysis: The recorded values were expressed as Mean ± SD of three replicate determinations using MS Excel software.

HPTLC was performed with the instrument CAMAG Linomat 5 "Linomat5_171118" S/N 171118 (1.00.12). HPTLC plates silica gel 60 F 254 with plate size 8.0 x 10.0 cm.

4. 1 HPTLC study of Methanol extracts

Methanol extract prepared used for the identification and quantification of Alkaloids, phenolics and flavonoids with the help of HPTLC.

HPTLC study of alkaloids

HPTLC study of alkaloids carried out with the solvent system Toluene: Ethyl Acetate: Methanol (7: 2: 1) for alkaloids, 5 Minutes run in Twin Trough Chamber 20x10cm made of glass, oven temperature at 105^0 C, Injection volume was 10.0 mL. Derivatization is done with the Drangendroff's reagent. Injection volume was 50.0 mL, oven temperature at 105^0 C, run time 20 Minutes. Vasicine, used as standard, also run along with the sample

HPTLC study of phenolics

Gallic acid used as standard for the identification of phenolics on HPTLC with the solvent system Chloroform: Ethyl acetate: Formic acid (5: 4: 1), run time 5-minute, application volume 10 mL, drying temperature 105^0 C .0.5% Ferric Chloride Solution in methanol used for derivatization for 20 minutes run time, injection volume 50 mL dried at 105^0 C.

HPTLC study of flavonoids

Quercetin was the standard used in the identification of flavonoids using HPTLC in the solvent system Toluene: Ethyl acetate: Formic acid (5: 3: 1) for 5 minutes run time, application volume 10 mL, drying temperature 105^0 C. Sodium acetate-aluminium chloride solutions used for the derivatization of the plate for 20minutes run time, application volume 50mL, drying temperature 105^0 C.

3.4. CONCLUSION

Preliminary phytochemical analysis of methanol extract of both the kashayam answered most of the phytochemical tests. Antioxidant study of Gulguluthikthakam kashayam and Prasarinyadi kashayam were carried out using methanol extract of its residue and filtrate as per

standard procedure with slight modification and found that methanol extract of Gulguluthikthakam kashayam residue has highest radical scavenging property than other extracts in all methods (FRAP, hydroxyl radical scavenging and DPPH assay). Quantitative analysis revealed the higher quantity of alkaloids, phenolics, flavonoids and steroids in the Gulguluthikthakam kashayam residue. Other extracts also contain alkaloids, phenolics, flavonoids and steroids in a sufficient amount to exhibit radical scavenging activity. Finally, the results in this study prove that both the kashayam are a good source of radical scavengers and have potent anti-inflammatory properties. The results obtained under each method depends on the chemical compounds present in each extract. The isolation and the identification of each compound will give insight towards the mechanism of the therapeutic effect of the kashayam in treating different types of oxidative stress related diseases.

HPTLC study of phenolics carried out along with the gallic acid as the reference standard. The R_f value of gallic acid obtained was 0.48. All the extracts got a spot very near to that of gallic acid. This indicates the presence of gallic acid in both the kashayam.

Gallic acid is a strong natural antioxidant, present in plants. It is a polyphenolic compound and has the ability to remove reactive oxygen species (ROS), e.g., hydrogen peroxide, superoxide anions, hypochlorous acid and hydroxyl radicals. It has been reported to prevent a number of disorders, including cardiovascular disease, cancer, inflammation, infection [19] diabetes [20], and neurological ailments [21].

HPTLC study of flavonoids carried out with quercetin as the standard. All the extracts confirmed the presence of quercetin with the comparable R_f value.

Quercetin is a natural flavonoid and a natural antioxidant. It is a pigment present in vegetables and fruits. It is a versatile antioxidant known to possess protective abilities against tissue injury induced by various drug toxicities.

HPTLC study of alkaloids carried out along with the standard vasicine. All the extracts confirmed the presence of vasicine.

Vasicine is the main constituent of Sida rhombifolia and Adhatoda vasica. It is an alkaloid having many medicinal properties, anti- inflammatory, bronchodilator, IgE-dependent mediator, respiratory stimulant, hypotensive, cardiac depressant, uterotonic, ant- tuberculosis [22].

Alkaloids, phenolics and flavonoids are the secondary metabolites quantified by HPTLC in this study. All the extracts contain the alkaloids, phenolics and flavonoids in a sufficient amount to satisfy its medicinal use.

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Chapter 4

Gas Chromatography -Mass spectrometry study of Prasarinyadi Kashayam and Gulguluthikthakam kashayam

4.1. Introduction

4.2. Results and Discussion

4.2.1. GCMS analysis of Prasarinyadi Kashayam

4.2.2. GCMS study of Gulguluthikthakam kashayam

4.3. Experimental

2.4. Conclusion

References

Abstract

Kashayam are prepared by mixing the ingredients in a particular ratio with the required amount of water and boiling for a long time to reduce the volume to a specified concentration. As the temperature is maintained at 80^0C majority of the volatile component may escape during preparation. However, the determination of the volatile oil principle by Clevenger apparatus gave volatile components. This was subjected to GCMS study to identify the volatile component present.

4.1. Introduction

Gas chromatography- mass spectrometry (GC-MS) is a technique which consists of two analytical procedures in sequence, namely a gas chromatography separation followed by mass spectroscopy detection (MS). This hyphenated analytical methodology is a widely used technique in many branches of science and technology. GC can separate volatile and semi-volatile compounds with great resolution, but it cannot identify them. To enable identification, the mass spectrometric detector (MS) is combined with the GC which helps to give the fingerprint of the molecule

i.e., its mass spectrum. This provides the information about molecular weight and the elemental composition. This hybrid instrument provides two separate dimensions of information about the components in the sample; retention time (RT) and ionization mass spectra of the separated component molecules based on their mass to charge ratio(m/z). Retention time is related to the specific chemical properties of the molecule (specificity of the capillary column, flow rate, temperature, characteristics of the components of the analyts) while molecular weight derived from the mass spectrum is indicative of atomic composition (the molecular ion), and the structural picture of the components.

In GC the separation of the components in the analytes is affected by the differential affinity of the components between the stationary phase and mobile phase, and so each component will elute out from the column at a different time interval called the retention time (RT). Identification is based on comparison of the retention time of the peaks in a sample to those from standards of known compounds, analyzed using the same analytical conditions. However, GC alone cannot be used for the specific identification of unknowns, which is where hyphenation to Mass Spectrometry works very well. MS can be used as a sole detector, or the column eluent can be split between the MS and GC detector(s).

MS is an analytical technique that measures the mass-to-charge ratio (m/z) of charged particles and therefore can be used to determine the molecular weight and elemental composition, as well as elucidating the chemical structures of molecules. Data from a GC-MS gives three diagnostic parameters, provides mass spectra that can be used for identification of unknown compounds plus the chromatogram that can be used for qualitative and quantitative analysis of the constituent compounds of the analytes.

The sample mixture is first separated by the GC before the analytes molecules are eluted into the MS for detection. They are transported by the carrier gas, which continuously flows through the GC and into the MS, where it is evacuated by the vacuum system. Within the mass spectrometer, the neutral molecules are first ionized, most commonly by electron ionization (EI). This high energy ionization can result in an unstable molecular ion and excess energy can be lost through fragmentation. Bond breakage(s) can lead to the loss of a radical or neutral molecule and molecular rearrangements can also occur. These reactions result in the formation of a number of ions of different masses, the heaviest being the molecular ion with fragment ions of various lower masses, depending on the structure of the component molecules.

There are numerous different mass analyzer types, and this is where the vast differences in mass resolution. Mass resolution is the ability of the mass analyzer to separate ions with very small differences in m/z. Unit mass resolution instruments can only separate nominal masses or those down to a single decimal place, whereas high mass resolution (HRMS) instruments can separate them to four or five decimal places.

The most common type of unit mass detector is the quadrupole mass analyzer, which is a scanning instrument and by changing the voltage allows only ions of a certain m/z to have a stable trajectory through the four poles to reach the ion detector. The two-dimensional chromatogram, as shown in Figure 4. 1 is produced by summing the abundances of all the ions at a single data point and plotting it against the retention time (RT) to produce a total ion chromatogram (TIC), which is more comparable to a chromatogram produced by a common GC detector. However, each data point in the total ion chromatogram is a

separate mass spectrum and can usually be opened in a separate window in the software.

In GC, the retention time is used for the identification of target analytes and usually the area under each peak is used for quantitation. For accurate quantitation, the peaks need to have good chromatographic separation with baseline resolution, as shown in Figure 4.1 peaks at RT1 and RT2. With GC-MS the mass spectrum provides an additional method for confirmation of the target analytes using the full mass spectrum or using the presence and relative ratios of a few of the ions. Quantitation using GC-MS data is usually from the area of a single, unique ion as it is less likely to have interferences from co-eluting peaks than by using the area under the TIC peak.

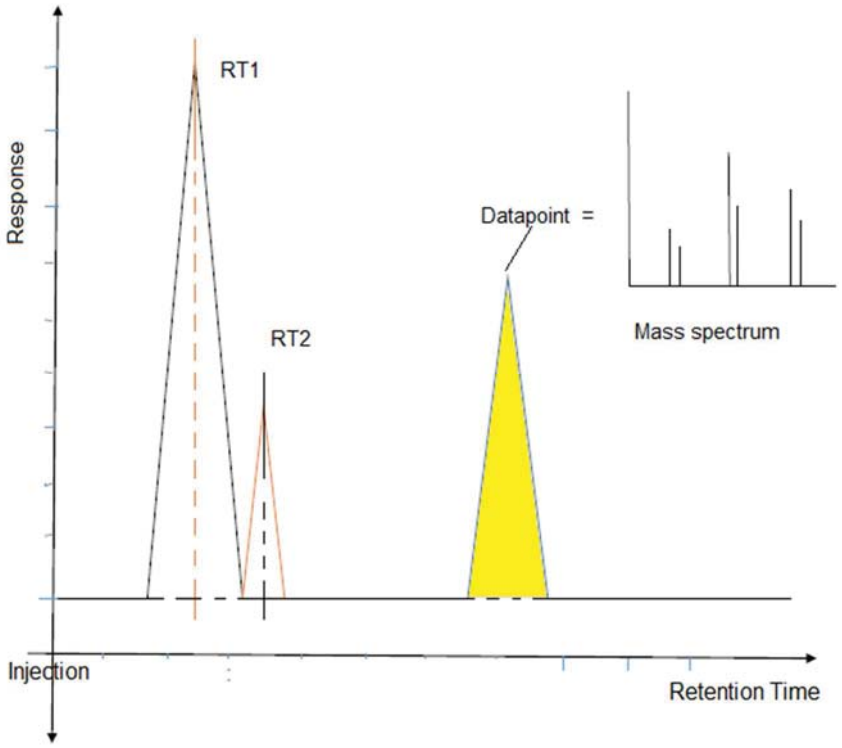


Figure 4. 1. Two-dimensional mass chromatogram

GCMS is a very valuable analytical method to separate and identify the volatile components in a multicomponent mixture/phytochemical extract. The number of peaks in the TIC chromatogram represents the individual chemical molecular entity. Each peak will appear at a specific retention time. The retention time (RT) is a diagnostic parameter. From the mass spectral degradation pattern of individual peaks, the structure of the compounds could be deduced, by comparing a query mass spectrum with reference to MS in a library utilizing the spectrum matching. Moreover, from the m/z value of the molecular ion and secondary ions in the spectra the molecular structure also could be derived.

4.2. Results and Discussion

Determination of volatile oil: As per the literature the plants used for the preparation of kashayam contain high amounts of different volatile constituents. But the total amount of volatile constituents of both the kashayam obtained in our study is only 0.05%. That may be due to the loss of highly volatile compounds during the preparation of the kashayam.

4.2.1. GCMS analysis of Prasarinyadi Kashayam

The volatile principle obtained on hydro distillation was subjected to GCMS analysis that revealed the presence of many compounds, and the ion chromatogram is shown in Fig 4.2. On comparison of the mass spectra of the constituents with the NIST MS libraries the eight phyto - constituents were characterized and identified (Table 4.1). The source plant from which these constituents obtained were also listed in table 4.1.

The eight compounds that have been identified by spectrum comparison were analyzed with the degradation pattern of the molecular structures. The degradation scheme depicted for all the eight compounds of the EI spectrum fully agrees with the m/z values of the spectrum obtained for each of the peaks. Thus, the structures of the compounds are confirmed.

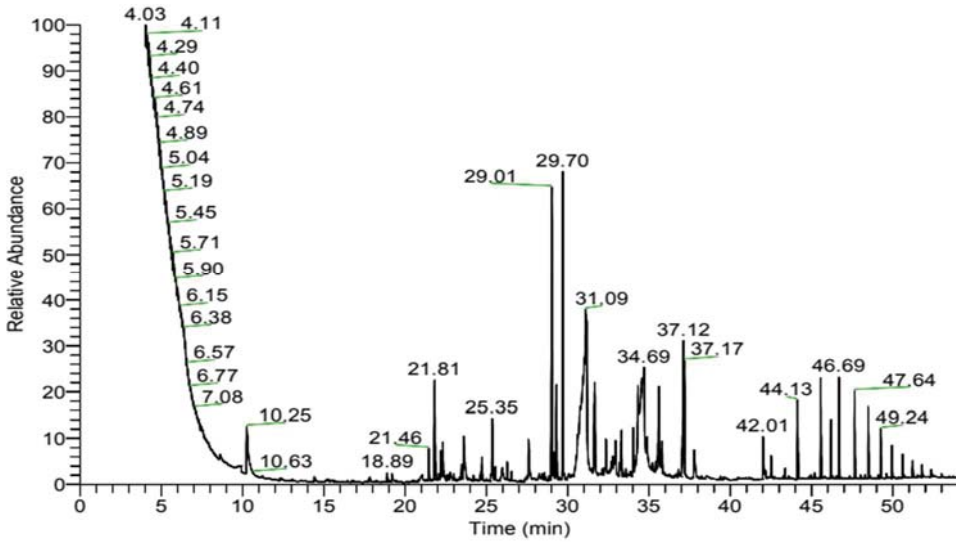


Figure 4.2.TIC (Total Ion Chromatogram) of volatile oil of Prasarinyadi kashayam.

Table 4.1 Compounds identified from the volatile oil of Prasarinyadi kashayam.

SI No.	Retention time	Compound name	IUPAC name	Molecular formula	Molecular weight	Plant source
1.	21.46	Ar-Curcumene	1-methyl-4-(6-methylhept-5-ene-2-yl) benzene	$C_{15}H_{22}$	202.17	*Zingiber officinale* Rosc.
2.	21.81	Eudesmol	2- [(2R, 4aR, 8aS)-4a-methyl-8-methylidene -1, 2, 3, 4, 5, 6, 7, 8a-octahydronaphthalene-2-yl] propan-2-ol	$C_{15}H_{26}O$	222.18	*Zingiber officinale* Rosc.
3.	22.18	Carotol	(3R, 3aS, 8aR)-6, 8a-dimethyl-3-propan-2-yl-1, 2, 3, 4, 5, 8-hexahydro azulen-3a-ol	$C_{15}H_{26}O$	222.21	*Alpinia calcarata* Roscoe
4.	23.6	Calamenene	(1S, 4S)-1, 6-dimethyl-4-propan-2-yl-1, 2, 3, 4-tetrahydronaphthalene	$C_{15}H_{22}$	202.18	*Zingiber officinale* Rosc.
5.	25.35	4-O-methyl Syringic acid	*3,4,5-Trimethoxybenzoic acid*	$C_{10}H_{12}O_5$	212.07	*Alpinia calcarata* Roscoe
6.	29.70	Cembrene	(1E, 3Z, 6E, 10E)-3, 7, 11-trimethyl-14-propan-2-ylcyclotetradeca -1, 3, 6, 10-tetraene	$C_{20}H_{32}$	272.23	*Zingiber officinale* Rosc.
7.	31.66	Thunbergol	(2E, 7E, 11E)- 1, 7, 11-trimethyl-4-propan-2-yl cyclotetradeca -2, 7, 11 -trien-1-ol	$C_{20}H_{34}O$	290.23	*Zingiber officinale* Rosc.
8.	35.61	[6]-Paradol	1-(4-hydroxy-3-methoxyphenyl) decan-3-one	$C_{17}H_{26}O_3$	278.16	*Zingiber officinale* Rosc.

The compound with the Retention Time of 21.46 min. gave a molecular ion at m/z value of 202.17($C_{15}H_{22}$, calculated for 202.341 monoisotopic). This is identified as α - Curcumene or ar-Curcumene a sesquiterpenoid, 1-methyl-4-(6-methylhept-5-ene-2-yl) benzene. This molecule is a constituent of *Zingiber officinale* Rosc. [1] used in the Kashayam. It gave the base peaks at m/z 119.10 and other major peaks at m/z 91.06, 59.05. The mass spectrum of ar-Curcumene (Fig.4.3) and its fragment pattern (scheme 4.1) are given.

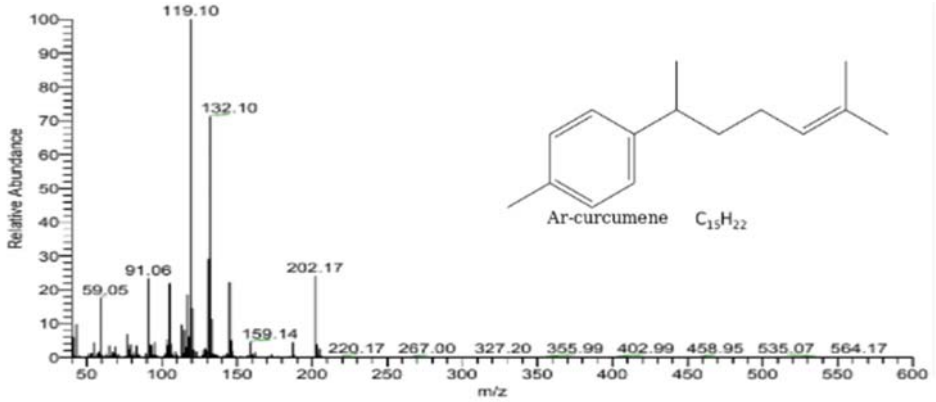
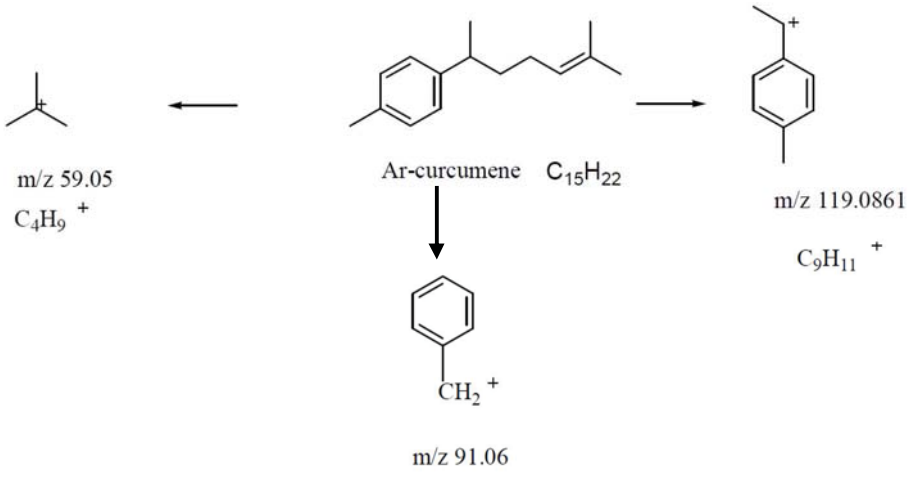


Figure.4.3. GCMS spectrum of Ar-Curcumene.



Scheme 4.1. Fragments leading to the determination of the structure of Ar-Curcumene.

The second compound with retention value of 21.81min. is identified as Eudesmol which gives molecular ions at m/z 222.18 (222.19, mono isotopic). This is identified as Eudesmol with a molecular formula $C_{15}H_{26}O$.It is a sesquiterpenoid. 2- [(2R, 4aR, 8aS)-4a-methyl-8-methylidene -1, 2, 3, 4, 5, 6, 7, 8a-octahydronaphthalene-2-yl] propan-2-ol, it gave the base peaks at m/z 189.16 and other major peaks at m/z 204.18, 133.11, 161.15 and 91.04. This molecule is a constituent of *Zingiber officinale* Rosc. [1] used in the Kashayam. The mass spectrum and fragmentation pattern are given in Fig.4.4 and scheme 4.2 respectively.

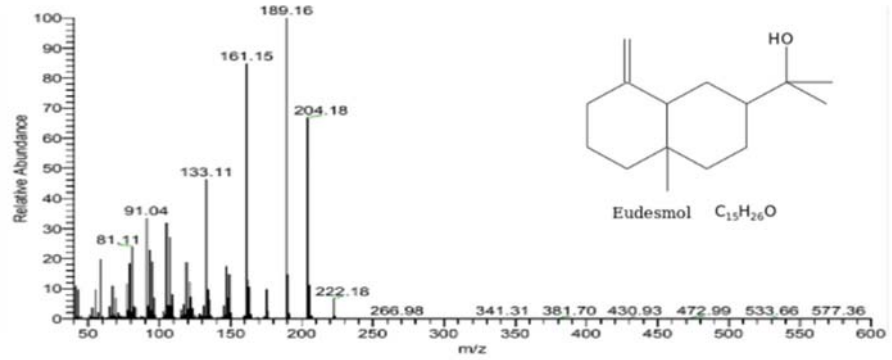
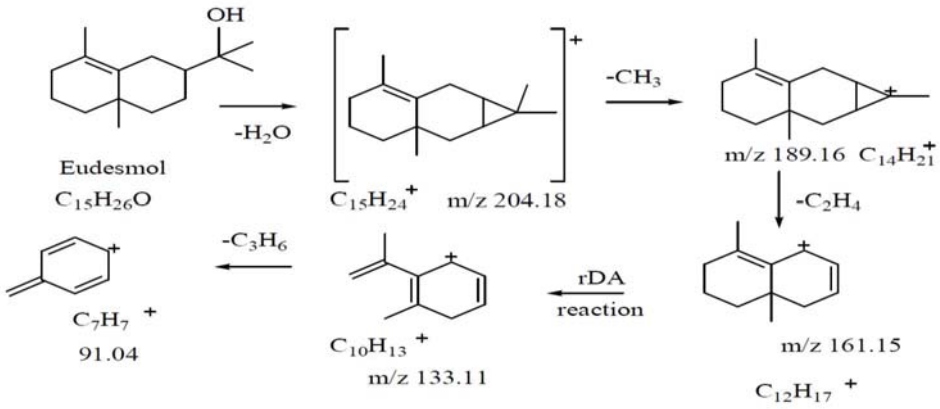


Figure 4.4. GCMS spectrum of Eudesmol



Scheme 4.2. Fragments leading to the determination of the structure of Eudesmol

The third compound with the retention time of 22.18 min. gave a molecular ion at m/z of 222.17($C_{15}H_{26}O$, calculated for 222.19, monoisotopic). This is identified as Carotol with the major peak at m/z 149.13 and fragments at m/z 189.16 and 161. It is a sesquiterpenoid, (3R, 3aS, 8aR)-6, 8a-dimethyl-3-propan-2-yl-1, 2, 3, 4, 5, 8-hexahydro azulen-3a-ol. This compound is a constituent of *Alpinia calcarata* Roscoe [2] used in the kashayam. The mass spectrum (Fig.4.5) and fragmentation pattern is given in scheme 4.3.

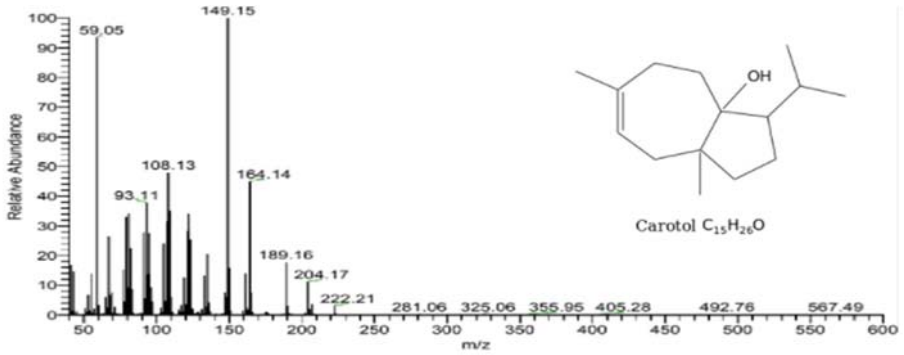
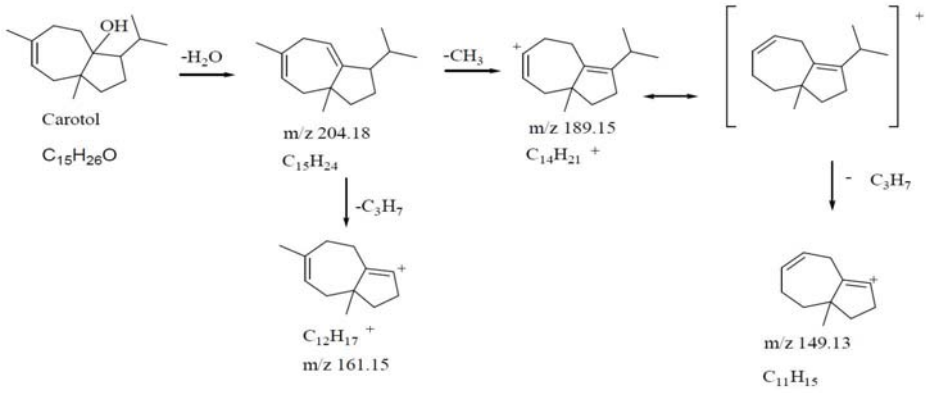


Figure 4.5. GCMS spectrum of Carotol



Scheme 4.3. Fragments leading to the determination of the structure of Carotol

The fourth compound with the Retention Time of 23.60 min. gave a molecular ion at m/z value of 202.18($C_{15}H_{22}$, calculated for 202.17 monoisotopic). This is identified as – Calamenene, a sesquiterpenoid, (1S, 4S)-1, 6-dimethyl-4-propan-2-yl-1, 2, 3, 4-tetrahydronaphthalene. This molecule is a constituent of *Zingiber officinale* Rosc. [1] used in the Kashayam. It gave the base peaks at m/z 69.08 and other major peaks at m/z 118.10 and 159.13 as shown in the figure 4.6. Its fragments are given in scheme 4.4.

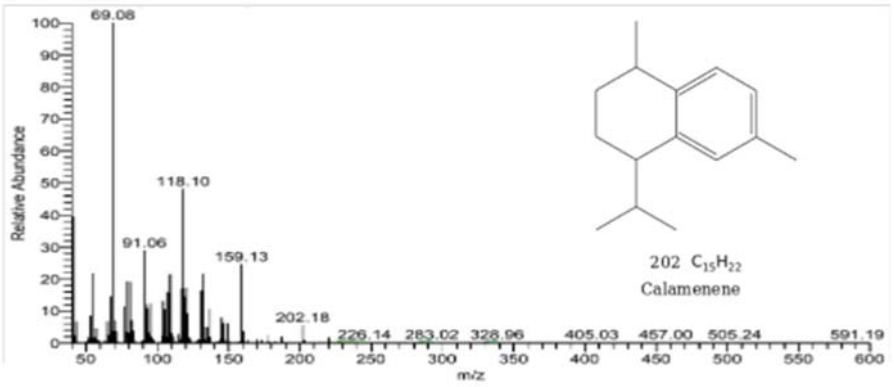
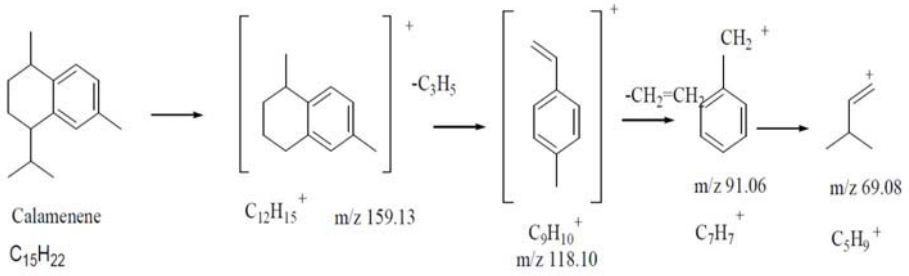


Figure 4.6. GCMS spectrum of Calamenene



Scheme 4.4. Fragments leading to the determination of the structure of Calamenene

The fifth compound with retention value of 25.35 min. is identified as 4-O-methyl syringic acid,3,4,5-Trimethoxybenzoicacid ,which gives molecular ions at m/z 212.07 ($C_{10}H_{12}O_5$, 202.07,

monoisotopic). This molecule is a constituent of *Alpinia calcarata* Roscoe [2] used in the kashayam. It gave the base peaks at m/z 105.03 and other major peaks at m/z 194.06 ,167.10 and 77.05 as shown in the figure 4.7. Its mass fragments are given in scheme 4. 5.

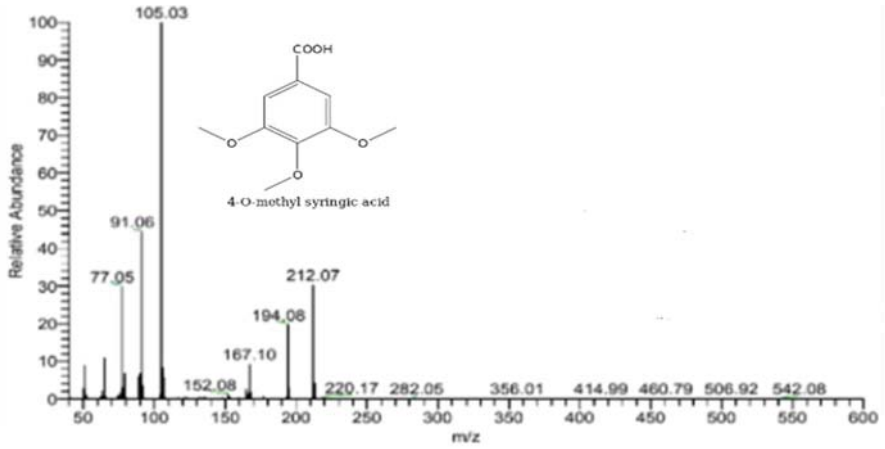
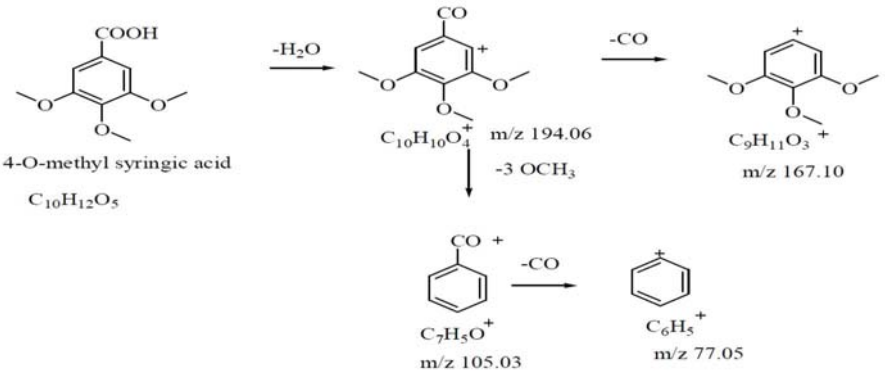


Figure 4. 7. GC-MS spectrum of 4-O-methyl syringic acid.



Scheme 4.5. Fragments leading to the determination of the structure of 4-O-methyl syringic acid.

The sixth compound with the Retention Time of 29.70 min. gave a molecular ion at m/z value of 272.23 ($C_{20}H_{32}$, calculated for 272.250 monoisotopic). This is identified as - Cembrene, a monocyclic diterpene, (1E, 3Z, 6E, 10E)-3, 7, 11-trimethyl-14-propan-2-ylcyclotetradeca -1, 3, 6, 10- tetraene. This molecule is a constituent of *Zingiber officinale* Rosc.

[1] used in the Kashayam. It gave the base peaks at m/z 93.09 and other major peaks at m/z 67.02 and 161.16. Its mass fragmentation pattern is given in scheme 4.6. It gave the base peaks at m/z 81.07 and other major peaks at m/z 229.20, 189.16 and 121.11 as shown in the figure 4.8.

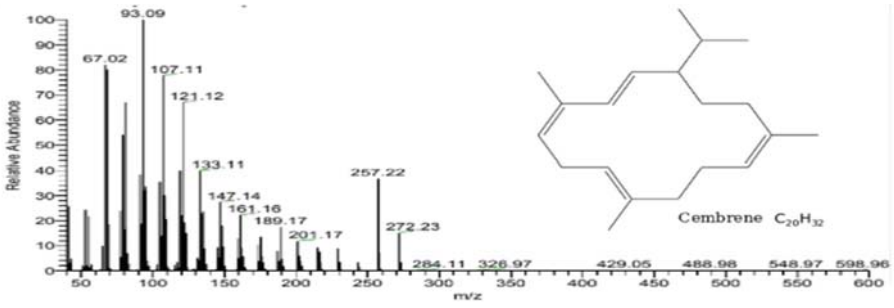
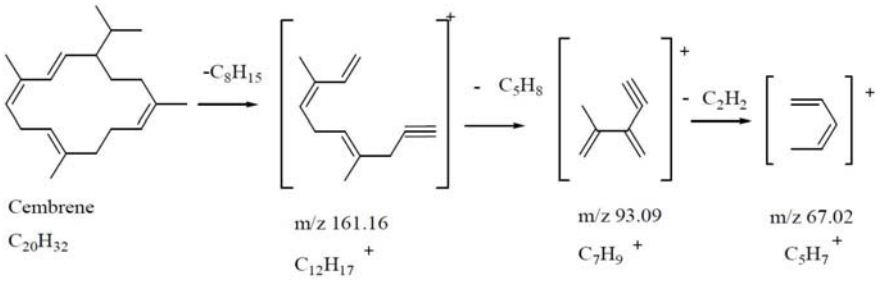


Figure 4.8 GC-MS spectrum of Cembrene



Scheme 4.6. Fragments leading to the determination of the structure of Cembrene.

The seventh compound with retention value of 31.66 min. is identified as Thunbergol which gives molecular ions at m/z 290.23 ($C_{10}H_{12}O_5$, 290.260, monoisotopic). It is a diterpene alcohol (2E, 7E, 11E)- 1, 7, 11-trimethyl-4-propan-2-yl cyclotetradeca -2, 7, 11 -trien-1-ol. This molecule is a constituent of *Zingiber officinale* Rosc. [1] used in the kashayam. It gave the base peaks at m/z 81.07 and other major peaks at m/z 229.20, 189.16 and 121.11 as shown in the figure 4.9. Its fragmentation pattern is given in scheme 4.7.

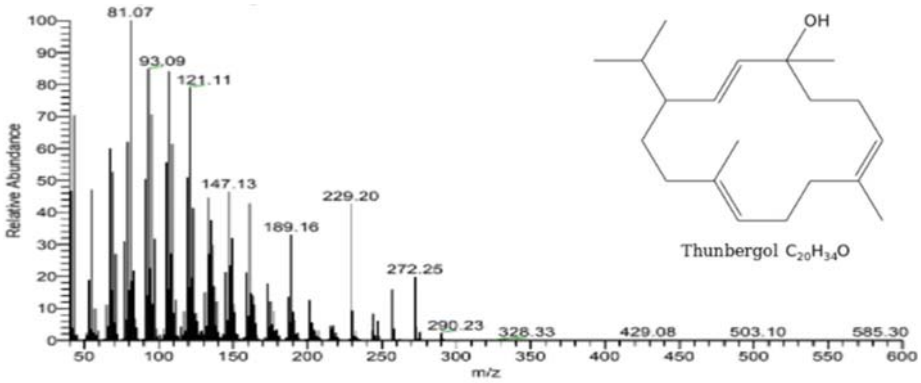
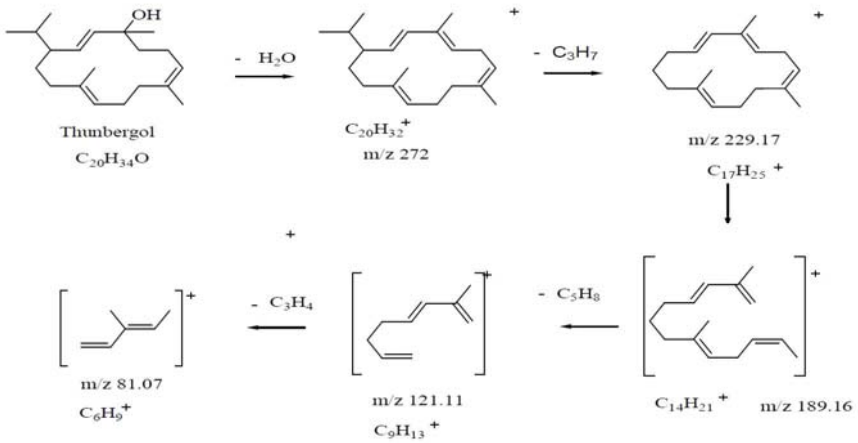


Figure 4.9. GCMS spectrum of Thunbergol



Scheme 4.7. Fragments leading to the determination of the structure of Thunbergol.

The eighth compound with the Retention Time of 35.61 min. gave a molecular ion at m/z value of 278.16($C_{17}H_{26}O_3$ calculated for 278.18, monoisotopic). This is identified as – [6]-Paradol, a monomethoxy benzene, 1-(4-hydroxy-3-methoxyphenyl) decan-3-one. This molecule is a constituent of *Zingiber officinale* Rosc. [1] used in the Kashayam. It gave the base peaks at m/z 137.05 and other major peaks at m/z 119.05 and 91.06. The mass spectrum and fragment pattern are given in Fig. 4.10 and Scheme 4.8 respectively.

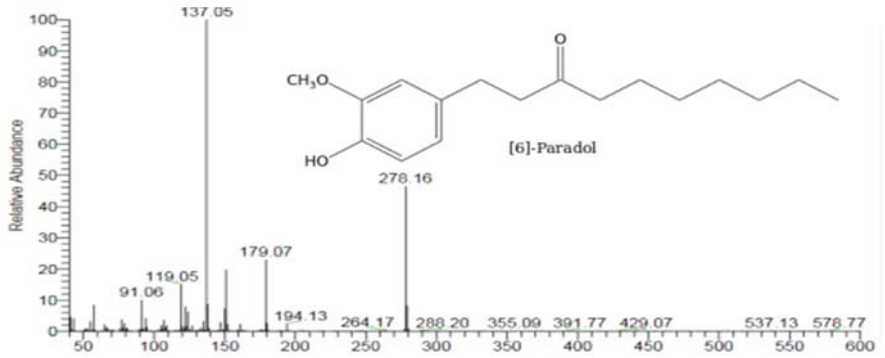
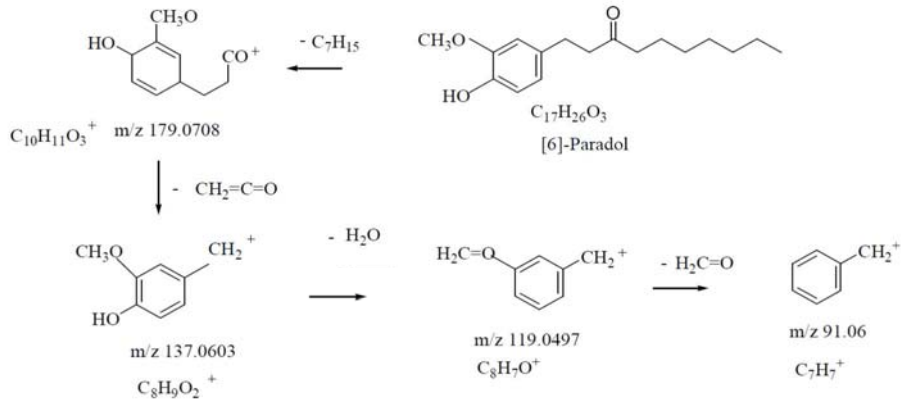


Figure 4.10. GCMS spectrum of [6]-Paradol.



Scheme 4.8. Fragments leading to the determination of the structure of [6]-Paradol.

In a detailed literature survey, large data was available on the pharmacological activities of these components that justifies the therapeutic use of Prasarinyadi kashayam. All these identified volatile compounds were found to be antioxidant, anti-inflammatory and immunostimulant. The results are presented in Table.4.2. It is interesting to note that out of the eight compounds six are the constituents of *Zingiber officinale* Rosc., and the other two are from *Alpinia calcarata* Roscoe.

Table 4.2. Pharmacology of Compounds identified from the volatile oil of Prasarinyadi kashayam.

SI No.	Name of the compounds	Pharmacological properties	References
1.	Ar-Curcumene	Anti-inflammatory, Antinociceptive [4], Antioxidant [5]	Julia A Podlogar et al 2012 , Jeena K. et al 2013
2.	Eudesmol	Regulation of cell mediated inflammatory response [6], Anti-inflammatory action through regulation of NF-KB signaling pathway[7]	Min-Jung Seo et al 2011, Kim. k. y. 2018
3.	Carotol	Anti-inflammatory [8], Antinociceptive [9]	Supriya Agnihotri et al 2013. Subhashree Singh et al 2020
4.	Calamenene	Immunotherapy for cancer [10]	MasaoTakei, et al
5.	4-O-methyl Syringic acid	Anti-inflammatory, Antioxidant [11], Neuro and hepatoprotective, alleviates oxidative stress [12]	Srinivasulu Cheemanapalli et al 2018. Shilpee Chanda et al 2008
6.	Cembrene	Anti-proliferation [13] Anti-inflammatory, Antioxidant [14]	Hegazy et al 2017 Prema Sarup et al 2015
7.	Thunbergol	Anti-inflammatory, Antioxidant, Nephroprotective [15]	Ayodele Jacob Akinyemi et al 2018
8.	[6]-Paradol	Anti-inflammatory [16], Neuro protective [17]	Arjun Sapkota et al 2019, Bhakta Prasad Gaire et al 2015

Kathirvel Poonkodi et al [3] reported the presence of Carotol and its amount calculated is found to be 7.79%. Calamenene 0.04% and Eudesmol 0.2% had been reported from *Zingiber officinale* Rosc.

In a detailed literature survey, large data was available on the pharmacological activities of these components that justifies the therapeutic use of Prasarinyadi kashayam. All these identified volatile compounds were found to be antioxidant, anti-inflammatory and immunostimulant. The results are presented in Table.4.2. It is interesting to note that out of the eight compounds six are the constituents of *Zingiber officinale* Rosc., and the other two are from *Alpinia calcarata* Roscoe.

4.2.2. GCMS study of Gulguluthikthakam kashayam

Mass spectrum of the volatile oil showed several peaks indicating that it still contains many phytochemical constituents out of which 12 could be identified by degradation pattern and comparison with Library search facility (see the ion chromatogram, Fig.4.11). The mass spectra of the constituents compared with the NIST MS libraries and the twelve phyto constituents were characterized and identified (Table 4.3). The plant source of the identified constituents was also listed in the table.4.3.

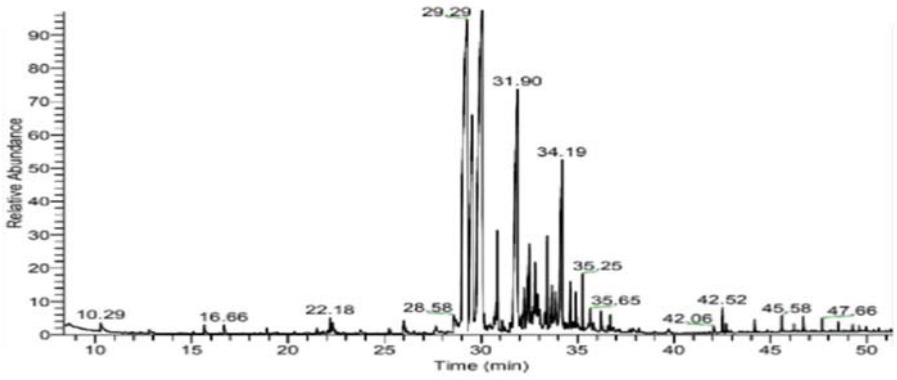


Figure 4. 11. TIC of the volatile oil of Gulguluthikthakam kashayam

Table 4.3 Compounds identified from the volatile oil of Gulguluthikthakam kashayam

SI No.	Retention time	Compound name	IUPAC name	Molecular formula	Molecular weight	Plant source
1.	10.29	Carvone	2-methyl-5-prop-1 -en-2-yl cyclohex-2-en-1-one	$C_{10}H_{14}O$	150.13	*Anethum graveolens* L. [18]
2.	15.65	Beta-caryophyllene	(1*R*, 4*E*, 9*S*)-4, 11, 11-trimethyl-8-methylidenebicyclo [7.2.0] undec-4-ene	$C_{15}H_{24}$	204.18	*Piper nigrum* L. [19]
3.	16.66	Valencene	(3*R*, 4*a S*, 5*R*)-4*a*, 5-dimethyl-3-prop-1-en-2-yl-2, 3, 4, 5, 6, 7-hexahydro-1*H*-naphthalene	$C_{15}H_{24}$	204.19	*Cyperus rotundus* L. [22], *Zingiber officinale* Rosc. [23]
4.	18.89	Cubenol	(1*S*, 4*R*, 4*aR*, 8*aR*)-4, 7-dimethyl-1-propan-2-yl-2, 3, 4, 5, 6, 8*a*-hexahydro-1*H*-naphthalen-4*a*-(2H) ol	$C_{15}H_{26}O$	222.18	*Zingiber officinale* Rosc. [23]
5.	21.47	Ar-Curcumene	1-methyl-4-(6-methylhept-5-ene-2-yl) benzene	$C_{15}H_{22}$	202.17	*Zingiber officinale* Rosc. [23], *Piper nigrum* L. [19], *Piper longum* L. [24] and *Curcuma longa* L. [20]
6.	22.18	Cedrol	(1*S*, 2*R*, 5*S*, 7*R*, 8*R*)-2, 6, 6, 8-tetramethyl tricyclo [5.3.1.01, 5] undecan-8-ol	$C_{15}H_{26}O$	222.17	*Zingiber officinale* Rosc. [23], *Piper nigrum* L. [19]and *Piper longum* L. [24]

7.	22.31	Guaiol	2-[(3*S*, 5*R*, 8*S*)-3, 8 -dimethyl-1, 2, 3, 4, 5, 6, 7, 8- octahydroazulen-5-yl] propan-2-ol	$C_{15}H_{26}O$	222.37	*Zingiber officinale* Rosc. [23]
8.	30.07	Cembrene	(1E, 3Z, 6E, 10E)-3, 7, 11-trimethyl-14-propan- 2-ylcyclotetradeca-1, 3, 6, 10-tetraene	$C_{20}H_{32}$	272.23	*Commiphora mukul* (Hook.ex Stocks) Engl. [25], *Zingiber officinale* Rosc. [23]
9.	31.90	Thunbergol	(2*E*, 7*E*, 11*E*)-1, 7, 11-trimethyl -4-propan-2-ylcyclo tetra deca -2, 7, 11-trien-1-ol	$C_{20}H_{34}O$	290.25	*Anethum graveolens* L. [18], *Piper nigrum* L. [19], *Commiphora mukul* (Hook.ex Stocks) Engl. [25]
10.	33.42	Mukulol	(1*R*, 2*Z*, 6*Z*, 10*Z*, 14*S*)-3, 7, 11-trimethyl-14 -propan-2-ylcyclo tetradeca-2, 6, 10-trien-1-ol	$C_{20}H_{34}O$	290.25	*Commiphora mukul* (Hook.ex Stocks) Engl. [25]
11.	35.65	[6]-Paradol	1-(4-hydroxy-3-methoxyphenyl) decan-3-one	$C_{17}H_{26}O_3$	278.16	*Zingiber officinale* Rosc. [23]
12	42.67	Guggulsterone VI	16- alpha hydroxy pregna 4-en-3-one	$C_{19}H_{24}O_4$	316.23	*Commiphora mukul*(Hook.ex Stocks) Engl. [25]

The peak with retention time of 10.29 min.is identified as Carvone with a molecular ion peak at m/z 150.13 ($C_{10}H_{14}O$ 150.10, monoisotopic). It is a monoterpene, 2-methyl-5-prop-1 -en-2-yl cyclohex-2-en-1-one. It is the constituent of *Anethum graveolens* L. [18] a medicinal herb used in the kashayam. It produced a base peak at m/z 82.04 and other fragments at m/z 54.04 and 93.04[Figure 4.12]. The fragment patterns are given in the scheme 4.9.

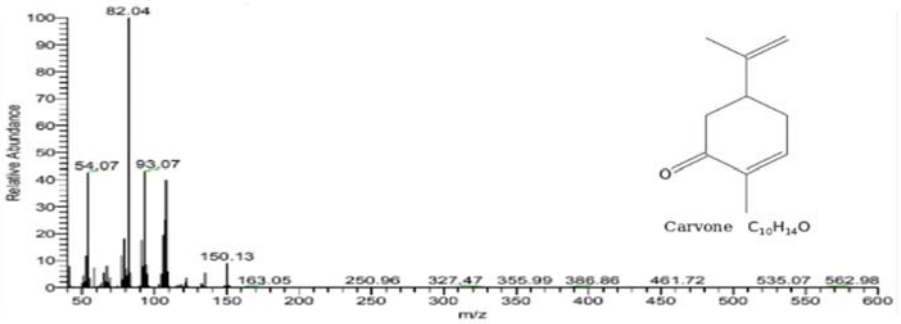
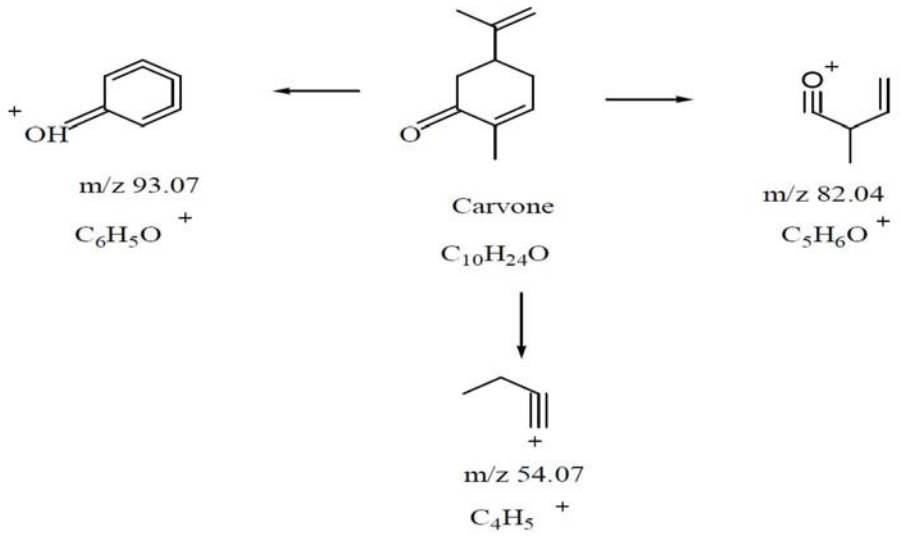


Figure 4.12. Mass spectrum of Carvone



Scheme 4.9. Fragment pattern of Carvone

The mass peak with retention time 15.65 min is identified as Beta-caryophyllene with the molecular ion at m/z 204.18($C_{15}H_{24}$ 204.187, monoisotopic). It gave the base peaks at m/z 133.11 and other fragments at m/z 189.16 and 161.14[Figure 4.13]. It is a sesquiterpenoid, (1*R*, 4*E*, 9*S*)-4, 11, 11-trimethyl-8-methylidenebicyclo [7.2.0] undec-4-ene. This compound is the constituent of *Piper nigrum* L. [19], *Curcuma longa* L [20] and *Cuminum cyminum* L. [21] used in the preparation of kashayam. Mass fragments are given in the scheme 4.10.

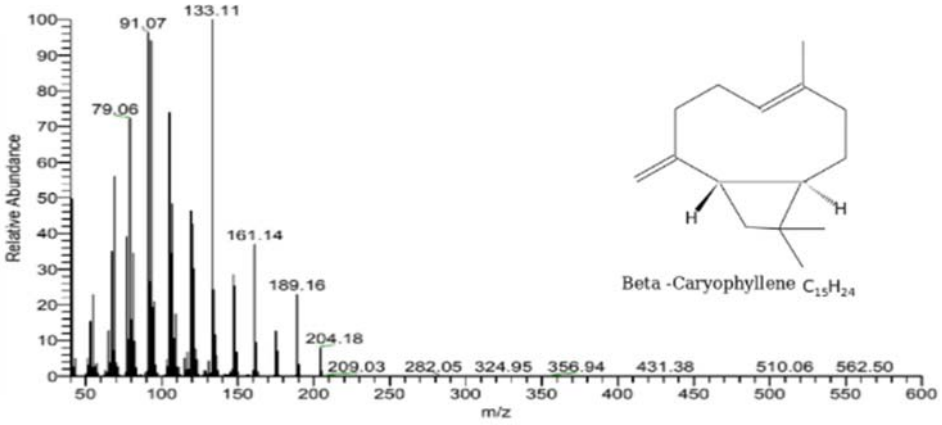
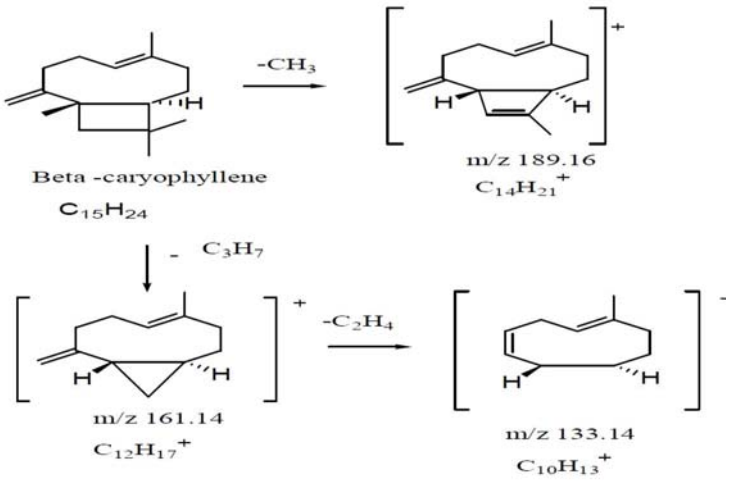


Figure 4.13. Mass spectrum of Beta-Caryophyllene



Scheme 4.10. Fragment pattern of Beta -Caryophyllene

The peak with retention time 16.66 min was identified as Valencene, $C_{15}H_{24}$, 204.19 (204.187, monoisotopic). It gave a base peak at m/z 93.08 and other fragments at m/z 80.07[Figure 4.14]. It is sesquiterpenoid, (3*R*, 4*a S*, 5*R*)-4*a*, 5-dimethyl-3-prop-1-en-2-yl-2, 3, 4, 5, 6, 7-hexahydro-1*H*-naphthalene. It is the constituent of *Cyperus rotundus* L. [22] and *Zingiber officinale* Rosc. [23] used in the kashayam. Mass fragments leading to the confirmation are given in the scheme 4.11.

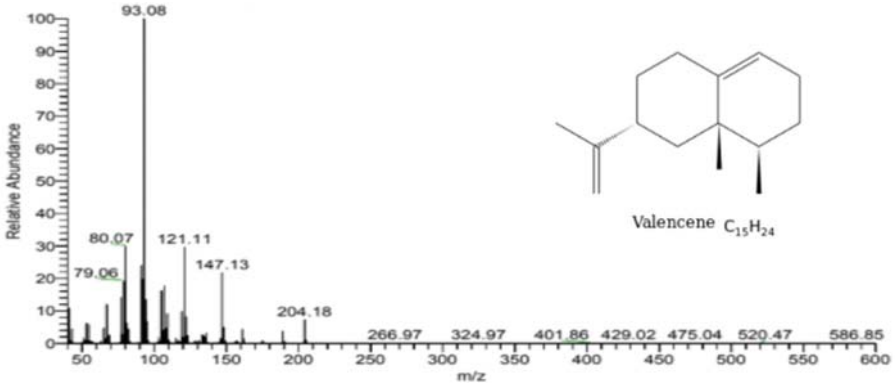
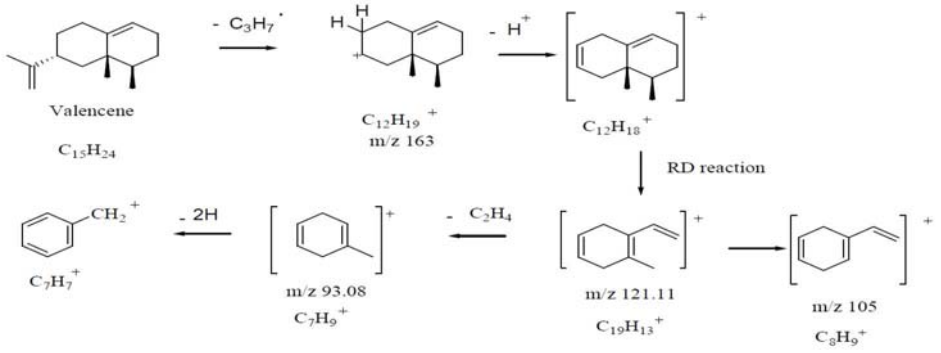


Figure 4.14. Mass spectrum of Valencene



Scheme 4.11 Fragment pattern of Valencene

The peak with retention time 18.89 min was identified as Cubenol according to the mass spectrum [Figure 4.15] and its fragmentation pattern [scheme 4.12]. Its molecular ion peak was found to be at m/z 222.18($C_{15}H_{26}O$, 222.198, monoisotopic). It produced a base peak at m/z

161.14 and other fragments at m/z 105.08, 119.08 and 204.19. It is a sesquiterpenoid, (1*S*, 4*R*, 4*aR*, 8*aR*)-4, 7-dimethyl-1-propan-2-yl-2, 3, 4, 5, 6, 8*a*-hexahydro-1*H*-naphthalen-4*a*-(2H) ol. This compound is the constituent of *Zingiber officinale* Rosc. [23] used in the kashayam.

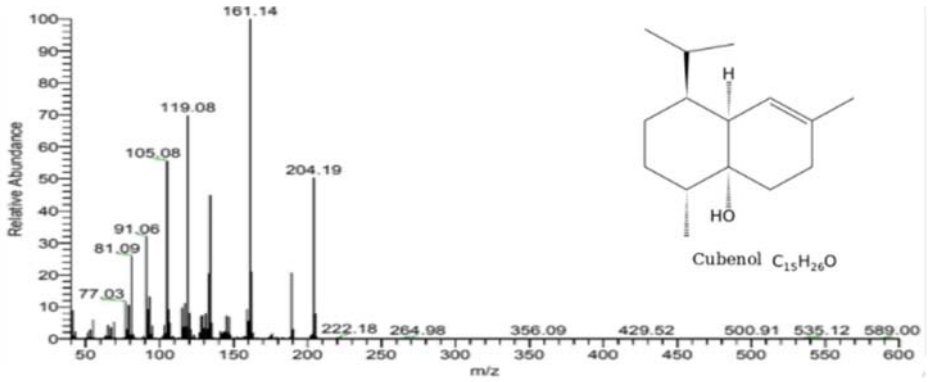
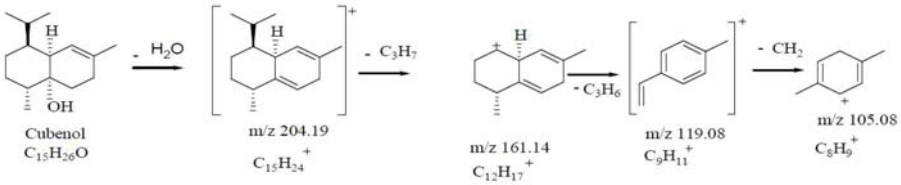


Figure 4.15. Mass spectrum of Cubenol



Scheme 4.12. Fragment pattern of Cubenol

The mass peak with retention time 21.47 min was identified as Ar-Curcumene; it gave a molecular ion at m/z value of 202.17($C_{15}H_{22}$, calculated for 202.341 monoisotopic). It is a sesquiterpenoid, 1-methyl-4-(6-methylhept-5-ene-2-yl) benzene. This molecule is a constituent of the medicinal plant *Zingiber officinale* Rosc. [23], *Piper nigrum* L. [19], *Piper longum* L. [24] and *Curcuma longa* L. [20] used in the kashayam. It gave the base peaks at m/z 119.10 and other major peaks at m/z 159.14, 132.10, 91, 59 [Figure 4.16]. The mass fragments are given in the scheme 4.13.

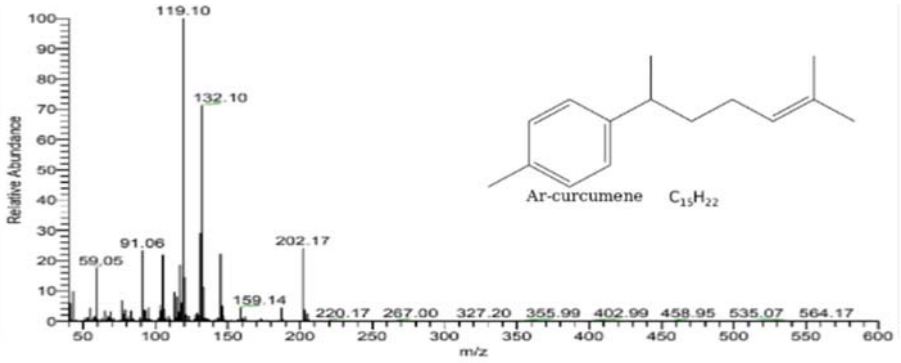
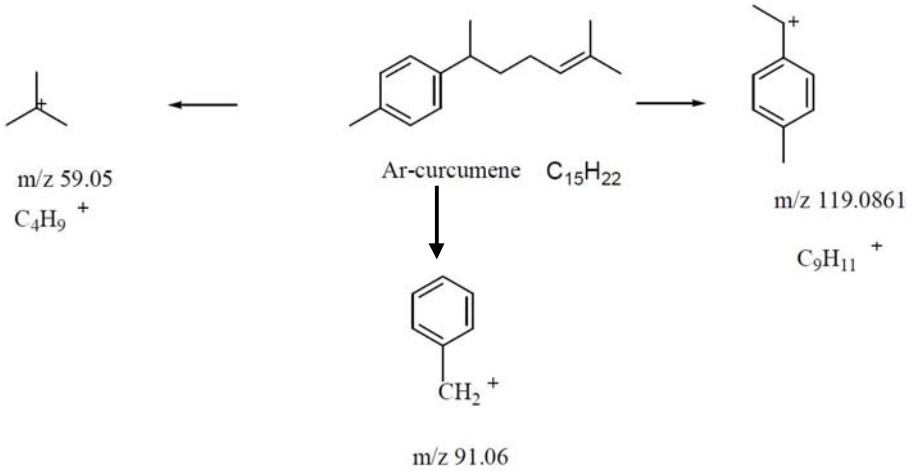


Figure 4.16. Mass spectrum of Ar- Curcumene



Scheme 4.13. Fragment pattern of Ar-Curcumene

The mass peak with retention time 22.18 min was identified as Cedrol with the molecular mass 222.17($C_{15}H_{26}O$, 222.198, monoisotopic mass). It gave a base peak at m/z 149.13 and other fragments at m/z 59.05[Figure 4.17]. It is a sesquiterpenoid alcohol, (1*S*, 2*R*, 5*S*, 7*R*, 8*R*)-2, 6, 6, 8-tetramethyl tricyclo [5.3.1.01, 5] undecan-8-ol. It is a constituent of *Zingiber officinale* Rosc. [23], *Piper nigrum* L. [19] and *Piper longum* L. [24]. The mass fragments leading to the confirmation of Cedrol are given in the Scheme 4.14.

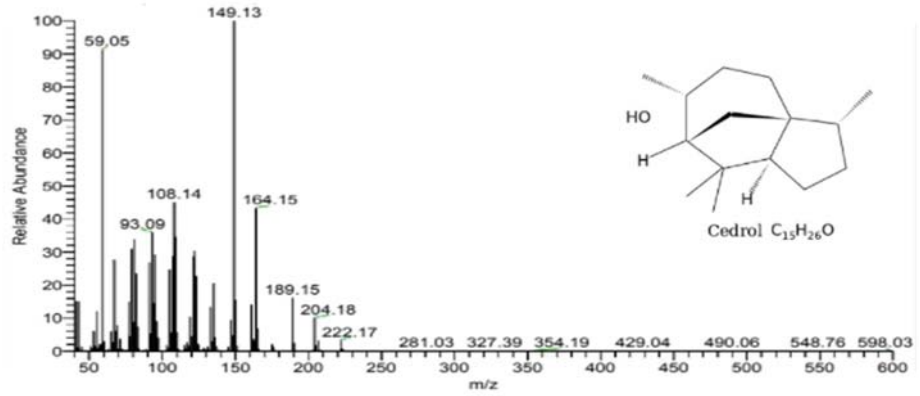
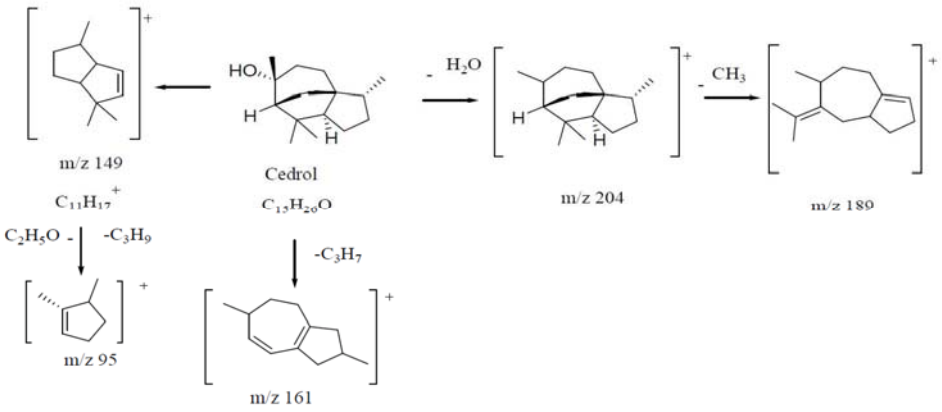


Figure 4.17. Mass spectrum of Cedrol



Scheme 4.14. Fragment pattern of Cedrol

The peak with retention time 22.31 min was identified as Guaiol with the molecular mass 222.14($C_{15}H_{26}O$, 222.198, monoisotopic mass) It is a sesquiterpenoid alcohol, 2-[(3*S*, 5*R*, 8*S*)-3, 8 -dimethyl-1, 2, 3, 4, 5, 6, 7, 8- octahydroazulen-5-yl] propan-2-ol. This molecule is a constituent of *Zingiber officinale* Rosc. [23] used in the Kashayam. It gave the base peaks at m/z 149.11 and other major peaks at m/z 161.11, 189.15, 107.10, 91.06 and 59.06[Figure 4.18]. The mass fragments are given in the scheme 4.15.

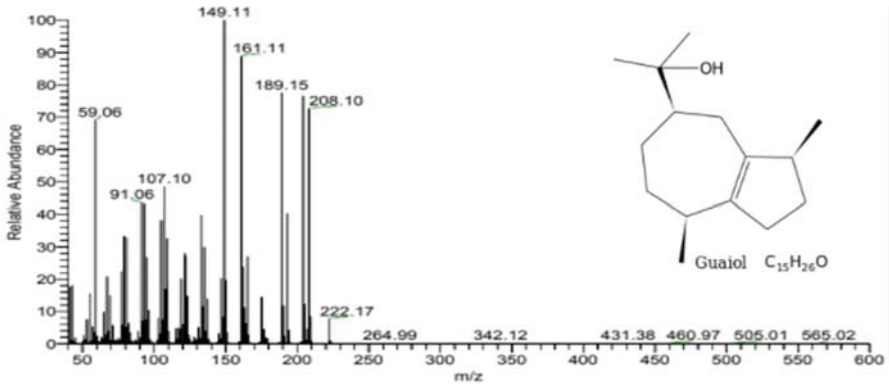
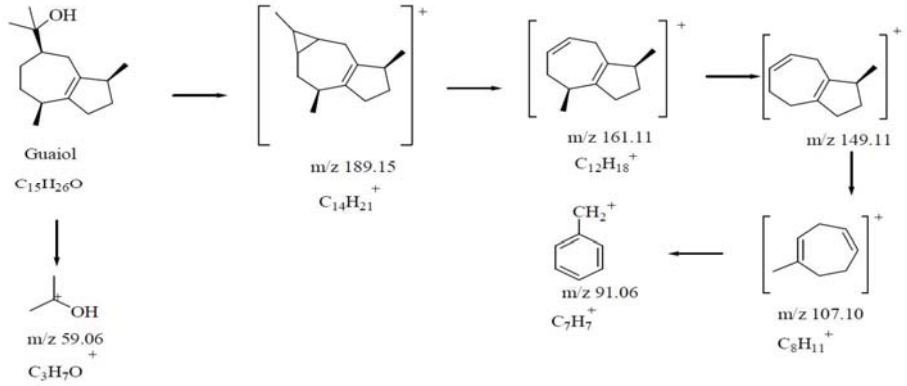


Figure 4.18. Mass spectrum of Guaiol



Scheme 4.15. Fragment pattern of Guaiol

The peak with retention time 30.07 min was identified as Cembrene [see the figure 4.19 and scheme 4.16] with the molecular mass 272.23($C_{20}H_{32}$, 272.25, monoisotopic mass). This is a diterpenoid, (1E, 3Z, 6E, 10E)-3, 7, 11-trimethyl-14-propan- 2-ylcyclotetradeca-1, 3, 6, 10-tetraene. This molecule is a constituent of *Zingiber officinale* Rosc. [23]and *Commiphora mukul* (Hook.ex Stocks) Engl. [25] used in the kashayam. It gave the base peaks at m/z 93.09 and other major peaks at m/z 67.02 and 161.14.

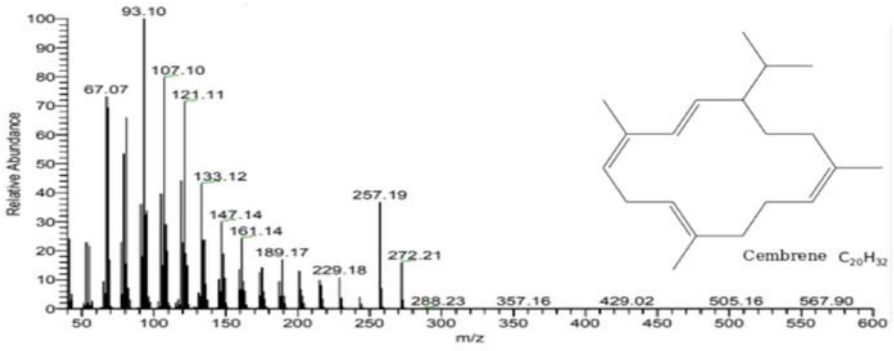
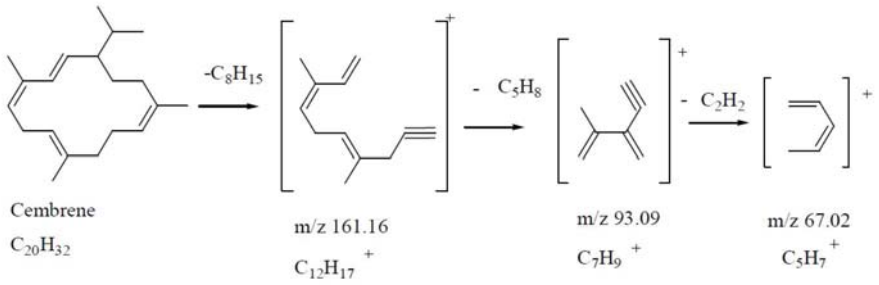


Figure 4.19. Mass spectrum of Cembrene



Scheme 4.16. Fragment pattern of Cembrene

The mass peak with retention time 31.90 min was identified as Thunbergol with the molecular mass 290.25($C_{20}H_{34}O$, 290.260, monoisotopic mass). This is a diterpenoid, (2*E*, 7*E*, 11*E*)-1, 7, 11-trimethyl -4-propan-2-ylcyclo tetra deca -2, 7, 11-trien-1-ol. This molecule is a constituent of *Zingiber officinale* Rosc. [23], *Anethum graveolens* L. [18], *Piper nigrum* L. [19] and *Commiphora mukul* (Hook.ex Stocks) Engl. [25] used in the Kashayam. It gave the base peaks at m/z 81.08 and other major peaks at m/z 272, 229.17, 189 and 121.11. The mass spectrum [Figure 4.20]and fragments pattern [scheme 4.17] are given.

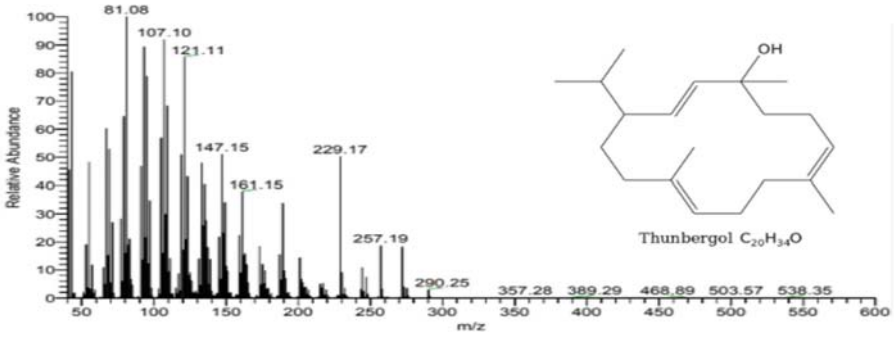
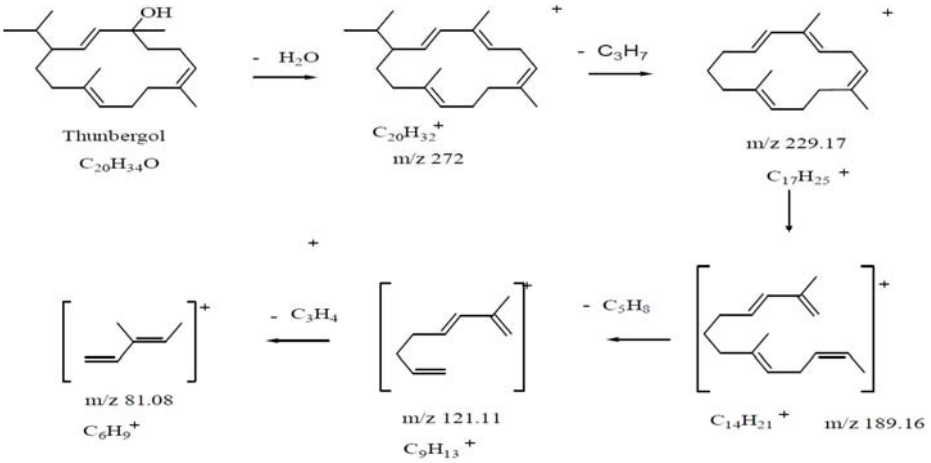


Figure 4. 20. Mass spectrum of Thunbergol



Scheme 4.17. Fragment pattern of Thunbergol

The peak with the retention Time of 33.42 min. gave a molecular ion at m/z value of 290.24 ($C_{20}H_{34}O$ calculated for 290.260, monoisotopic). This is identified as –Mukulol, (1*R*, 2*Z*, 6*Z*, 10*Z*, 14*S*)-3, 7, 11-trimethyl-14 -propan-2-ylcyclo tetradeca-2, 6, 10-trien-1-ol. This molecule is a constituent of *Commiphora mukul* (Hook.ex Stocks) Engl. [25] used in the kashayam. It gave the base peaks at m/z 109.12 and other major peaks at m/z 272.22, 257.22, 229.18, 189.19 and 81.08. The mass spectrum [Figure 4.21] and its fragment pattern [scheme 4.18] are given.

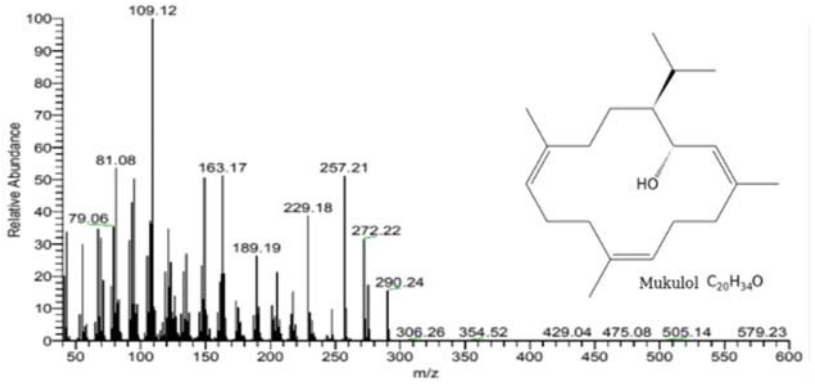
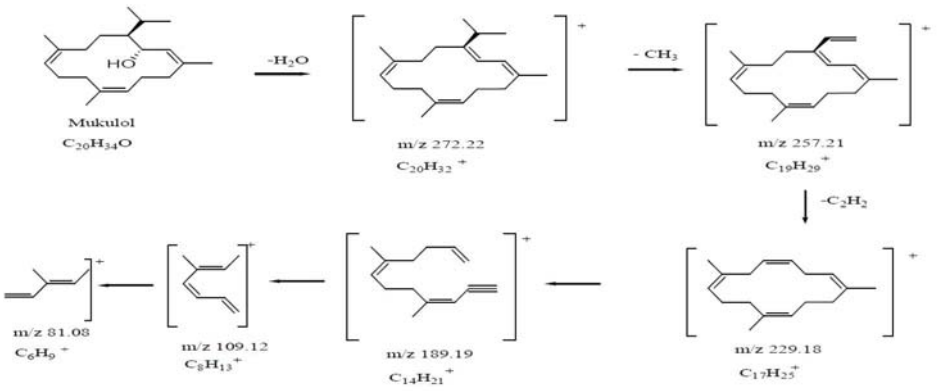


Figure 4.21. Mass spectrum of Mukulol



Scheme 4.18. Fragment pattern of Mukulol

The peak with the retention time 35.65 min. gave a molecular ion at m/z value of 278.16($C_{17}H_{26}O_3$ calculated for 278.18, monoisotopic). This is identified as – [6]-Paradol, a monomethoxy benzene, 1-(4-hydroxy-3-methoxyphenyl) decan-3-one. This molecule is a constituent of *Zingiber officinale* Rosc. [23] used in the Kashayam. It gave the base peaks at m/z 137.05 and other major peaks at m/z 179.07, 119.05 and 91.06[Figure 4.22]. The fragment pattern is given in the scheme 4.19.

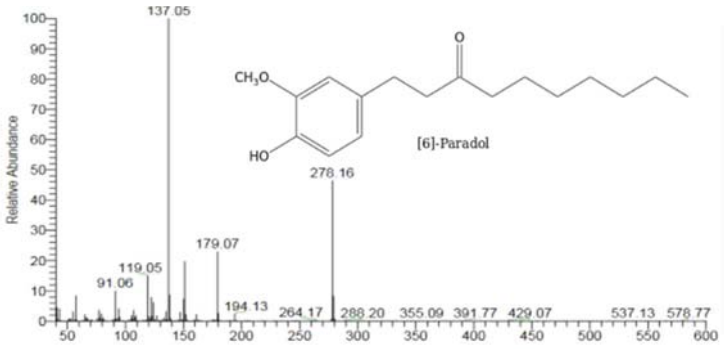
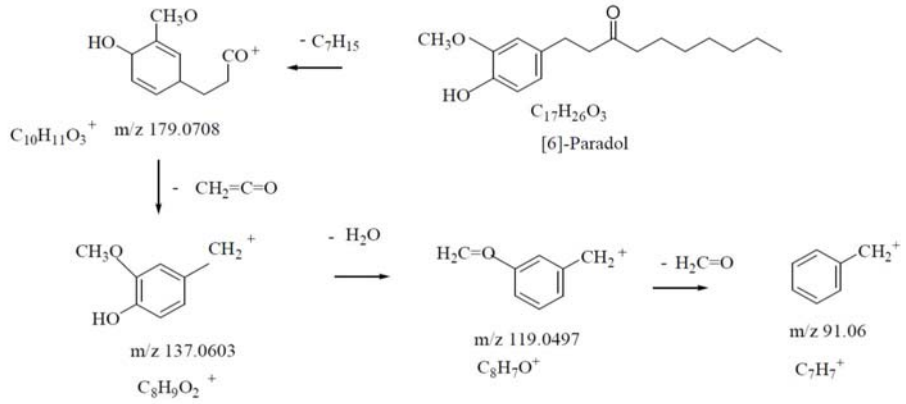


Figure 4.22. Mass spectrum of [6]-Paradol



Scheme 4.19 Fragment pattern of [6]-Paradol

The mass peak with the retention time of 42.67 min. gave a molecular ion at m/z value of 316.23($C_{19}H_{24}O_4$ calculated for 278.18, monoiso topic). This is identified as –Guggulusterone, 16 alpha hydroxy pregna 4-en-3-one, a hormone. This molecule is a constituent of *Commiphora mukul* (Hook.ex Stocks) Engl. [25] used in the kashayam. It gave the base peaks at m/z 190.17 and other major peaks at m/z 207.13, 135.13, 108.12 and 79.08[Figure 4.23]. The mass fragments are given in the scheme 4.20.

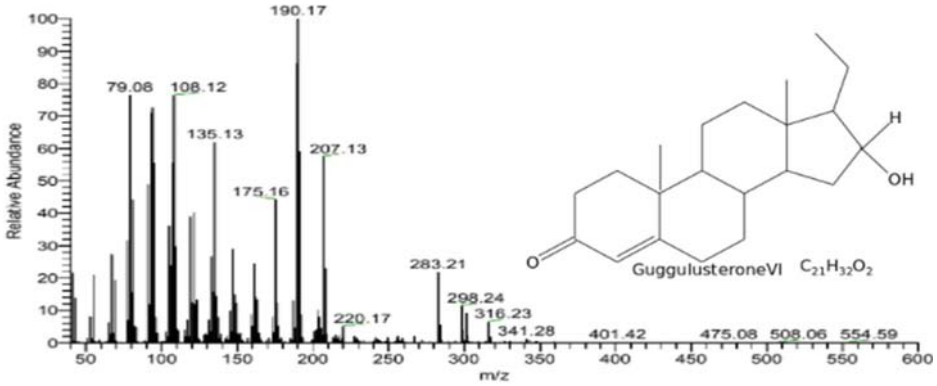
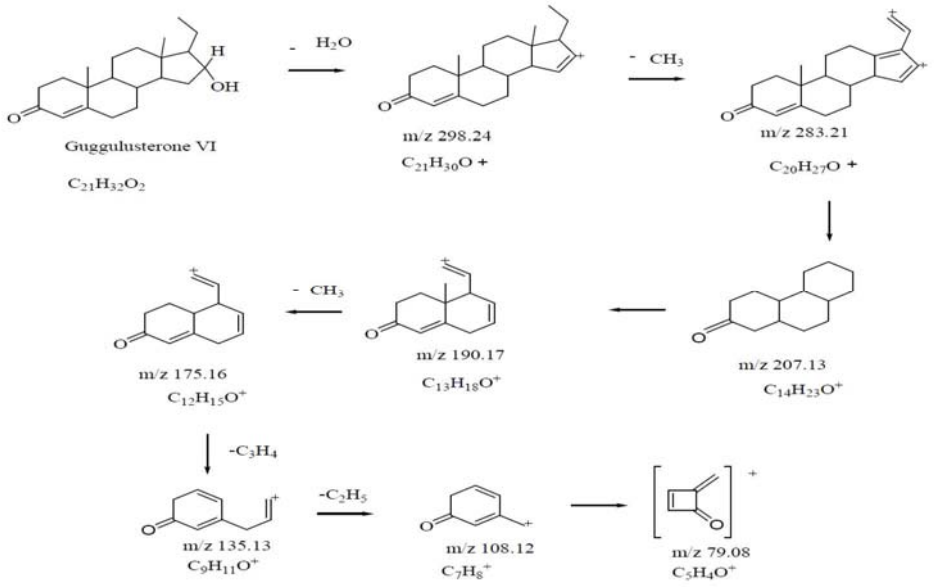


Figure 4.23. Mass spectrum of Guggulusterone VI



Scheme 4.20. Fragment pattern of Guggulusterone VI

Carvone is the main component of dill seed oil. As per Kapoor et al [26] it is found to be in the 48-89.98%. The sesquiterpenoid, caryophyllene reported from Piper species, *Curcuma longa* L. and *Cuminum cyminum* L., Caryophyllene (0.8%) was one of the main components of Piper longum L. [27]. Valencene has been reported from *Cyperus rotundus* L. and *Zingiber officinale* Rosc. Pradeep Kumar Sharma et al [28] reported Valencene about 7.61% from *Zingiber officinale* Rosc. Ar- Curcumene has been reported from *Zingiber officinale* Rosc., Piper species and *Curcuma longa* L. Ar- Curcumene (15.9%) [29] is one of the main components of fresh ginger. Nayana et al [30] had reported 0.9-1.3% Cedrol from *Zingiber officinale* Rosc. Cembrene, Thunbergol, Mukulol and Guggulusterone are the compounds reported from the *Commiphora mukul* (Hook.ex Stocks) Engl.

All the identified compounds had been reported as antioxidant, anti-inflammatory, analgesic, antipyretic, nephroprotective, anticholinesterase, antiarthritic, antispasmodic, antibacterial and immunostimulant. The results are presented in Table.4.4.

Table 4.4. Pharmacology of identified compounds

SI No.	Name of the compounds	Pharmacological properties
1.	Carvone	Antispasmodic [31], Antioxidant and free radical scavenging [32]
2.	Beta caryophyllene	Anti-inflammatory and analgesic effect on different inflammatory conditions [33]. Attenuates oxidative stress and neuroinflammation [34].
3.	Valencene	Inhibits inflammatory signaling process mediated by NF-kB. Antipyretic [35] and Anti-inflammatory [36]
4.	Cubenol	Anti-inflammatory, Antioxidant and Antimicrobial [37]. Anti-inflammatory and anticholinesterase activity [38]
5.	Ar-Curcumene	Anti-inflammatory, Antioxidant [39] Anti-inflammatory, Antinociceptive [40]
6.	Cedrol	Inhibits oxidative stress and inflammation [41] Reduces inflammation in Rheumatoid arthritis [42]
7.	Guaiol	Anti-inflammatory [43,44]
8.	Cembrene	Anti-inflammatory, Antioxidant [45] Treatment of Rheumatism [46]
9.	Thunbergol	Anti-inflammatory, Antioxidant [47] Nephroprotective [48]
10.	[6]-Paradol	Attenuation of neuroinflammatory process [49] Enhances Drug metabolism [50]
11.	Mukulol	Antiarthritic and Anti-inflammatory [51] Treatment for osteoarthritis inflammation [52]
12.	Guggulusterone VI	Anti-inflammatory and Antiarthritic [53] Anti-inflammatory [54]

The study is only a qualitative one and hence, the exact percentage of the volatile components that are present in the formulation could not be ascertained. The twelve compounds were identified by comparison with NIST- MS library. A probable degradation pattern of the molecular

structure was formulated from the m/z values of the secondary ions in each of the spectrum. This completely matches with the molecular weight of the fragment ions. All of the twelve compounds identified and confirmed show pharmacological activities validating its use in the Ayurveda therapeutics. A literature survey of the past ten years shows the results of a large number of pharmacological studies on the individual compounds and also of the medicinal herbs that confirms the therapeutic utility of these compounds in inflammatory related ailments.

4.3. Experimental

Apparatus used: Clevenger apparatus used for the quantification of volatile oil. Identification of compounds done with GCMS apparatus

Quantification of Volatile oil: 200 mL of kashayam obtained from the market used for the determination of volatile compounds. Clevenger apparatus used for the quantification of volatile oil. 200 mL of kashayam was placed into a 1000 mL volume flask connected directly to the Clevenger apparatus and heated with the heating mantle for 3Hrs.The yield of volatile oil obtained (%) is calculated by the following formula:

% Yield of volatile oil= (Volume of oil obtained/volume of kashayam taken) x100

GCMS analysis:

The sample was analyzed by thermo scientific ISQ QD, Trace 1300 GC. Helium was used as the carrier gas (99.99%) with a flow rate of 1mL/minute. The GC oven temperature program was as follows. Initial temperature at 60°c and held for 5, then increased to 180°c at a rate of 4°c/min. Held for 5 min. and temperature was ramped to 280°c at a rate of 10°c/min. and kept for 2 min. The MS was operated in EI mode. The column used was TG5MS (30 x 0.25mm x 0.25 micrometer). MS transfer

line temperature was 290°c and ion source temp was 230°c with a mass range of 40-600 amu. The database used is NIST MS.

4.4. CONCLUSION

The present study is only a qualitative workup. Because the decoction is prepared by the ingredient plants in a large volume of water and concentrating the extract to specified volume as per the Ayurveda text. The quantity of each component is found to vary. However, the identified components were in higher quantity as compared to the other components as is evident from the ion chromatogram. All the components were found to be either having anti-inflammatory, antioxidant, immunostimulant, neuroprotective and other pharmacologic activity support the use of this Ayurvedic formulation for the treatment of diseases that are caused by chronic inflammatory conditions.

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Chapter 5
LCMS study of Prasarinyadi Kashayam

5.1 Introduction

5.2 Result and Discussion

5.2.1. LCMS study on methanol extract

5.2.2. HR LCMS study

5.2.3. UPLC-QTOFMS analysis

5.3. Experimental

5.4. Conclusion

References

Abstract

During the preparation of kashayam phytochemicals present in plant parts may undergo different types of reaction such as condensation, decomposition, hydrolysis, dimerization, substitution, polymerization and metal complex formation. The easily volatile phytochemicals already present or formed during the kashayam preparation may vaporize. So, the kashayam is the complex mixture of various compounds or phytochemicals with different or same polarities and molecular mass. Their separation still remains a big challenge for the process of identification and characterization. The identification of such a complex mixture can be done easily and accurately by LCMS MS analysis. In this study an attempt is made to analyze the phytochemical components of the kashayam that have been prepared using polyherbal formulations by LC MSMS analysis

5.1. Introduction

Mass spectrometry (MS), with its high sensitivity and capability of providing molecular weight and additional structural information, is an efficient tool in the analysis and structural elucidation of natural products [1]. MS is a powerful and very complete technique that can be used at every stage of natural product study, from discovery and structural characterization of new compounds to biosynthetic enzyme identification and manipulation. MS is based on the production of gaseous, positively or negatively charged ions that are subsequently separated according to their mass-to-charge (*m/z*) ratio and detected. New generation ion sources and analyzers have been introduced in MS and have enhanced its application in different fields such as natural product identification, pharmaceutical and drug research, forensic science, proteomics, genomics and many other fields of analytical chemistry.

In traditional MS, purified samples were subjected to high-energy ionization under ultra-high vacuum conditions ("hard" ionization) e.g., electron impact [EI]). The development of electrospray ionization (ESI) MS has revolutionized the analysis of complex mixtures of natural products. In contrast to earlier ionization techniques such as EI, which were applicable only to thermally stable, low molecular weight volatile compounds, virtually any ion (from inorganic salts to large macromolecules) can be analyzed by ESI-MS. It can be coupled to High Performance Liquid Chromatography (HPLC), and this hyphenated technique is called LCMS. In LCMS the eluate from the HPLC is sprayed into the ion source of the MS as a solution. In the source, the solvent is evaporated under atmospheric pressure in the presence of an electric field, which generates charged ions to be separated by the mass analyzer. In this way, the mass analyzer has become a unique kind of chromatographic

detector, able to give information about the molecular mass, the chemical formula and structure of several components of complex biological samples. As a result, the interface of HPLC coupled with ESI-MS has provided an excellent method in the detection, isolation and identification of secondary metabolites from complex extracts.

Soft ionization techniques, including electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are routinely used to ionize thermally labile and moderately polar organic analytes. These methods usually yield ions with no unpaired electrons resulting in $[M + H]^+$ species, referred to as protonated molecules. These ions possess low internal energy, therefore fragmentation in a single stage MS experiment is very low. This way, the intact accurate mass for sensitive, fragile compounds and large biomolecules as well as their detailed structures could be determined, even when high pressure liquid chromatography is used as a prefractionation method [2,3, 4].

In ESI, the sample is dissolved in a polar, volatile solvent and pumped through a narrow stainless-steel capillary (75-150 m i. d.). A sufficiently high voltage, commonly 3-5 kV, is applied at the capillary tip, which is situated within the ionization source of the spectrometer. This high voltage causes charge migration of the species in solution to the metal/solution interface, resulting in an electric double layer. In the capillary tip, electrostatic forces acting on the ion performed in the double layer counterbalance the surface tension, culminating in a conical surface known as Taylor cone. The small charged-droplets of the fine spray that emerge from the capillary tip are diminished in size due to a co-axially introduced nebulizing gas (usually nitrogen, helium or argon) flowing around the outside of the capillary, thus leading to solvent evaporation [5, 6,7]. Finally, ions are produced by the droplets and flow into the mass

analyzer [5, 8]. As a result of ESI, protonated ($[M+H]^+$)/deprotonated ($[M-H]^-$) molecules, and cationized (usually $[M+Na]^+$ or $[M+K]^+$)/ anionized molecules (i.e., $[M+Cl]^-$) can be produced, depending on the molecular structure of the analytes [7]. Natural products exhibiting acidic groups (i.e., flavonoids, and caffeoyl quinic acid derivatives) are easily deprotonated, which enables their analysis in the negative ion mode, whereas for NPs with basic groups (i.e., alkaloids), which can be easily protonated, the positive ion mode is more adequate [5]. The major advantage of interfacing ESI with LC is related to the ion formation, which occurs at atmospheric pressure in the condensed phase outside the high vacuum of the mass spectrometer, thereby eliminating one of the greatest difficulties inherent to the analysis, the negative influence of the vacuum system.

ESI methodology can be used in either positive (ES+) and negative ion mode (ES^-), depending on the proton affinity of the compound. In ES+, only analytes with at least one positive elementary charge, and in ES^-, only analytes with at least one negative elementary charge, can be detected. Molecules possessing basic characteristics could be easily ionized in ES^+ via making adducts with proton(s), while molecules having acidic functional groups form ES^- ions. Only electrospray-compatible solvents (e.g., acetonitrile, water, methanol, ethanol)—containing 0.1% formic or acetic acid to enhance protonation—or solvent mixtures are required to be used.

The first and most important step in the identification of a compound is the determination of its molecular mass. ESI is quite efficient at providing this information. In contrast to “hard” ionization techniques ESI rarely generates fragments. Fragmentation is achieved by collision-induced dissociation (CID). Molecules are ionized by

protonation, cationization, or deprotonation to form pseudo molecular ions. During protonation and cationization, positively charged ions are formed such as $[M+H]^+$ and $[M+Na]^+$ /$[M+K]^+$, respectively. During deprotonation, a proton is removed from the molecule resulting in negatively charged ions $[M-H]^-$ [9, 10]. Performing both positive ion and negative ion ESI-MS experiments can give reliable evidence for the molecular weight of a compound. In addition, depending on the mass analyser used, high accuracy in the determination of the molecular weight can be achieved. From this accurately determined molecular mass (±0.001 mass units), the chemical formula and the number of double bonds, rings or heteroatoms can be inferred. The rule of 13 in mass spectrometry could also be used to ascertain the molecular formula. Once the molecular weight has been determined, further structural information may be obtained by tandem MS [11].

LCMS provides identification of unknown compounds through efficient separation capabilities of HPLC and exact structural characterization by mass spectrometer. Reverse phase columns used in HPLC are more desirable and widely used for the analysis of multiple phytochemical constituents. In Reverse phase HPLC, the stationary phase used is non polar and the mobile phase is polar. It separates the components with the help of HPLC and each separated compounds detected with the help of Mass Spectrometry.

LC-MS-MS is becoming a very efficient isolation and purification tool for the identification of natural products in crude plant extracts. For an efficient use of this technique in the identification of natural products, a careful study of the parameters used to generate informative MS/MS spectra is needed. Molecular weight information and fragment

information also is necessary for partial identification or for replication of known constituents.

Tandem mass spectrometry (MS/MS), coupling of two MS-MS, is a powerful technique for detecting a target compound in complex plant extracts. In a tandem mass spectrometer, ions are formed in the ion source and separated by mass-to-charge ratio in the first stage of mass spectrometry (MS1). Ions of a particular mass-to-charge ratio (precursor ions) are selected and fragment ions (product ions) are created by collision-induced dissociation, ion-molecule reaction, photo-dissociation, or other process. The resulting ions are then separated and detected in a second stage of mass spectrometry (MS2). The second ionization offers the possibility of acquiring valuable structural information and through it achieve high possibility to determine the structure of new molecules. MS-MS could be performed on instruments such as triple quadrupole (QQQ), ion trap, time of flight (QTOF), Fourier transform, etc. In tandem mass spectrometry (MS-MS), ions generated by ESI can be selected in the first stage of MS to collide with molecules of an inert collision gas [8] This step is very fast (between 10^{14} to 10^{16} s) and leads to an increase in the ion's internal energy, promoting it to an excited state [9]. Immediately after the ion excitation, the unimolecular decomposition is initiated and, as result, the activated selected ions (the precursor ions) are converted into fragment ions (product ions) in a collision energy-dependent manner.

The triple quadrupole is the most frequently used mass spectrometer for MS/MS analysis. Using a triple quadrupole mass analyser, a single ion is selected using the first quadrupole, Q1. Subsequently, only the selected ion (precursor ion) enters the second quadrupole (Q2, collision chamber) where it collides with argon atoms

and fragments are generated. These fragments (product ions) can then be analyzed by the third quadrupole (Q3). Since the fragments generated are characteristic of the chemical structure of the parent ion, structural information about the parent ion can be obtained by analyzing the product ions.

HRMS and its hyphenated techniques have become the major platform for chemical profiling, metabolomics and quality control of plant natural products. The high analytical power provided by the new HRMS instruments (working in the MS or MS/MS mode, stand alone or hyphenated with separation techniques) is making the characterization of natural products more feasible. High resolution mass spectrometry is capable of answering many questions regarding analytical characterization of these compounds, usually found in very complex matrices, including their structure, composition and concentration in a very fast and sensitive way. In high resolution mass spectrometry (HRMS) the mass can be measured to many decimal places. Ordinary MS measures nominal mass and HRMS can measure exact mass so precisely that it can detect minute differences in masses between two complexes. The mass resolution specifies capability to discriminate peaks and high resolution produces more separation. Considering the qualitative analysis time of flight MS (TOF-MS) is more precise on the basis of resolution and mass accuracy. This is very significant for determining the structure of molecules [11].

HRMS instruments based on TOF-MS analyzers are the most extensively reported in literature for the analysis of plant natural products. In particular, the hybrid Q-TOF-MS instruments have been largely adopted as powerful tools for untargeted analysis of complex plant extracts, due to their capability of providing accurate mass data, and

structural information from HR-MS/MS fragmentation [12,13]. The coupling of time-of-flight mass spectrometry (TOF-MS) with electrospray ionization (ESI) has been achieved only recently. This method combines high accuracy and sensitivity due to the high frequency sampling of all ions simultaneously across the full mass range. The high accuracy of masses determined by HPLC–ESI-QTOF MS enables calculations of elemental composition. HPLC–ESI-MS typically will provide the $[M+H]^+$ or $[M-H]^+$ ions from which the molecular weight can be directly inferred.

During the preparation of kashayam phytochemicals present in plant parts may undergo different types of reaction such as condensation, decomposition, hydrolysis, dimerization, substitution, polymerization and metal complex formation. The easily volatile phytochemicals already present or formed during the kashayam preparation may vaporize. So, the kashayam is the complex mixture of various compounds or phytochemicals with different or same polarities and molecular mass. Their separation still remains a big challenge for the process of identification and characterization. The identification of such a complex mixture can be done easily and accurately by LCMS analysis. In this study an attempt is made to analyze the phytochemical components of the kashayam that have been prepared using polyherbal formulations by LC MS analysis.

5.2. Result and Discussion

5.2.1. LCMS study on methanol extracts

LCMS study was carried out on C18 column with the linear gradient elution with A (0.1%formic acid) and B (acetonitrile) as the solvent system. The gradient used as follows 0-15min 20%B, 15-35 min 80%B, 35-37 min 20%B, 35-37 min 20%B as the mobile phase system. The mobile phase was filtered through a 0.45µm membrane filter. The solvent flow rate was 0.4mL/min.

Highly packed mass peaks are observed in the total ion chromatogram of Prasarinyadi kashayam (Figure 5.1) with the relative intensity varying from 5 to 100. Number of ions per peak is 5 to 6.

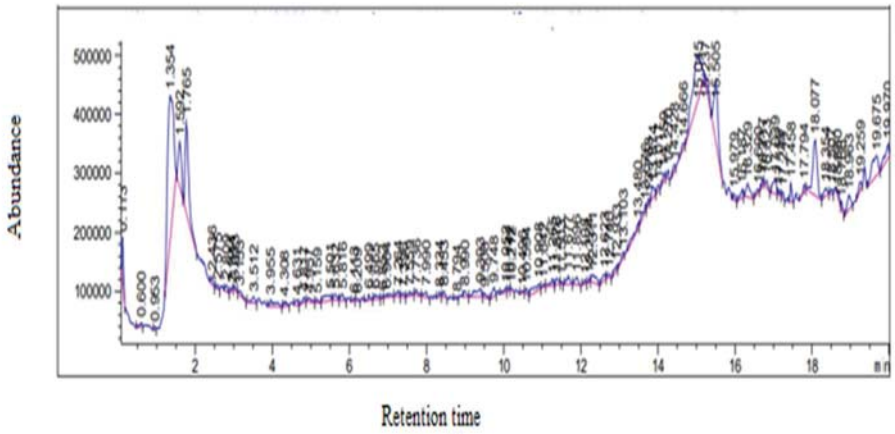


Figure 5.1 TIC of methanol extract of Prasarinyadi kashayam

5.2.2. Identification of compounds with HRLCMS

HR LCMS spectrum of methanol extract of Prasarinyadi kashayam contains more than 100 peaks. Out of them 42 compounds identified tentatively (Table 5.1). Out of 42 compounds identified 24 are phenolic compounds, 11 alkaloids, 5 terpenes, 1 alkane and an acid. A detailed literature survey revealed that all these constituent compounds

exhibited pharmacological activities. They include anti-inflammatory, antioxidant, antiradical, antidiabetic, antiviral, antibacterial, anti-cancer, anti -Alzheimer's, chemopreventive, anti-allergic, antispasmodic, anti-proliferative, wound healing, anti-malaria properties. Out of the identified compounds, 28 compounds have been reported as anti- inflammatory and antioxidant. List of compounds with anti-inflammatory activity are given in Table 5.2.

Table 5.1 List of identified compounds from methanol extract of Prasarinyadi kashayam using HRLCMS

SI No	Rt	Molecular formula	Molecular weight	Compound name	IUPAC Name of the componend	Class of compound	Molecular ion	Plant source
1	1.073	$C_{11}H_{10}N_2O_2$	202.213	Vasicinone	(3S)-3-Hydroxy-2, 3-dihydro-1H-pyrrolo [2, 1-b] quinazolin-9-one)	**Alkaloid**	203.0519 $[M+H]^+$	*Sida rhombifolia* L. [14]
2	5.47	$C_{21}H_{20}O_6$	368.35	Curcumin	(1E,6E)-1,7-bis (4-hydroxy- 3-methoxyphenyl) -1,6- heptadiene-3,5-dione	Phenolics	368.147$[M+]^+$	*Zingiber officinale* Rosc. [15]
3	5.797	$C_{27}H_{44}O_7$	480.642	20-Hydroxyecdysone	(22*R*)-2β,3β,14α,20,22,25-hexahydroxy-5β-cholest-7-en-6-one	Terpenes	481.3743$[M+H]^+$	*Sida rhombifolia* L.[16]
4	6.263	$C_{20}H_{27}NO_3$	329.3434	Merresectin E	[4-hydroxy-3-isoprenylbenzoic acid]tropan	Alkaloid	329.0439$[M+]^+$	*Merremia tridentate* (L.) Hallier f. [17]
5	6.531	$C_{21}H_{20}O_{11}$	448.4	Isoorientin	2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-6-[(2S,3R,4R,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]-4H-1-benzopyran-4-one	Phenolics	447.3081$[M-H]^-$	*Merremia tridentate*(L.) Hallier f. [15]
6	6.627	$C_{23}H_{26}N_4O_3$	406.5	Argentine	(1S,9R)-11-[(1S,9R)-6-oxo-7,11-diazatricyclo[7.3.1.02,7]trideca-2,4-diene-11-carbonyl]-7,11-diazatricyclo[7.3.1.02,7]trideca-2,4-dien-6-one	Alkaloid	429.2980 $[M+Na]^+$	*Zingiber officinale* Rosc. [15]

SI No	Rt	Molecular formula	Molecular weight	Compound name	IUPAC Name of the componend	Class of compound	Molecular ion	Plant source
7	7.366	$C_{20}H_{20}O_6$	356.4	Kievitone	3-(2,4-dihydroxyphenyl)-5,7-dihydroxy-8-(3-methylbut-2-enyl)-2,3-dihydrochromen-4-one	Phenolics	357.1326$[M+H]^+$	*Vigna mungo* (L.) Hepper [18]
8	9.333	$C_{29}H_{48}O$	412.69	Stigmasterol	(3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-17-[(*E*,2*R*,5*S*)-5-ethyl-6-methylhept-3-en-2-yl]-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1*H*-cyclopenta[a]phenanthren-3-ol	Terpenes	413.2126$[M+H]^+$	*Vigna mungo* (L.) Hepper [18]
9	9.455	$C_{20}H_{20}O_5$	340.375	8-Prenylnaringenin	(2S)-5,7-Dihydroxy-2-(4-hydroxyphenyl)-8-(3-methylbut-2-en-1-yl)-2,3-dihydro-4H-1-benzopyran-4-one	Phenolics	379.1137 $[M+K]^+$	*Merremia tridentate*(L.) Hallier f. [17]
10	4.156	$C_{21}H_{32}O_4$	348.5	10-Gingerdione	(*Z*)-3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)tetradec-3-en-5-one	Phenolics	349.1779$[M+H]^+$	*Zingiber officinale* Rosc. [15]
11	4.483	$C_{15}H_{22}O_3$	250.33	Procurcumadiol	(3S,3aR,8aS)-3,3a-dihydroxy-3,8-dimethyl-5-propan-2-ylidene-1,2,4,8a-tetrahydroazulen-6-one	Phenolics	251$[M+H]^+$	*Zingiber officinale* Rosc. [15]
12	7.609	$C_{15}H_{10}O_6$	286.24	Luteolin	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4H-chromen-4-one	Phenolics	309$[M+Na]^+$	*Merremia tridentate*(L.) Hallier f. [17]

SI No	Rt	Molecular formula	Molecular weight	Compound name	IUPAC Name of the componend	Class of compound	Molecular ion	Plant source
13	8.858	$C_{25}H_{35}NO_3$	397.5	Merresectin C	[4-Hydroxy-3,5-bis isoprenylbenzoic acid]tropan	Alkaloid	413[M+CH_4]	*Merremia tridentate*(L.) Hallier f. [17]
14	5.712	$C_{20}H_{18}O_7$	370.1062	Hydroxyhinokinin	6-Hydroxy(3R,4R)-3,4bis[(2H-1,3-benzodioxo)7'-methyl]oxalane-2-one	Phenolics	371.0948 $[M+H]^+$	*Piper longum* L.
15	9.678	$C_{17}H_{26}O_4$	294.38	6-Gingerol	(5S)- 1-(3,4-dimethoxyphenyl)-5-hydroxydecan-3-one	Phenolics	295.0554 $[M+H]^+$	*Zingiber officinale* Rosc. [15]
16	9.927	$C_{31}H_{64}$	436.85	Hentriacontane	Hentriacontane	Alkane	437.2008 $[M+H]^+$	*Sida rhombifolia* L. [16]
17	12.135	$C_{18}H_{30}O_2$	278.43	Alpha-linolenic acid	Alpha- linolenic acid	Acid	279.033 $[M+H]^+$	*Vigna mungo* (L.) Hepper [18]
18	12.208	$C_{19}H_{16}O_4$	308.314	Bisdemethoxycurcumin	(1E,6E)-1,7-Bis(4-hydroxyphenyl)hepta-1,6-diene-3,5-dione	Phenolics	309 $[M+H]^+$	*Zingiber officinale* Rosc. [15]
19	19.602	$C_{20}H_{22}O_8$	390.388	8-Hydroxytinosporide	(1S,2S,3R,4R,5R,8S,10R,12S)-4-Hydroxy-2,3:15,16-diepoxycleroda-13(H),14-dieno-(17,12:18,1)-biscarbolactone-8-ol	Terpenes	391.2794 $[M+H]^+$	*Tinospora cordifolia*(Willd) Miers ex Hook.f.&Thoms.
20	1.127	$C_{19}H_{19}N_3O$	305.4	Vasicolinone	3-[2-(dimethylamino)phenyl]-2,3-dihydro-1*H*-pyrrolo[2,1-b]quinazolin-9-one	Alkaloid	305.1298 $[M+]^+$	*Sida rhombifolia* L. [16]

SI No	Rt	Molecular formula	Molecular weight	Compound name	IUPAC Name of the componend	Class of compound	Molecular ion	Plant source
21	1.422	$C_{17}H_{22}O_4$	290.4	6-Dehydrogingerdione	(E)-1-(4-hydroxy-3-methoxyphenyl)dec-1-ene-3,5-dione	Phenolics	291$[M+H]^+$	*Zingiber officinale* Rosc. [15]
22	3.06	$C_{15}H_{24}O_2$	236.35	Curdione	(3S,6E,10S)-6,10-Dimethyl-3-(propan-2-yl)cyclodec-6-ene-1,4-dione	Terpenes	237.15$[M+H]^+$	*Zingiber officinale* Rosc. [15]
23	3.792	$C_{14}H_8O_4$	240.214	1, 4-Dihydroxyanthraquinone	1,4-Dihydroxyanthracene-9,10-dione	Phenolics	241.114 $[M+H]^+$	*Sida rhombifolia* L. [16]
24	3.93	$C_{15}H_{12}O_5$	272.25	Glycinol	(6*aS*,11*aS*)-6,11*a*-dihydro-[1]benzofuro[3,2-c]chromene-3,6*a*,9-triol	Phenolics	295.15 [M+Na]+	*Vigna mungo* (L.)Hepper [18]
25	4.348	$C_{18}H_{30}O_4$	310.4284	4-methyl 6-gingerdiol	(E)-1-(3,4-dimethoxyphenyl)dec-3,5-diol	Phenolics	311.1700 M+H+	*Zingiber officinale* Rosc. [15]
26.	7.516	$C_{20}H_{18}NO_4+$	336.3612	Berberine	5,6-dihydro-9,10-dimethoxybenzo[g]-1,3-benzodioxolo[5,b-a]quinolizirium	Alkaloid	371 M+Cl-	*Alpinia calcarata* Roscoe [20]
27	10.661	$C_{16}H_{19}NO_3$	274.35	Sanguinine	(1*S*,12*S*,14*R*)-4-methyl-11-oxa-4-azatetracyclo[8.6.1.$0^{1,12}$.$0^{6,17}$]heptadeca-6(17),7,9,15-tetraene-9,14-diol	Alkaloid	274.2705 $M+H^+$	*Sida rhombifolia* L. [14]
28	3.788	$C_{15}H_{10}O_7$	302.24	Quercetin	2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one	Phenolics	303 $M+H^+$	*Alpinia calcarata* Roscoe [19]

SI No	Rt	Molecular formula	Molecular weight	Compound name	IUPAC Name of the componend	Class of compound	Molecular ion	Plant source
29	27.08	$C_{14}H_{14}O_4$	246.26	Marmesin	(2S)-2-(2-Hydroxypropan-2-yl)-2,3-dihydro-7H-furo[3,2-g][1]benzopyran-7-one	Phenolics	247.1623 $M+H^+$	*Zingiber officinale* Rosc. [15]
30	3.674	$C_{18}H_{19}NO_5$	330.37	Trans feruloylctapamine	(*E*)-*N*-[2-hydroxy-2-(4-hydroxyphenyl)ethyl]-3-(4-hydroxy-3-methoxyphenyl)prop-2-enamide	Alkaloid	330.2007 M^+	*Allium sativum* L.[21]
31	7.301	$C_{21}H_{22}O_6$	372.41	Tetrahydrocurcumin	1,7-bis(4-hydroxy-3-methoxyphenyl)heptane-3,5-dione	Phenolics	372.2120 $M+2H^+$	*Zingiber officinale* Rosc. [15]
32	3.792	$C_{11}H_{10}N_2O_3$	218.2	Vasicinolone	3, 7-dihydroxy-2, 3-dihydro-1*H*-pyrrolo [2, 1-b] quinazolin-9-one	Alkaloid	241.114 $M+Na^+$	*Sida rhombifolia* L.[16]
33	6.627	$C_{22}H_{22}O_9$	430.40	Techtochrysin 5-β-glucoside	5-beta glucosyl -7-methoxy-2-phenyl-4*H*-1-benzopyran-4-one	Phenolics	429.2980 $M-H^+$	*Alpinia calcarata* Roscoe [19]
34	7.301	$C_{21}H_{26}O_6$	374.34	Hexahydrocurcumin	(RS)-5-Hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-3-heptanone	Phenolics	373.1297 $[M-H]^+$	*Zingiber officinale* Rosc. [15]
35	7.660	$C_{10}H_{12}O_4$	196.20	Asaraldehyde	2,4,5 trimethoxybenzaldehyde	Terpenes	196.991 M^-	*Zingiber officinale* Rosc. [15]
36	4.657	$C_{20}H_{18}O_8$	386.35	7-Hydroxyhinokinin	(3R,4R)-3,4-bis[(2H-1,3-benzodioxo) 7-hydroxy -7'-methyl]oxalane-2-one	Phenolics	386.837 M^+	

SI No	Rt	Molecular formula	Molecular weight	Compound name	IUPAC Name of the componend	Class of compound	Molecular ion	Plant source
37	3.490	$C_8H_{11}N$	121.18	Phenethylamine	Phenylethan-2 amine	Alkaloid	121.0293 M^+	*Sida rhombifolia* L.[16]
38	4.003	$C_{13}H_{12}N_2O$	212.25	4-O-Methylsyringic acid	3,4,5-trimethoxybenzoic acid	Alkaloid	212.0206 M^+	*Alpinia calcarata* Roscoe [17]
39	1.687	$C_7H_6O_5$	170.12	Gallic acid	3,4,5-trihydroxybenzoic acid	Phenolics	169.0119 $M\text{-}H^+$	*Alpinia calcarata* Roscoe [17]
40	3.55	$C_{22}H_{26}O_8$	418.44	Syringaresinol	4,4′-[(1S,3aR,4S,6aR)-Tetrahydro-1H,3H-furo[3,4-c]furan-1,4-diyl]bis(2,6-dimethoxyphenol)	Phenolics	417.2116 $M\text{-}H^+$	
41	4.990	$C_{15}H_{18}O_2$	230.30	Curzerenone	(5R,6R)-6-Ethenyl-3,6-dimethyl-5-(prop-1-en-2-yl)-6,7-dihydro-1-benzofuran-4(5H)-one	Terpenes	230.2486 M^+	*Zingiber officinale* Rosc. [15]
42	3.274	$C_{33}H_{43}O_{21}$	775.68	Keamferol-8C-glc-3, 5-glc-glc	3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one -8C-glc-3, 5-glc-glc	Phenolics	775.4722	*Vigna mungo* (L.) Hepper [18]

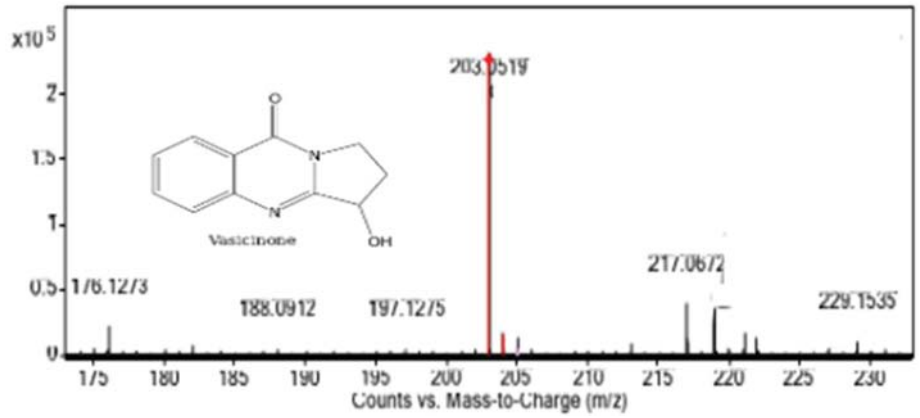


Figure 5.2. Mass spectrum of Vasicinone

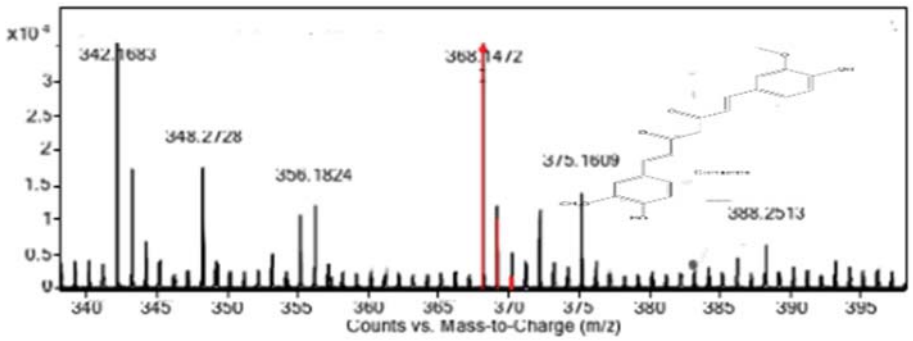


Figure 5.3 Mass spectrum of Curcumin

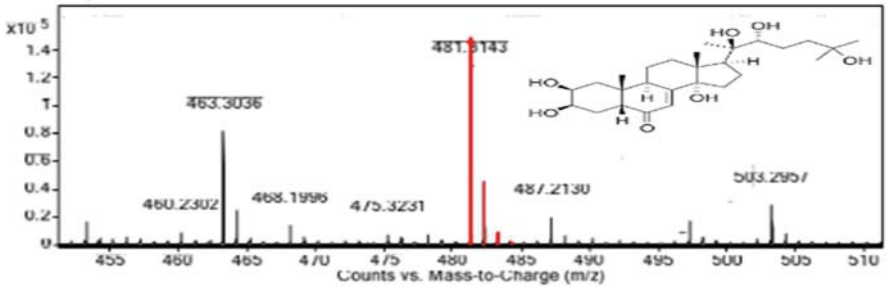


Figure 5.4 Mass spectrum of 20-Hydroxyecdysone

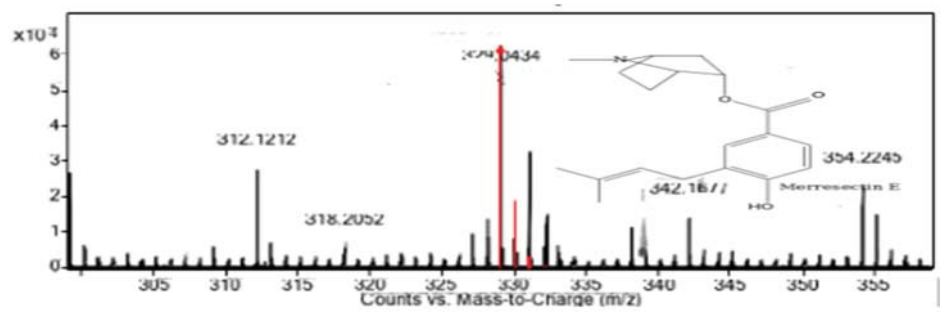


Figure 5.5 Mass spectrum of Merresectin E

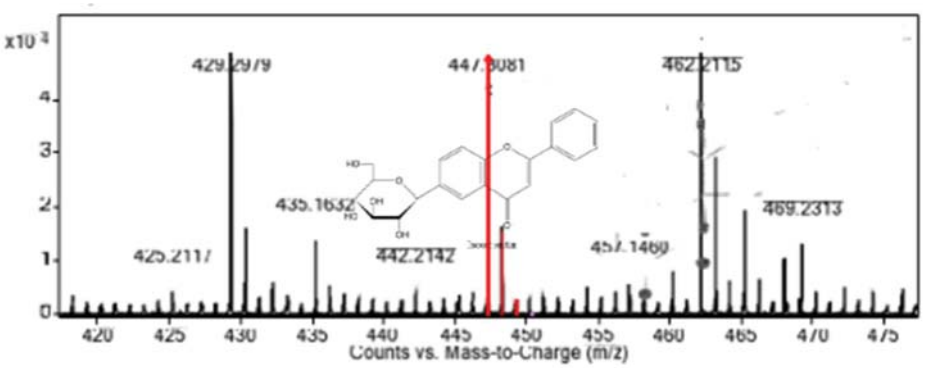


Figure 5.6 Mass spectrum of Isoorientin

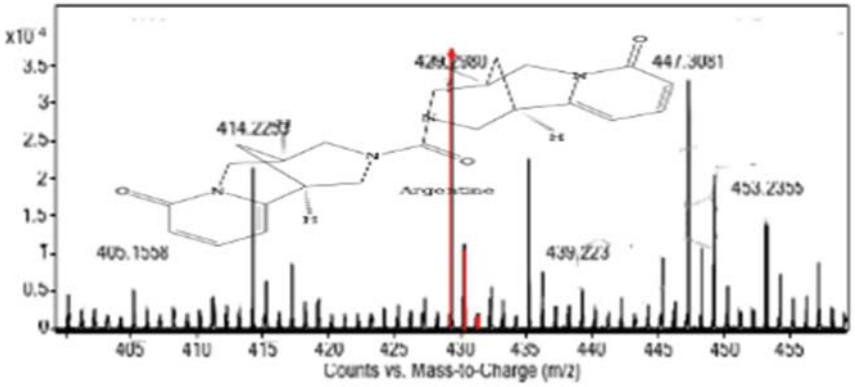


Figure 5.7 Mass spectrum of Argentine

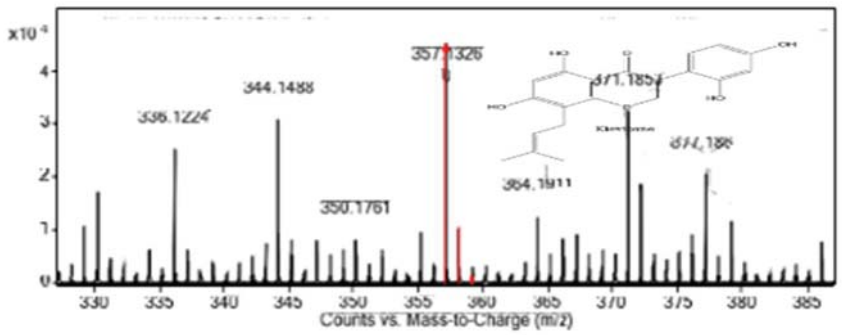


Figure 5.8 Mass spectrum of Kievitone

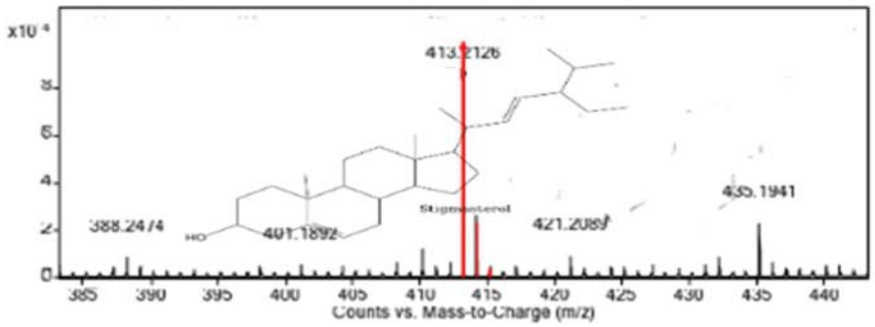


Figure 5.9 Mass spectrum of Stigmasterol

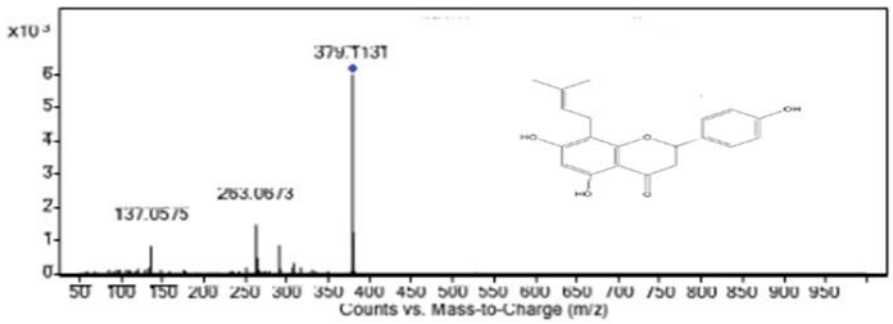


Figure 5.10 Mass spectrum of 8-Prenylnaringenin

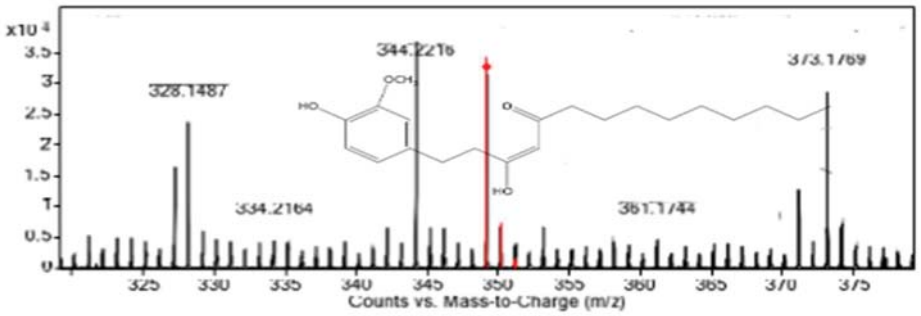


Figure 5.11 Mass spectrum of 10-Gingerdione

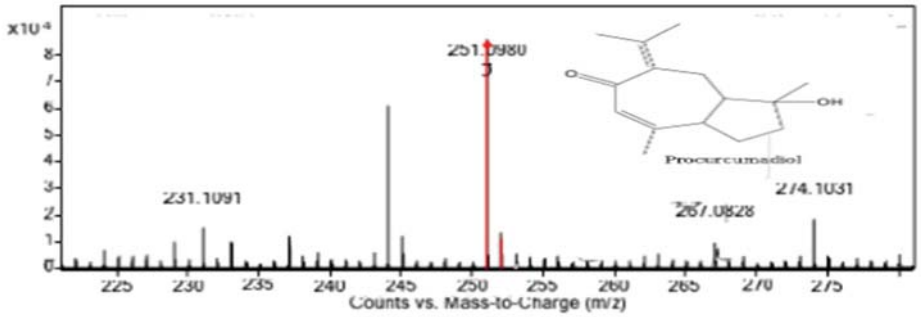


Figure 5.12 Mass spectrum of Procurcumadiol

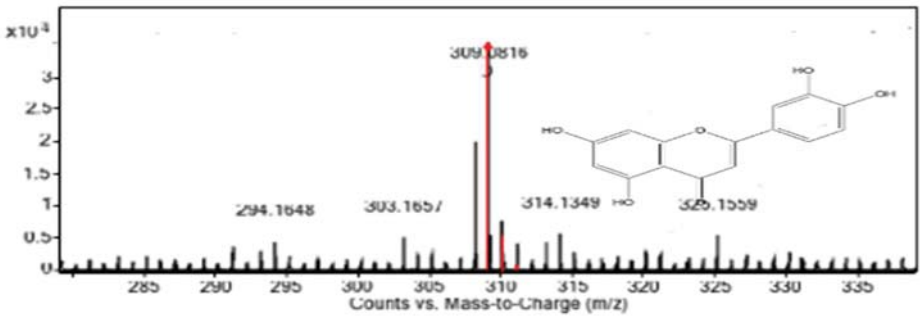


Figure 5.13 Mass spectrum of Luteolin

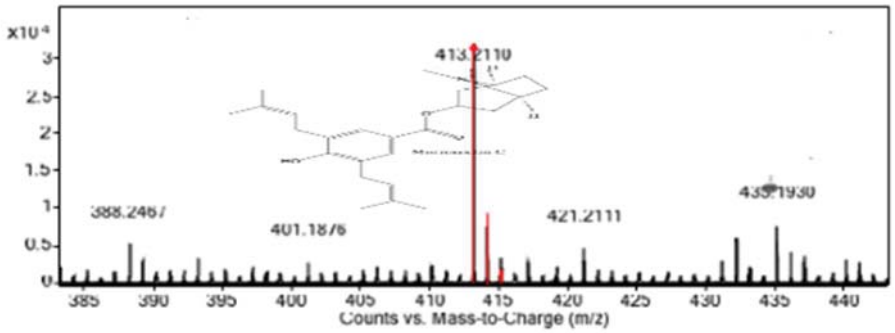


Figure 5.14 Mass spectrum of Merresectin C

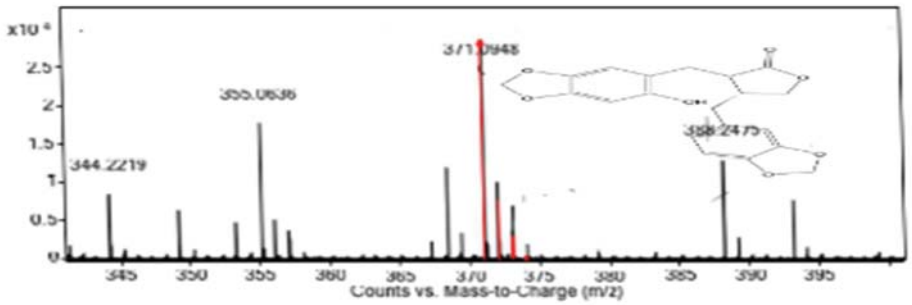


Figure 5.15 Mass spectrum of Hydroxyhinokinin

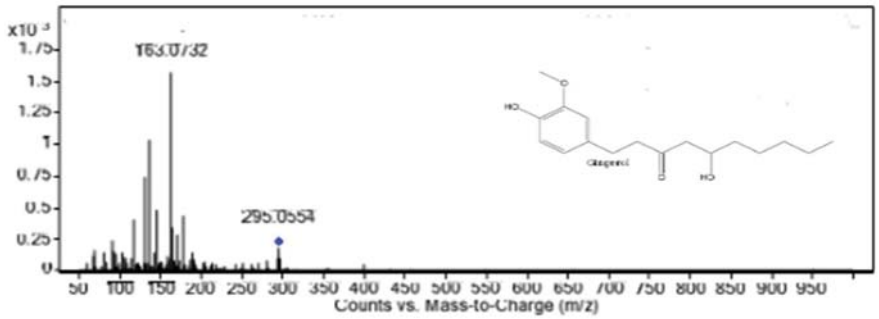


Figure 5.16 Mass spectrum of 6 Gingerol

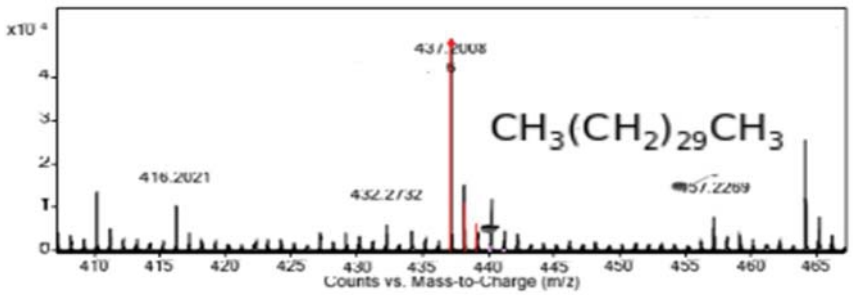


Figure 5.17 Mass spectrum of Hentriacontane

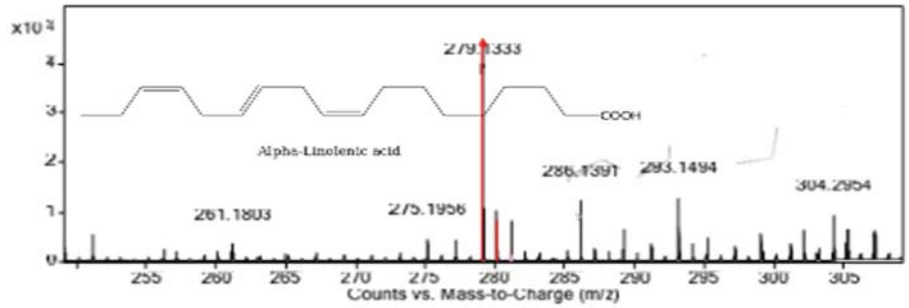


Figure 5.18 Mass spectrum of Alpha-Linolenic acid

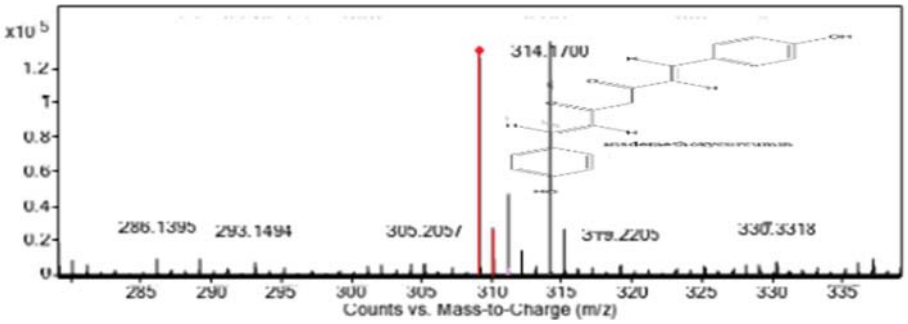


Figure 5.19 Mass spectrum of Bisdemethoxycurcumin

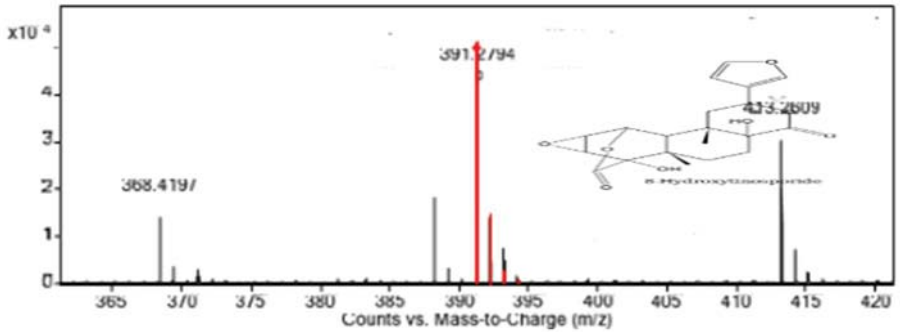


Figure 5.20 Mass spectrum of 8-Hydroxytinosporide

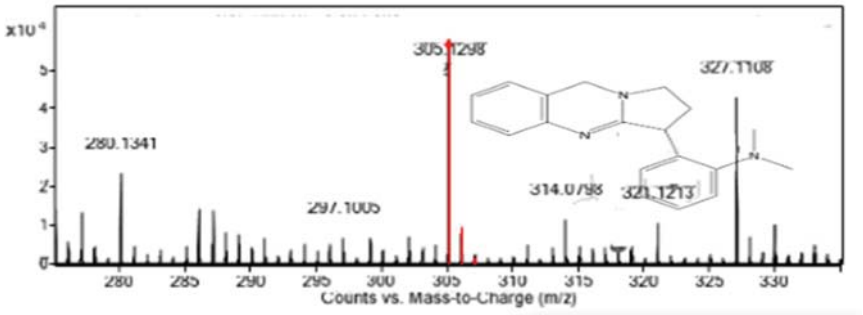


Figure 5.21 Mass spectrum of Vasicolinone

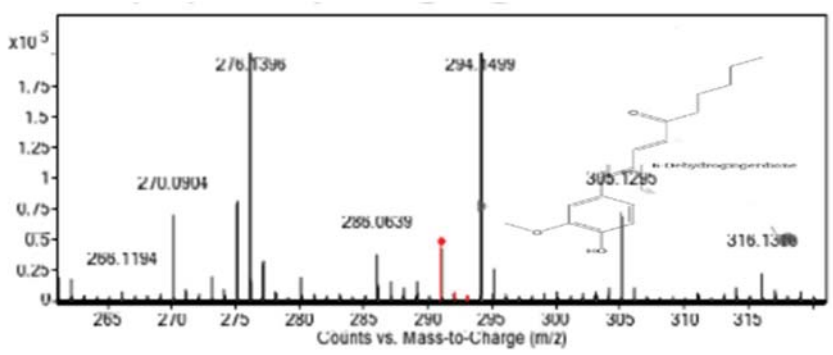


Figure 5.22 Mass spectrum of 6-Dehydrogingerdione

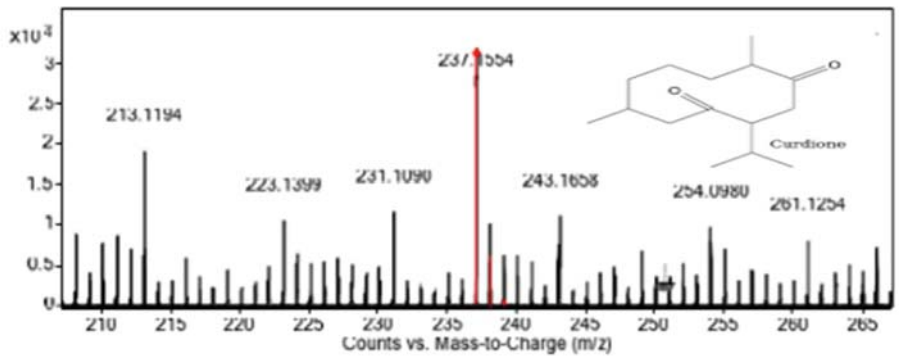


Figure 5.23 Mass spectrum of Curdione

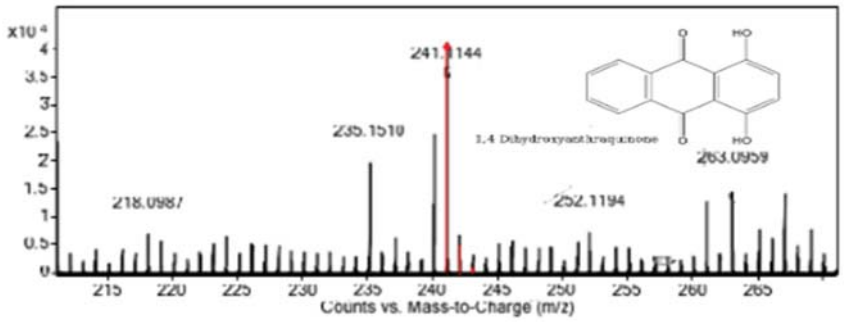


Figure 5.24 Mass spectrum of 1, 4-Dihydroxyanthraquinone

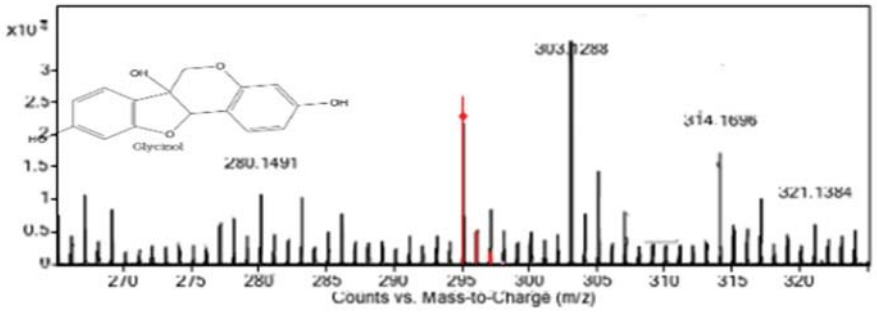


Figure 5.25 Mass spectrum of Glycinol

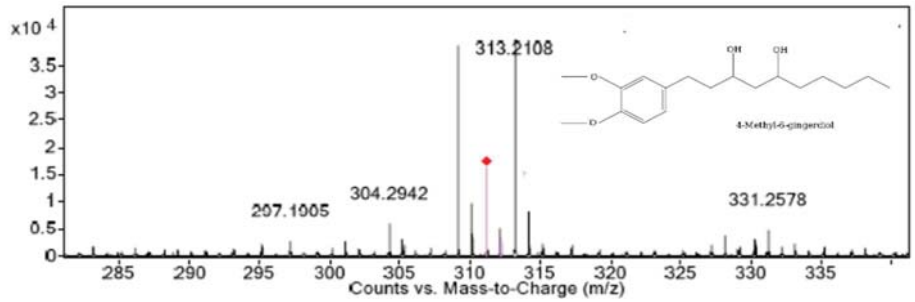


Figure 5. 26 Mass spectrum of 4-Methylgingerdiol

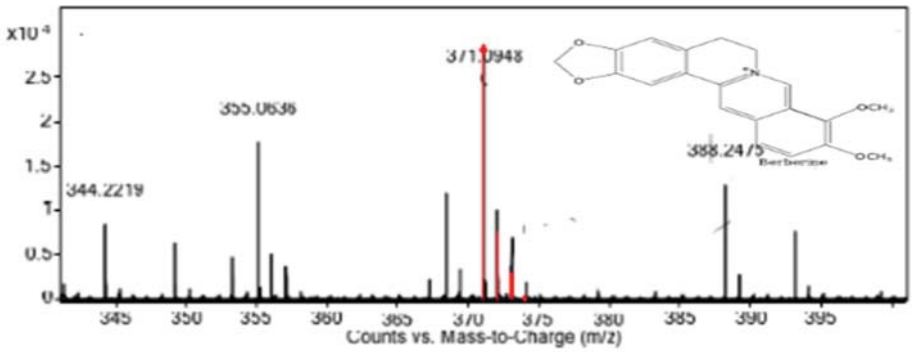


Figure 5.27 Mass spectrum of Berberine

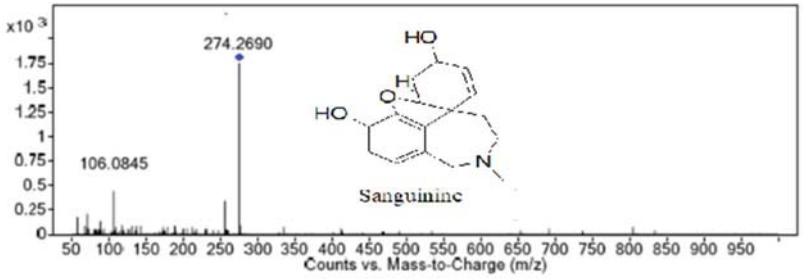


Figure 5.28 Mass spectrum of Sanguinine

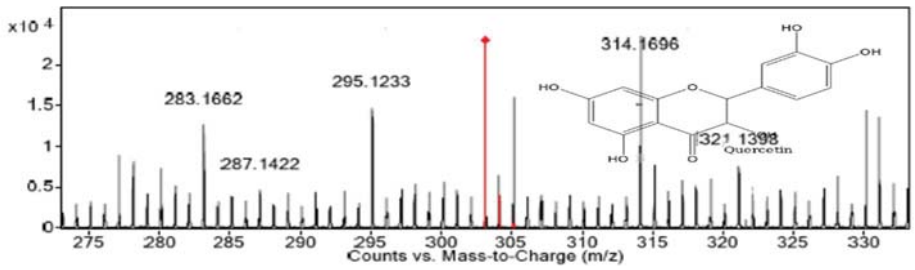


Figure 5.29 Mass spectrum of Quercetin

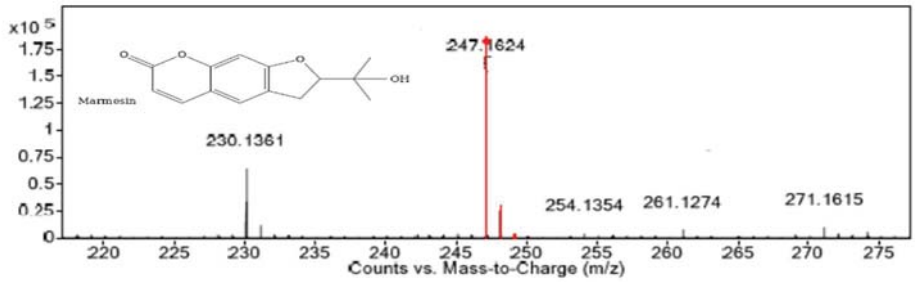


Figure 5.30 Mass spectrum of Marmesin

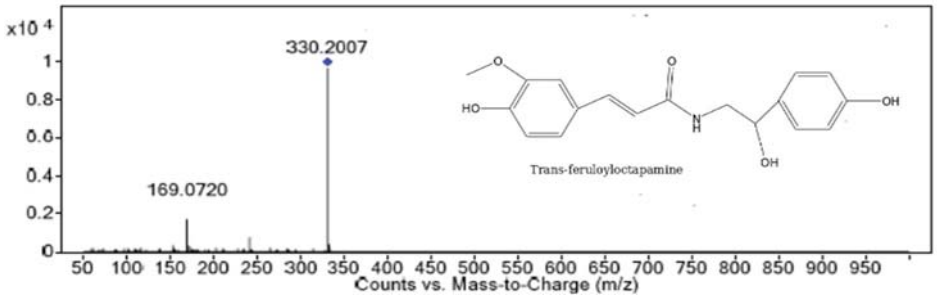


Figure 5.31 Mass spectrum of Trans feruloylctapamine

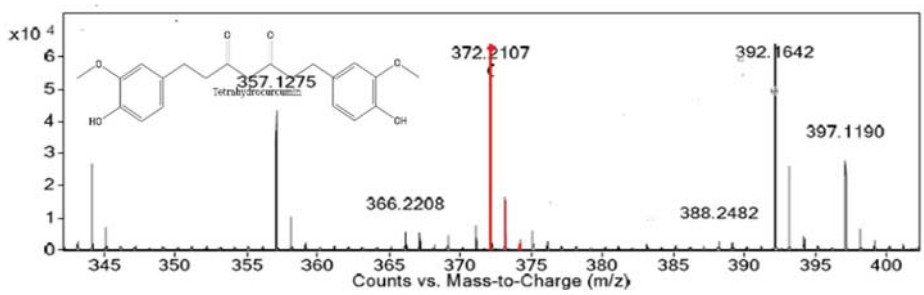


Figure 5.32 Mass spectrum of Tetrahydrocurcumin

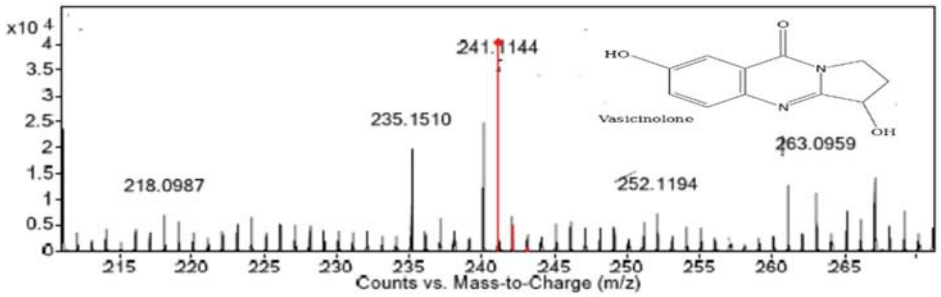


Figure 5.33 Mass spectrum of Vasicinolone

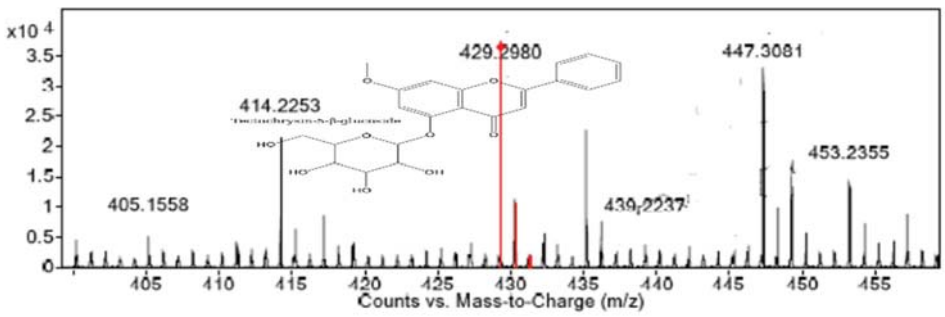


Figure 5.34 Mass spectrum of Techtochrysin -5-β-glucoside

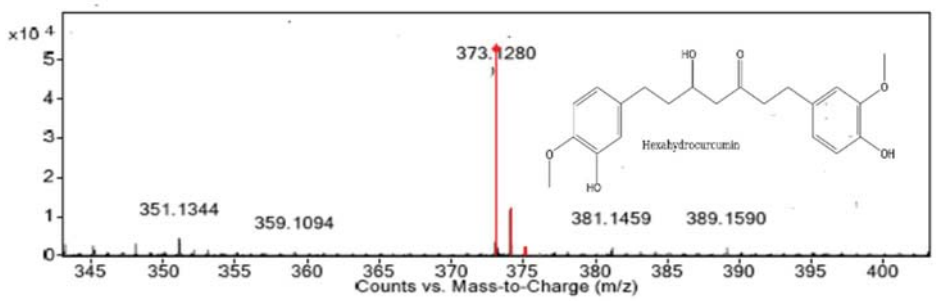


Figure 5.35 Mass spectrum of Hexahydrocurcumin

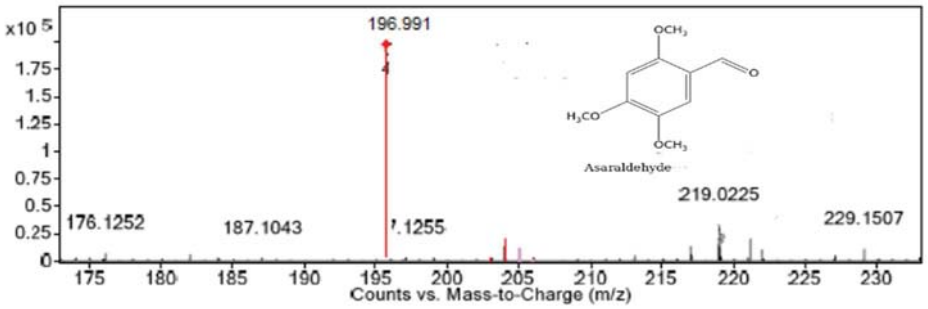


Figure 5.36 Mass spectrum of Asaraldehyde

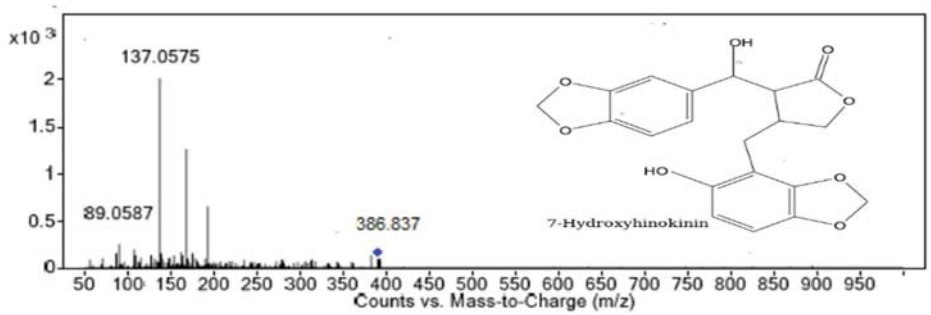


Figure 5.37 Mass spectrum of 7-Hydroxyhinokinin

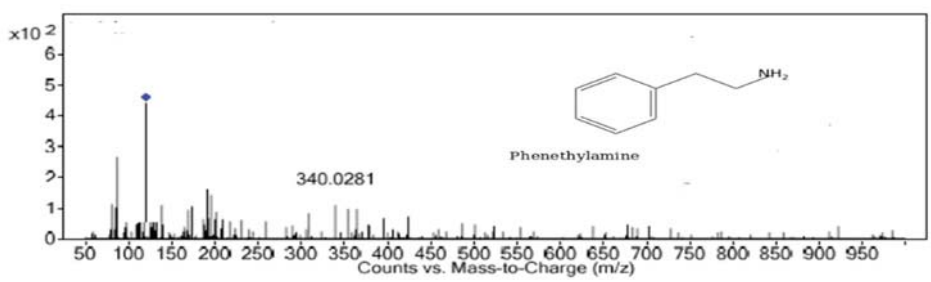


Figure 5.38 Mass spectrum of Phenethylamine

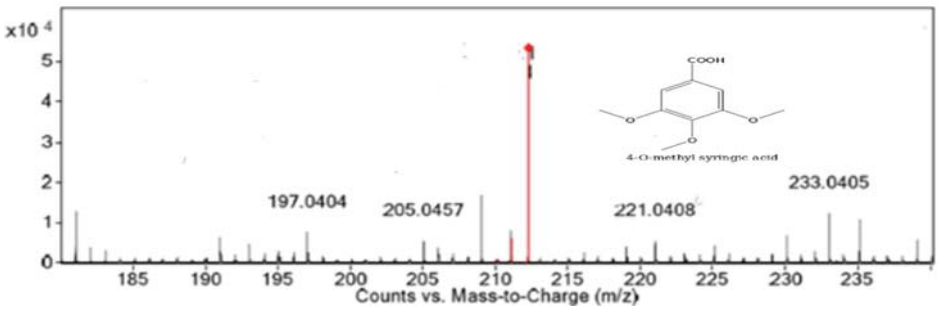


Figure 5.39 Mass spectrum of 4-O –Methylsyringic acid

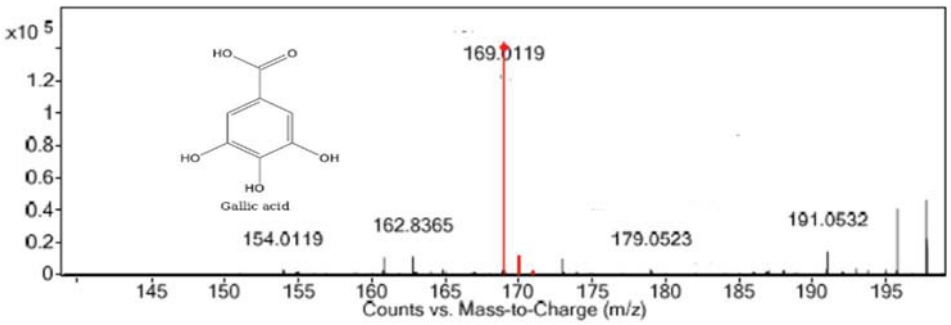


Figure 5.40 Mass spectrum of Gallic acid

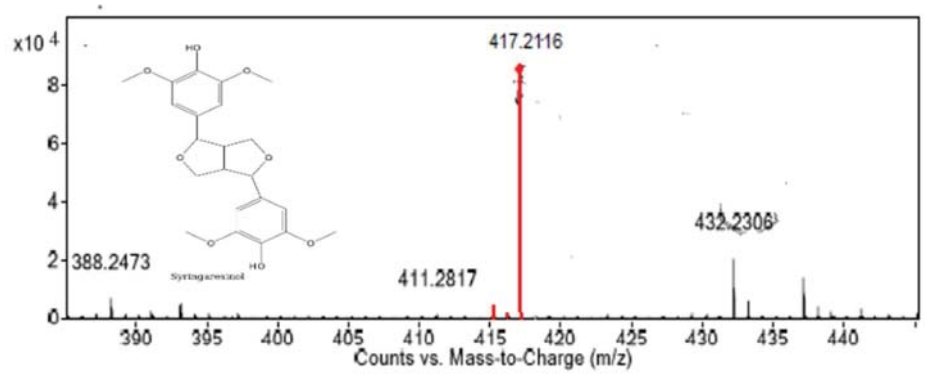


Figure 5.41 Mass spectrum of Syringaresinol

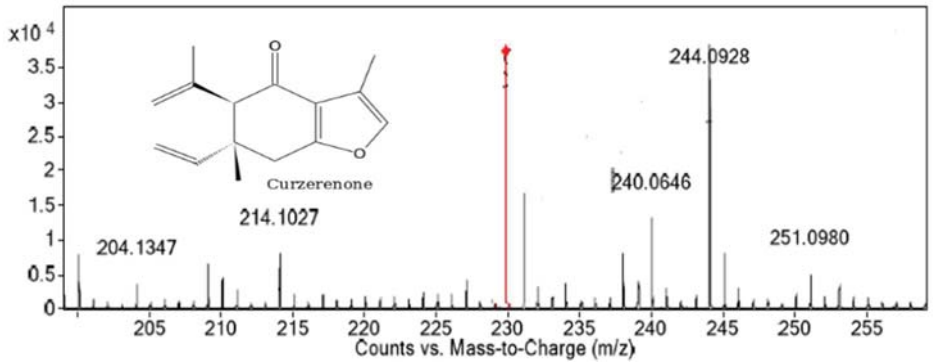


Figure 5.42 Mass spectrum of Curzerenone

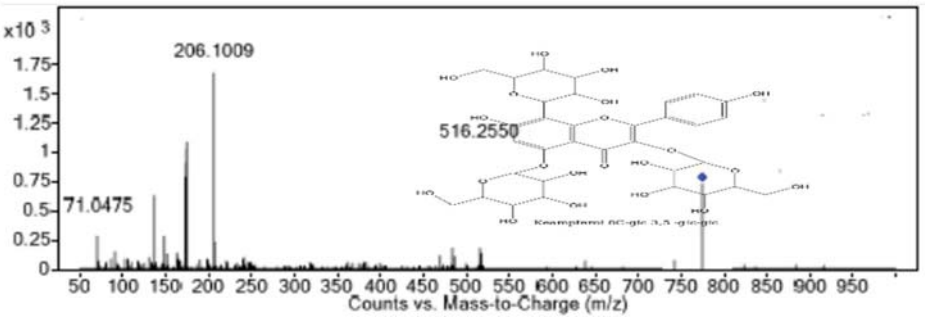


Figure 5.43 Mass spectrum of Kaempferol 8C-glc 3, 5 –glc-glc

Table 5.2. List of identified compounds having anti-inflammatory, antioxidant and immunostimulant activity from the methanol extract of Prasarinyadi kashayam

SI No.	Compound name	Biological activity	Plant source
1.	Vasicinone	Anti-inflammatory[22]	*Sida rhombifolia* L.
2.	Curcumin	Anti-inflammatory[23], Anti-oxidant, Immunomodulatory activity,	*Zingiber officinale* Rosc.
3.	20-Hydroxyecdysone	Anti-inflammatory[24], Antioxidant, Immunomodulatory medicine	*Sida rhombifolia* L.
4.	Isoorientin	Anti-inflammatory [25]	*Merremia tridentata*(L.) Hallier f.
5.	Kievitone	Anti-inflammatory[26]	*Vigna mungo* (L.) Hepper
6.	8-Prenylnaringenin	Antioxidant[27]	*Merremia tridentata* (L.) Hallier f.
7.	10-Gingerdione	Anti-inflammatory [28]	*Zingiber officinale* Rosc.
8.	Stigmasterol	Anti-inflammatory [29]	*Vigna mungo* (L.) Hepper
9.	Luteolin	Anti-inflammatory[30], enhances immunity, antioxidant,	*Merremia tridentata* (L.) Hallier f.
10.	6-Gingerol	Anti-inflammatory [28]	*Zingiber officinale* Rosc.
11.	Hentriacontane	Anti-inflammatory [31]	*Sida rhombifolia* L.
12.	Alpha-linolenic acid	Anti-inflammatory [32]	*Vigna mungo* (L.) Hepper
13.	Bisdemethoxycurcumin	Anti-inflammatory [33]	*Zingiber officinale* Rosc.
14.	Vasicolinone	Anti-inflammatory effects[22]	*Sida rhombifolia* L.
15.	6-Dehydrogingerdione	Anti-inflammatory, antioxidant [34]	*Zingiber officinale* Rosc.
16.	Curdione	initiate immunity[35]	*Zingiber officinale* Rosc.
17.	1, 4-Dihydroxyanthraquinone	Anti-inflammatory effects[38], anti-oxidative activity	*Sida rhombifolia* L.

SI No.	Compound name	Biological activity	Plant source
18.	Quercetin	Anti-inflammatory[36], Immunostimulant, Antioxidant	*Alpinia calcarata* Roscoe
19.	Gallic acid	Anti-inflammatory[37], Antioxidant	*Alpinia calcarata* Roscoe
20.	Vasicinolone	Anti-inflammatory effects[22]	*Sida rhombifolia* L.
21.	Tetrahydrocurcumin	Anti-inflammatory[34], Anti-oxidant, Immunomodulatory activity,	*Zingiber officinale* Rosc.
22.	4-Methyl-6-gingerdiol	Anti-inflammatory[34], antioxidant	*Zingiber officinale* Rosc.
23.	Berberine	Anti-inflammatory[39], Antioxidant	*Alpinia calcarata* Roscoe
24.	Asaraldehyde	Anti-inflammatory[40]	*Zingiber officinale* Rosc.
25.	Curzerenone	Anti-inflammatory effects[41]	*Zingiber officinale* Rosc.
26.	Keampferol -8C-glc-3, 5-glc-glc	Anti-inflammatory[42]	*Vigna mungo*(L.) Hepper
27.	Syringaresinol	Anti-inflammatory[43]	
28.	4-O-methylsyringic acid	Anti-inflammatory[44]	*Alpinia calcarata* Roscoe

Among the different biological activities that have been extensively investigated in medicinal plants and herbal drugs, anti-inflammatory and associated properties stand the highest. Alkaloids, flavonoids, polyphenols and terpenoids are the main classes of plant derived compounds that are potent in healing several inflammatory diseases.

The HRLCMS identified compounds in this formulation revealed that the phytochemicals identified in this study have been found to exhibit potent immunostimulant, antioxidant and anti-inflammatory properties. Hence, this study forms a sort of scientific validation for the therapeutic

use of this medicinal preparation. Polyphenols regulate immunity by interfering with immune cell regulation, proinflammatory cytokines synthesis, and gene expression. They inhibit certain enzymes involved in reactive oxygen species (ROS) production like xanthine oxidase and NADPH oxidase (NOX) while they upregulate other endogenous antioxidant enzymes like superoxide dismutase (SOD), catalase, and glutathione (GSH) peroxidase (Px) [45]. Furthermore, they inhibit phospholipase A2 (PLA2), cyclooxygenase (COX) and lipoxygenase (LOX) activities leading to a reduction in the production of prostaglandins (PGs) and leukotrienes (LTs) resulting in inflammation antagonism. The effects of these biologically active compounds on the immune system are associated with extended health benefits for different chronic inflammatory diseases [46]. Studies of plant extracts and compounds show that polyphenols can play a beneficial role in the prevention and the progress of chronic diseases related to inflammation such as diabetes, obesity, neurodegeneration, cancers, and cardiovascular diseases, among other conditions. From the table above, it can be seen that many of the compounds diagnosed from the HRLCMS analysis are polyphenols.

5.2.3. UPLC-ESI Q- TOF/MS Studies of Prasarinyadi kashayam

In the structure elucidation process based on ESI-QTOF/MS data, the knowledge of the fragmentation reactions involved in the formation of the peaks of the mass spectrum may provide important information about the molecular structure and the connectivity between the atoms. These studies showed major reactions, and the mechanisms were derived from the functional groups, which are the fundamental keys to the mechanisms. For studies with natural products, however, the overall system must be considered, so that the mechanism involved cannot be suggested based solely on the functional groups. When performing the rationalization of a

fragmentation pathway, it is important to remember that the proposed fragmentation processes must be based on organic chemistry principles. The ESI MS fragmentation reactions of natural products can be divided into two main groups: charge retention fragmentations (CRF) and charge migration fragmentations (CMF) [47].

Charge retention fragmentations (CRF) are composed of a class of reactions that results in fragment ions with the charge located at the site identical to its precursor ion. Because CRF typically occurs at a location that is physically remote from the location of the charge and without its direct participation in the mechanism. Normally, CRF reactions proceed through concerted mechanisms and may occur even at low collision energies, depending on the chemical structure, ionization method, mass collision target and other equipment parameters. CRF can be classified into nine main reaction mechanisms: remote hydrogen rearrangements, Retro-Diels–Alder (RDA) reactions, retro-ene reactions, retro hetero ene reactions, charge remote fragmentations, aromatic eliminations, other pericyclic processes, carbon monoxide eliminations from cyclic carbonyl compounds and radical eliminations.

The CMF are fragmentation reactions in which the charge is displaced from the precursor ion. In general, CRF gas-phase reactions of negative ions are similar to those of positive ions because they occur in positions remote from the charge site. However, CMF in negative ions significantly differs from those of positive ions. In positive ions, CMF reactions usually eliminate a leaving group for which the charge site was initially the location of a neutral molecule. However, the neutral loss from negative ions typically occurs at the part of the ion structure that was initially charged.

Table 5.3 List of compounds identified with the help of UPLC-Q-TOF-MS analysis.

SI No.	Retention Time	Compound name	Type of Compound	Molecular formula	IUPAC name of the Compound	Molecular weight	Molecular ion	Fragment ions
1.	7.660	Asaraldehyde	Terpenes	$C_{10}H_{12}O_4$	2, 4, 5-trimethoxy benzaldehyde	196.20	196.8947(2%) M^+	178.8843(100%)
2.	4.573	6-Gingerol	Phenolics	$C_{17}H_{26}O_4$	(5*S*)-1-(3,4-dimethoxyphenyl)-5-hydroxy decan-3-one	294.38	293M-H^+	193.04084, 136
3.	4.892	Berberine	Alkaloid	$C_{20}H_{18}NO_4$	5,6-Dihydro-9,10-dimethoxybenzo[g]1,3-benzodioxolo5,b-a]quinolizirium	336.36	337.1630(100 %) M+H^+	320.0945(2%), 279.16153
4.	5.511	Tetrahydrocurcumin	Phenolics	$C_{21}H_{24}O_6$	1,7-bis(4-hydroxy-3-methoxyphenyl)-3,5-heptanedione is a beta-diketone	372.41	372.8219(100 %) M^+	357.0864, 217.9250(2%)
5.	6.076	8-Hydroxytinosporide	Terpenes	$C_{20}H_{22}O_8$	(1S,2S,3R,4R,5R,8R,10R,12S)-4-hydroxy-2,3:15,16-diepoxycleroda-13(H),14-dieno(17,12:18,1-biscarbolactone-8-ol	390.38	389.8125(100 %) M-H^+	262.9072(16%), 234.9126(28%)

SI No.	Retention Time	Compound name	Type of Compound	Molecular formula	IUPAC name of the Compound	Molecular weight	Molecular ion	Fragment ions
6.	4.581	4-Methyl-6-gingerdiol	Phenolics	$C_{18}H_{30}O_4$	(E)-1-(3,4-dimethoxyphenyl)dec-3,5-diol	310.43	311.1859(100 %) $M+H^+$	293.1753(48%), 219.1034
7.	4.657	7-Hydroxyhinokinin	Phenolics	$C_{20}H_{18}O_8$	(3R,4R)-3,4-bis[(2H-1,3-benzodioxo)7-hydroxy-7'-methyl-] oxalane-2-one	386.35	386.8377(100 %) M^+	343.0959, 283.2594
8.	7.660	Tectochrysin-5-β-glc	Phenolics	$C_{22}H_{22}O_9$	7-methoxy-2-phenyl-4*H*-1-benzopyran-4-one-5-beta glucoside	430.40	429.1953(32%) $M-H^+$	172.9535(100%), 339.1648(11%)
9.	3.274	Kaempferol-8C-glc-3, 5-glc, glc	Phenolics	$C_{33}H_{43}O_{21}$	3,4',5,7-tetrahydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one-8C-glc-3, 5-glc, glc	775.68	775.4722(100 %) M^+	757.4591(8%), 677.4946(10%), 581.3900(1%)
10.	3.561	Phenethylamine	Alkaloid	$C_8H_{11}N$	2-phenylethanamine	121.18	121.0293 M^+	77.0397
11.	4.003	4-O-methyl syringic acid	Phenolics	$C_{13}H_{12}N_2O$	3,4,5-trimethoxybenzoic acid	212.25	212.0216(12%) M^+	167.9952(100%), 194.0108(42%), 136.0230(8%)
12.	3.655	Vasicinolone	Alkaloid	$C_{11}H_{10}N_2O_3$	(3S)-3, 7-dihydroxy-2, 3-dihydro-1*H*-pyrrolo [2, 1-b] quinazolin-9-one	218.21	218.2120(100 %) M^+	200.2010(31%), 174.1853(5%), 156.1750(2%)

SI No.	Retention Time	Compound name	Type of Compound	Molecular formula	IUPAC name of the Compound	Molecular weight	Molecular ion	Fragment ions
13.	4.990	Curzerenone	Terpenes	$C_{15}H_{18}O_2$	(5R,6R)-6-Ethenyl-3,6-dimethyl-5-(prop-1-en-2-yl)-6,7-dihydro-1-benzofuran-4(5H)-one	230.30	230.2486(100 %) M^+	212.2381(62%)
14.	3.902	Marmesin	Phenolics	$C_{14}H_{14}O_4$	(2*S*)-2-(2-hydroxypropan-2-yl)-2, 3-dihydrofuro [3, 2-g] chromen-7-one	246.26	246.2434(100 %) M^+	228.2328,202.2174, 184.2066
15.	3.553	Sanguinine	Alkaloid	$C_{16}H_{19}NO_3$	(1*S*,12*S*,14*R*)-4-methyl-11-oxa-4-azatetracyclo[8.6.1.$0^{1,12}$.$0^{6,17}$]heptadeca-6(17),7,9,15-tetraene-9,14-diol	273.33	274.2751(100 %) $M+H^+$	256.2643(28%), 230.2484(3%), 212.2374, 106.0869(28%), 88.0764(40%)70.0656(12%)
16.	5.015	Quercetin	Phenolics	$C_{15}H_{10}O_7$	2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxychromen-4-one	302.24	302.3064(100 %) M^+	284.2957(21%), 137.0082

Sl No.	**Retention Time**	**Compound name**	**Type of Compound**	**Molecular formula**	**IUPAC name of the Compound**	**Molecular weight**	**Molecular ion**	**Fragment ions**
17.	3.553	Syringaresinol	Phenolics	$C_{22}H_{26}O_8$	4,4′-[(1S,3aR,4S,6aR)-Tetrahydro-1H,3H-furo[3,4-c]furan-1,4-diylbis(2,6-dimethoxyphenol)	418.44	419.0219(76%) $M+H^+$	375.0328(50%), 298.9830(6%)
18.	3.264	Ecdysterone	Terpenes	$C_{27}H_{44}O_7$	(22*R*)-2β,3β,14α,20,22,25-hexahydroxy-5β-cholest-7-en-6-one	480.63	497.3337 (4%)M+17	488.4002, 462.6576, 451.3288(100%), 433.3113, 415.8143, 398.2262
19	5.493	Trans-feruloylctopamine	Alkaloid	$C_{18}H_{19}NO_5$	(*E*)-*N*-[2-hydroxy-2-(4-hydroxyphenyl) ethyl]-3-(4-hydroxy-3-methoxyphenyl) prop-2-enamide	329.35	330.3372(100%) $M+H^+$	312.3258(20%), 286.3114(2%)

Identification of the compound present in the supernatant of the kashayam obtained from centrifugation has been done with the UPLC-QTOF-MS analysis. Identified compounds are given in Table 5. 3. 19 compounds have been identified. Out of the 19 compounds include 4 terpenes, 5 alkaloids and 10 phenolic compounds. The fragmentation reactions of fourteen of the identified compounds has been elucidated and described in the schemes below.

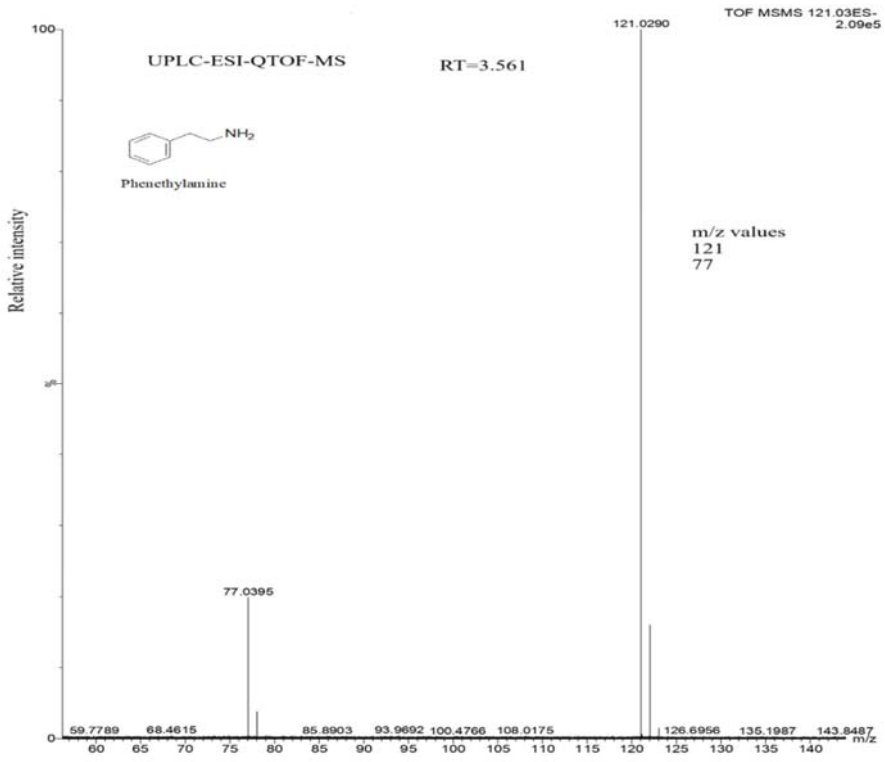
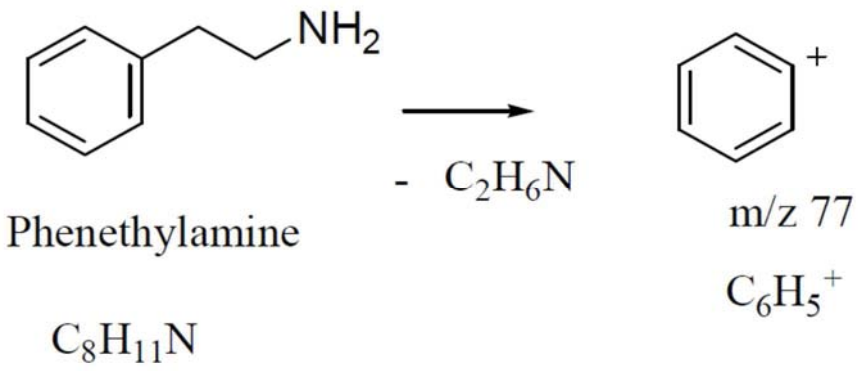


Figure 5. 44. UPLC-ESI-QTOF-Mass Spectrum of Phenethylamine



Scheme 5.1. Mass fragments of Phenethylamine

Compound with the retention time 3.561 produces a molecular ion M^+ at m/z 121.0293 and fragment at m/z 77. 0397.The fragment at m/z 77.0397 formed by the removal of (C_2H_6 N) side chain. Benzoic acid and Phenethylamine are considered for the molecular mass 121.The elemental analysis and fragments formed identified the compound as Phenethylamine or 2-phenylethanamine with the molecular formula $C_8H_{11}N$.It is the constituent of *Sida rhombifolia* L. used in the kashayam. In the HR LCMS analysis also, the presence of Phenethylamine in the methanol extract is obtained.

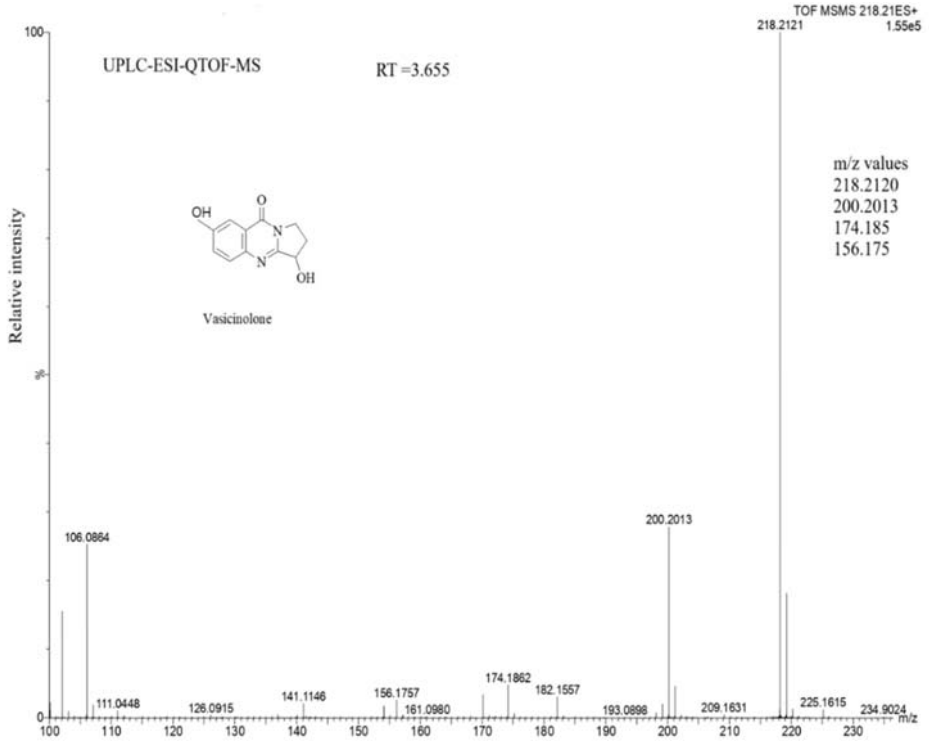
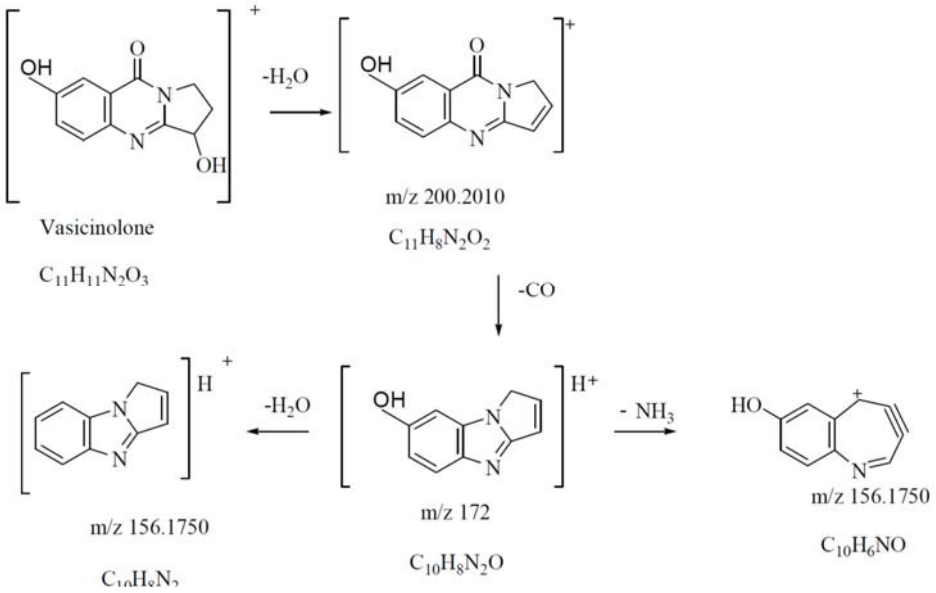


Figure 5.45. UPLC-ESI-QTOF-Mass Spectrum of Vasicinolone



Scheme 5.2. Fragmentation reactions of Vasicinolone

Compound with the retention time 3.655 form a molecular ion M^+ peak at m/z 218.2120(100%). Secondary fragments formed are at m/z 200.2010 (31%), 174.1853 (5%) and 156.1750(2%). Zerumbone, Nuciferol, Vasicinolone, Quindoline and Bisacumol are the compounds with the molecular mass 218. Considering Vasicinolone as the molecule, the fragmentation reaction could be explained. The peak at m/z 200.2010 formed by the loss of water molecules from the molecular ion and m/z at 174.1853 formed by the removal of CO molecules from the ring containing carbonyl group. The fragment at m/z 156.1750 formed from 174.1853 by the removal of water molecules. Alternatively, the product ion peak with the mass m/z 156 could also be formed by a lone pair-induced inductive cleavage that eliminates a molecule of ammonia (NH_3). Fragments formed are similar to that reported by Liu et al [48]. Its molecular mass and its fragmentation pattern confirmed the presence of Vasicinolone, 3, 7-dihydroxy-2, 3-dihydro-1*H*-pyrrolo [2, 1-b] quinazolin-9-one, with the molecular formula $C_{11}H_{10}N_2O_3$. Jam and Sharma [49] have reported the oxidation of Vasicinol to Vasicinolone. Vasicinol has been identified in the plant *Sida rhombifolia* L. used in the preparation of kashayam. This is in agreement with the identification of Vasicinolone with HRLCMS.

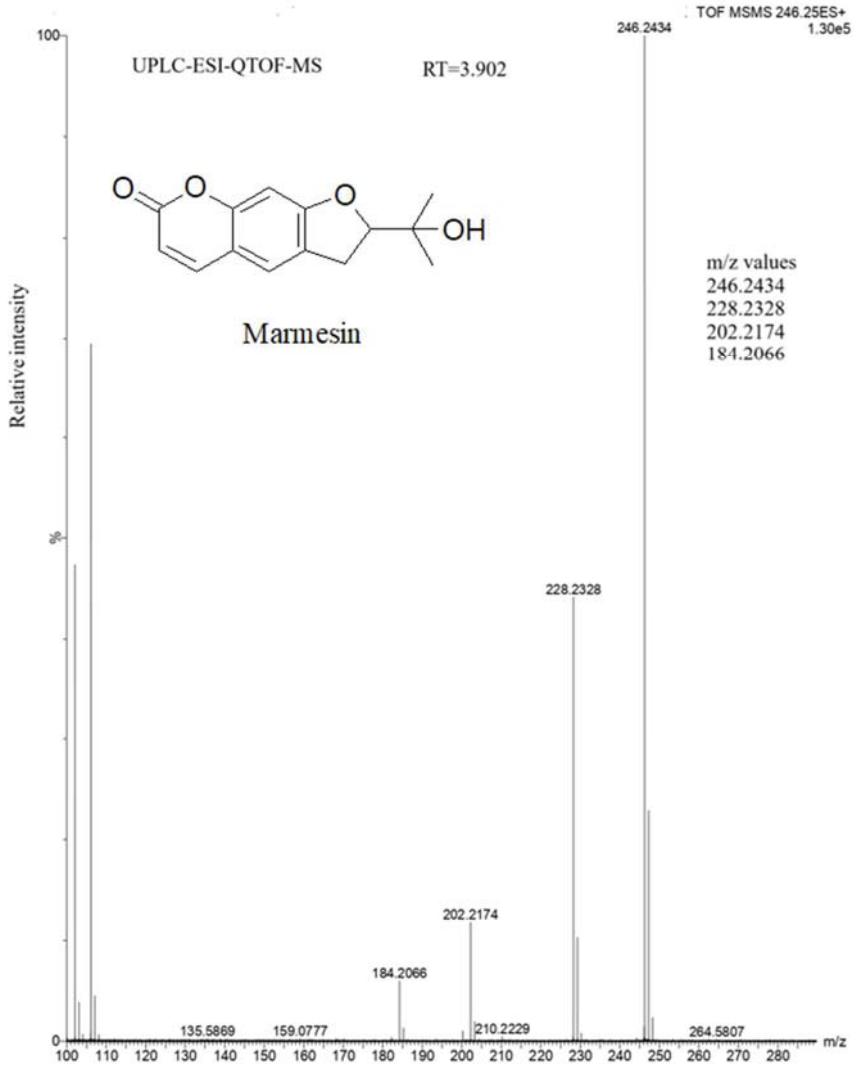
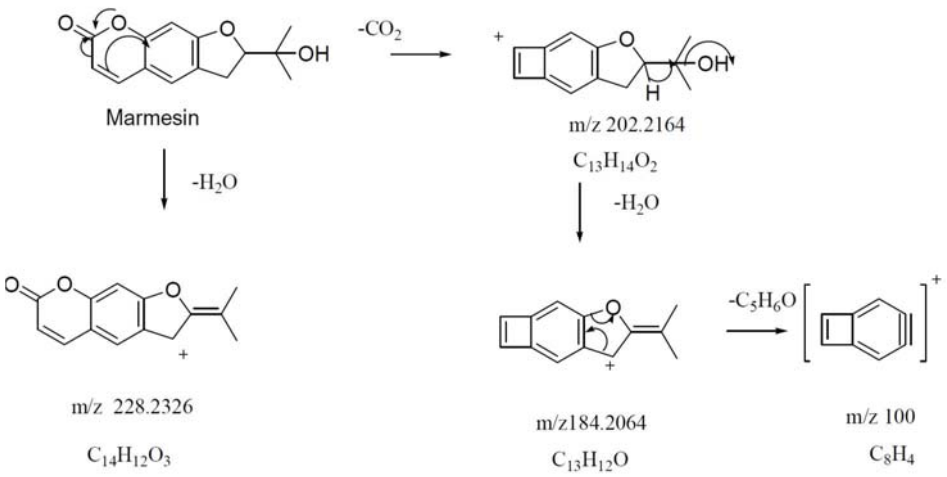


Figure 5.46. UPLC-ESI-QTOF-Mass Spectrum of Marmesin



Scheme 5.3. Mass fragments of Marmesin

Compound with retention time 3.902 produces a molecular ion (M^+) peak at m/z 246.2434 (100%). Secondary fragments formed at m/z 228.2328 (42%) and 202.2174 and 184. 2066. Marmesin, Merrekentrones D and Hypaphorine are the molecules considered for the molecular mass of 246.The m/z values of the product ion agree with that of the fragmentation reactions of Marmesin. The molecular ion losses a carbon dioxide moiety to form the product ion at m/z 202.2164 which subsequently loses a molecule of water to give the ion at m/z 184. The CO_2 loss is a characteristic reaction of benzopyrene-2-one derivatives in ESI spectra. This ion undergoes a reaction to fragment a 2-Methylbut-1-ene-3-one moiety to get an ion at m/z 100. The mass peak at 228 is formed by the loss of a water molecule from the molecular ion peak. The elemental analysis and the reactions given above confirmed this compound as Marmesin, (2*S*)-2-(2-hydroxypropan-2-yl)-2, 3-dihydrofuro [3, 2-g] chromen-7-one, with the molecular formula $C_{14}H_{14}O_4$. It is the additional confirmation of HRMS identification of Marmesin.

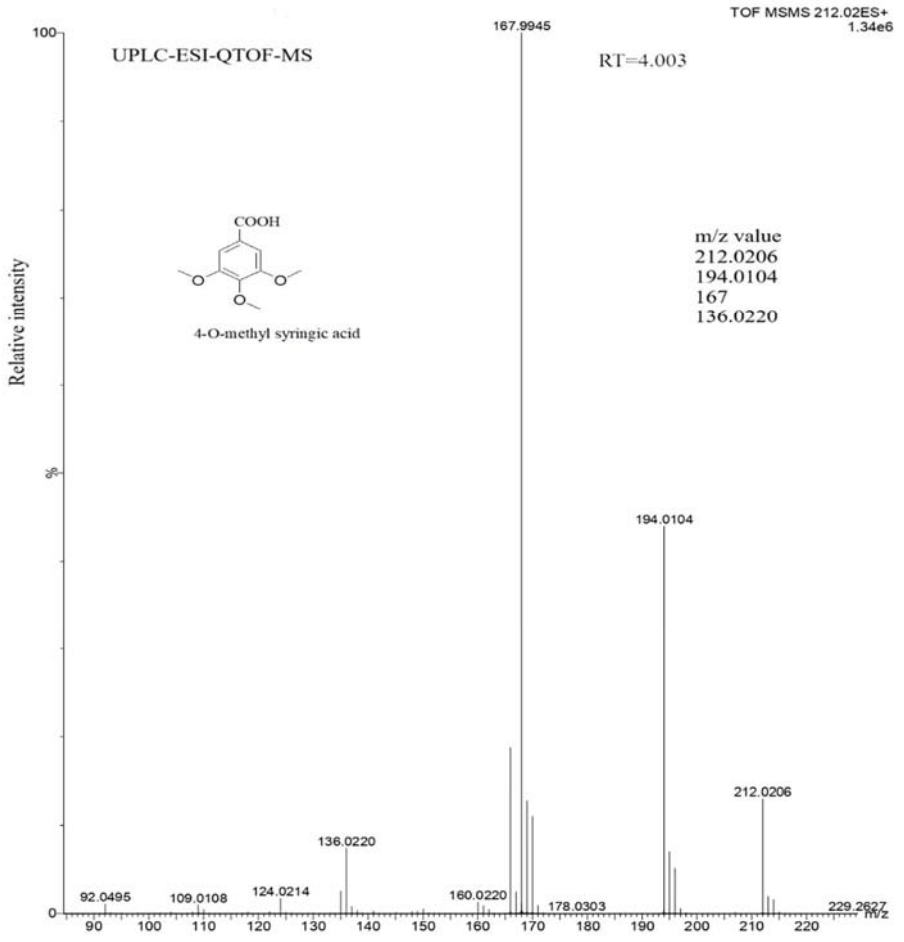
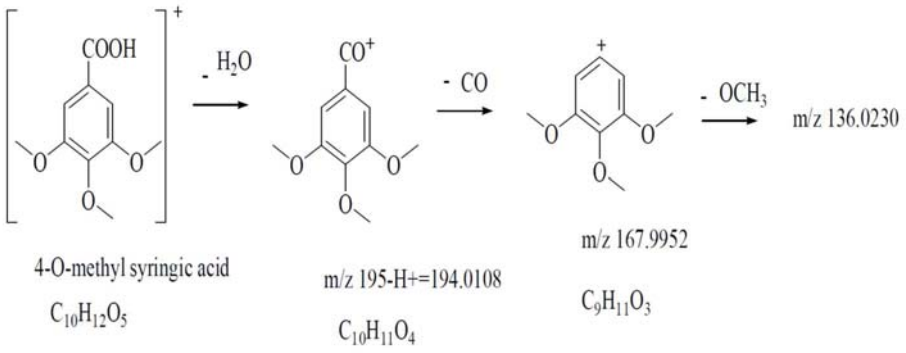


Figure 5.47. UPLC-ESI-QTOF-Mass Spectrum of 4-O- methyl syringic acid



Scheme 5.4. Mass fragments of 4-O- methyl syringic acid

Compound with retention time 4.003 form a molecular ion, M^+ peak at m/z 212.0206 (12%) and base peak at m/z 167.9945 (100%). Other fragment peaks are at m/z 194.0104 and 136.0220. Harmine and 4-O-methyl syringic acid are the compounds considered for the molecular mass 212.The peak at m/z 194 is the result of loss of -OH of the acid functional group by the addition of an H^+. The product ion at m/z 167.9945 is due to the elimination of -CO which subsequently eliminates a $-OCH_3$ group to give the product ion at m/z 136. The molecule is identified as 4-O-Methyl syringic acid (3,4,5-trimethoxybenzoic acid).

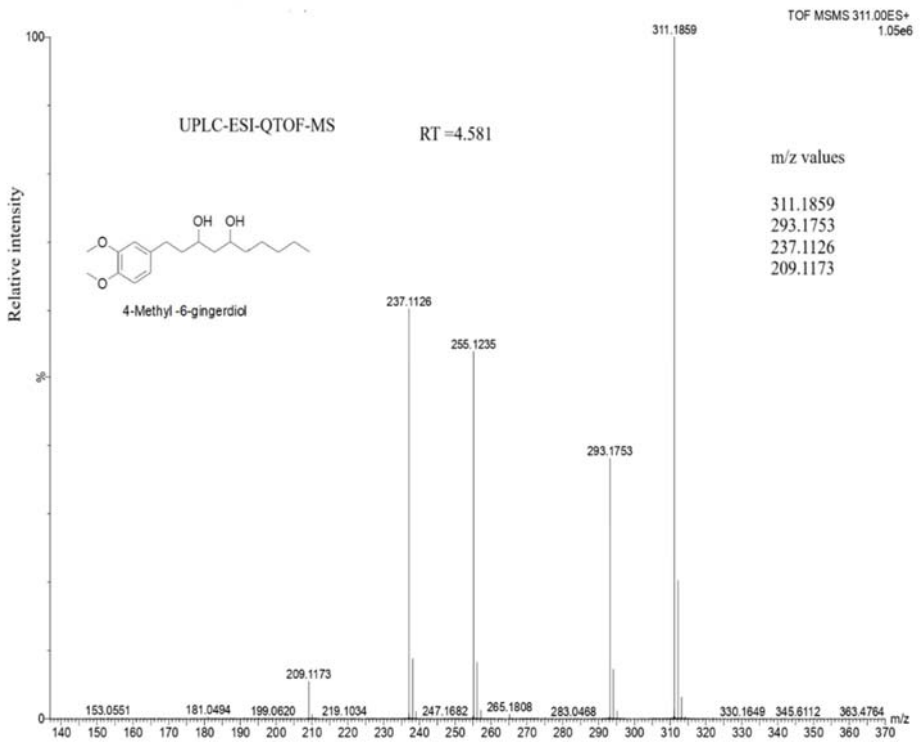
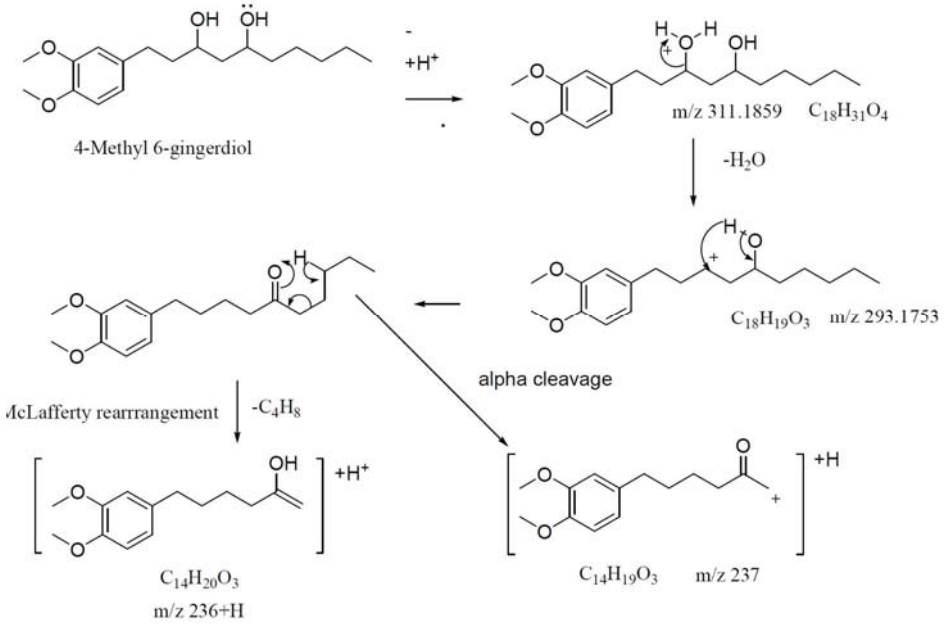


Figure 5.48. UPLC-ESI-QTOF-Mass Spectrum of 4-Methyl-6-gingerdiol



Scheme 5.5. Mass fragments of 4-Methyl-6-gingerdiol

The compound eluted with a retention time 4.581 produces a protonated molecular ion at m/z 311. 1859. Secondary fragments are formed at m/z 209.1173,237.1126,255.1235 and 293. 1753.The peak at m/z 293.1753 was formed by the loss of C3-OH as a water molecule. This molecule undergoes the transfer of a hydrogen from C-5 OH to C-3. The resulting molecule undergoes two types of reaction. One is Mc-Lafferty rearrangement and the other one is α-cleavage. Both these fragmentation pathway results in the formation an ion at m/z 237.

Removal of a water molecule from 3,5 diol, the molecular ion produces an oxetane ring structure. This oxetane ring structure produces the peak at m/z 209 and 192 by alpha cleavage and loss of $CH_3(CH_2)_4CHO$ respectively. The fragment ion at m/z 192 loses a C_3H_6 to form a peak at m/z 153[53].

The elemental analysis and the information given above confirmed this compound as 4-Methyl-6-gingerdiol with the molecular formula $C_{18}H_{30}O_4$.4-Methyl-6-gingerdiol((E)-1-(3,4-Dimethoxyphenyl) dec-3,5 diol) is a phenolic compound identified from the plant *Zingiber officinale* Rosc. used in the kashayam. Identification of 4-Methyl -6-gingerdiol with UPLC QTOF is the confirmation of it with HRMS.

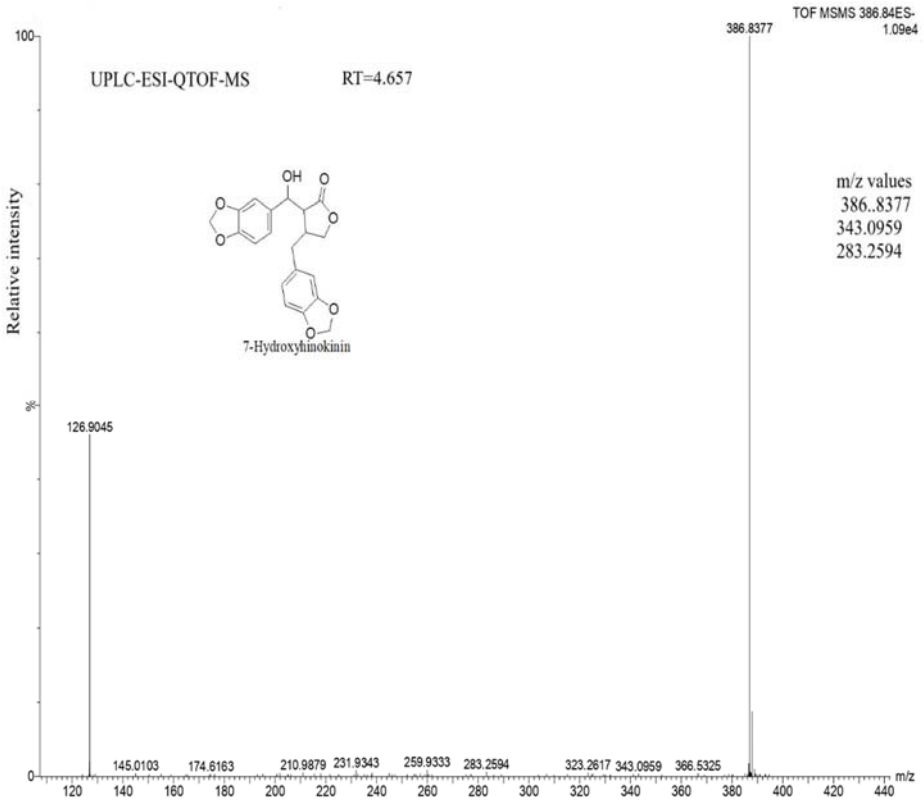
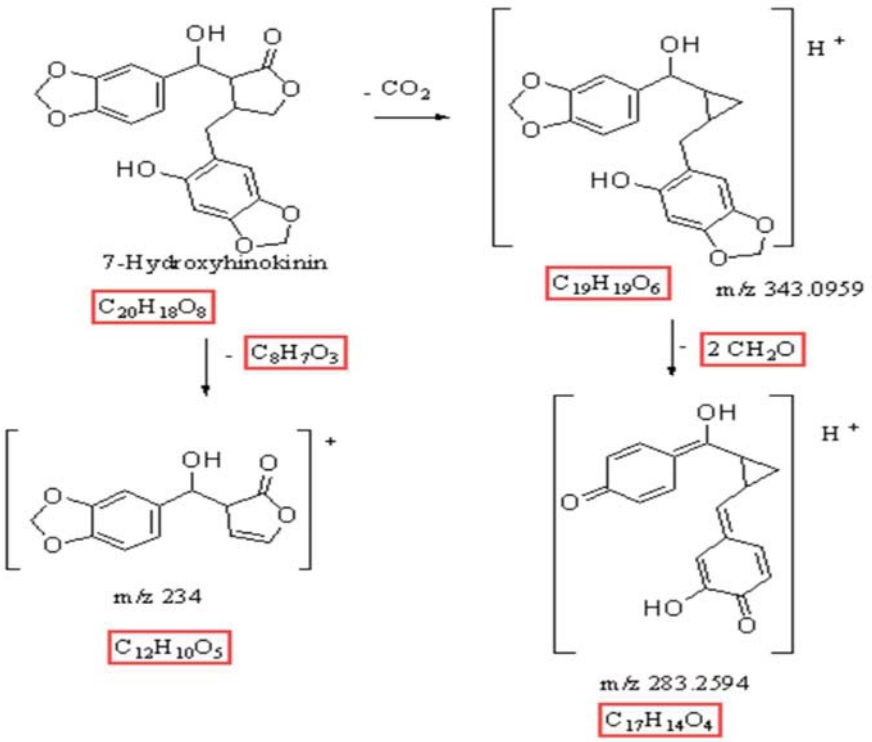


Figure 5.49. UPLC-ESI-QTOF-Mass Spectrum of 7-Hydroxy hinokinin



Scheme 5.6 Mass fragments of 7-Hydroxyhinokinin

Compound with retention time 4.657 formed a molecular ion (M^+) peak at m/z 386.8377(100%). The characteristic ions formed at m/z 343.0959 and 283. 2594.Cholesterol and 7-Hydroxyhinokinin are the compounds considered. The elimination of CO_2 from the lactone ring produces a product ion at m/z 343.0959. The elemental analysis and the mass fragments given above confirmed the compound as 7-Hydroxyhinokinin, (3R,4R)-3,4-bis[(2H-1,3-benzodioxo)7'-methyl-7-hydroxy] oxalane-2-one, with the molecular formula $C_{20}H_{18}O_8$.This compound identified by HRMS validated by the UPLC-QTOF-Ms analysis.

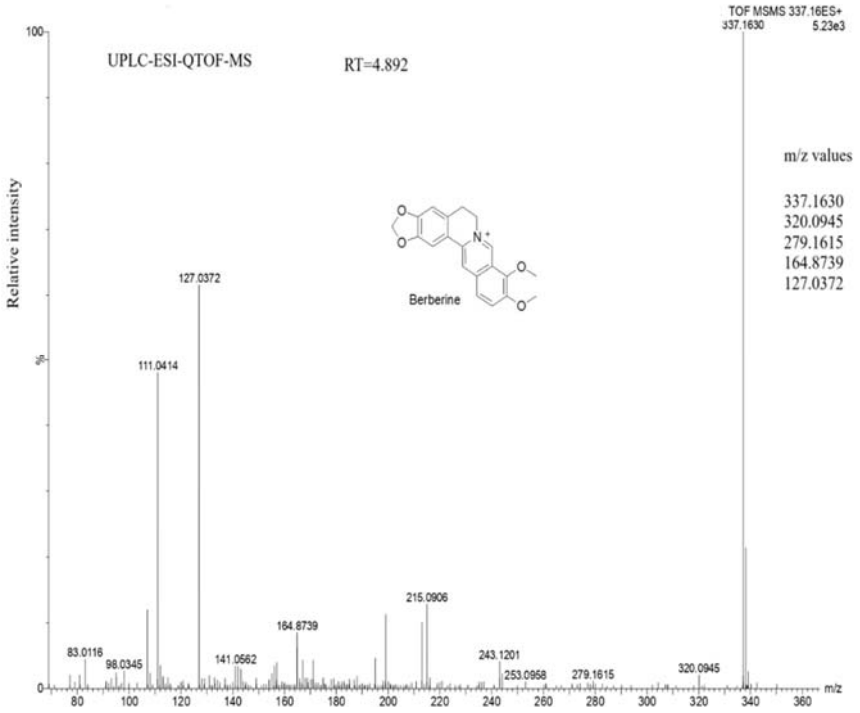
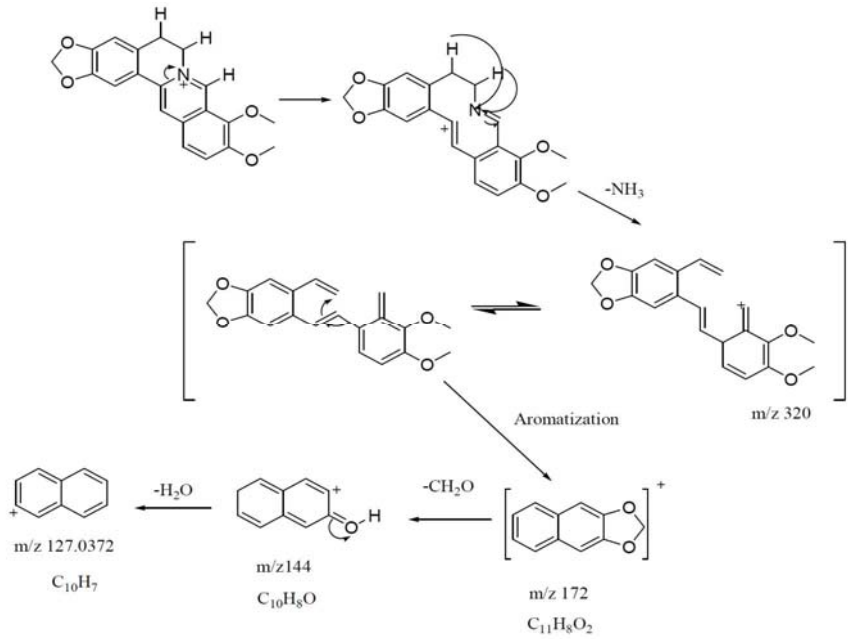
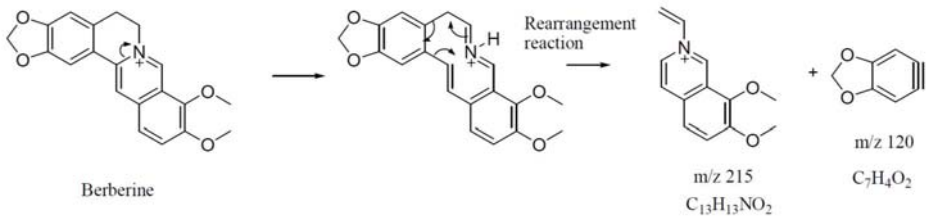


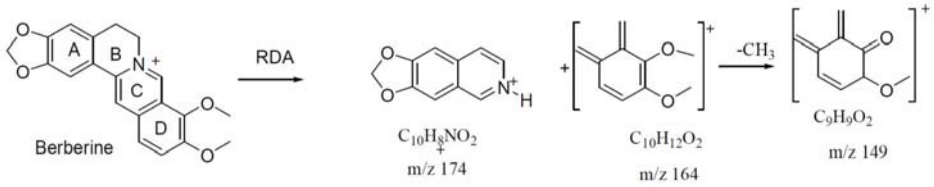
Figure 5.50. UPLC-ESI-QTOF-Mass Spectrum of Berberine



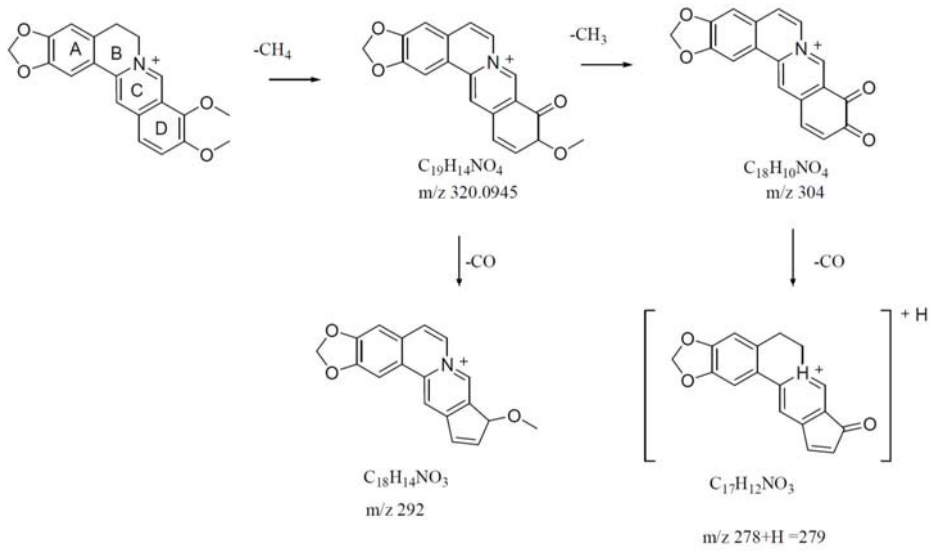
Scheme a



Scheme b



Scheme c



Scheme d

Scheme 5.7. Mass fragments of Berberine

The compound eluted with the retention time 4.892 form a protonated molecular ion peak at m/z 337. 1630. The product ion at m/z 320.0945 results from the elimination of nitrogen as ammonia (NH_3) by hydrogen abstraction from C5 and C6. the neutral loss of ammonia from protonated nitrogen from iso-quinoline alkaloids has been supported by the fact that nitrogen is the most favored protonation site, because the protonated molecule has the lowest energy. This fragment undergoes aromatization and produces a fragment with mass 172.This fragment sequence is based on the study by Zhixing suing et al [52,53]. They have studied 19 types of iso-quinoline alkaloids by Q TOF-MSMS and ascertained this type of reaction sequence by computational studies also. This fragment with mass 172 subsequently undergoes loss of CH_2O and H_2O to form an ion at m/z 127 that is depicted in the reaction sequence in the scheme. Ring-B cleavage takes place by a rearrangement sequence involving quaternary nitrogen of the iso-quinoline ring system which resulted in the formation of an alkene substituted iso-quinoline moiety with an m/z value of 215. 0006.An RDA reaction in the C-ring of berberine produces a product ion at m/z164.8739 by ring cleavage [54]. The peak at m/z 321 (320.0945) also could be formed by the loss of -CH_3(CH_4) from the methyl at ring D. This further undergoes degradation as shown in the scheme to produce a product ion at 279.The elemental analysis and mass fragments obtained leads the identification of the compound as Berberine with the molecular formula $C_{20}H_{18}NO_4$.Berberine, 5,6-Dihydro-9,10-dimethoxybenzo[g]1,3-benzodioxolo5,b-a]quinolizirium,is a quaternary ammonium salt.

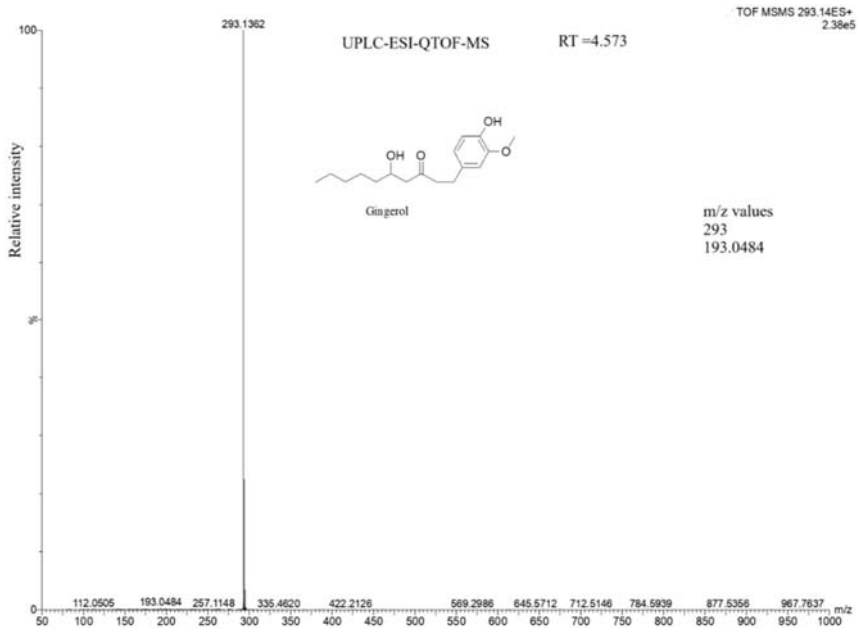
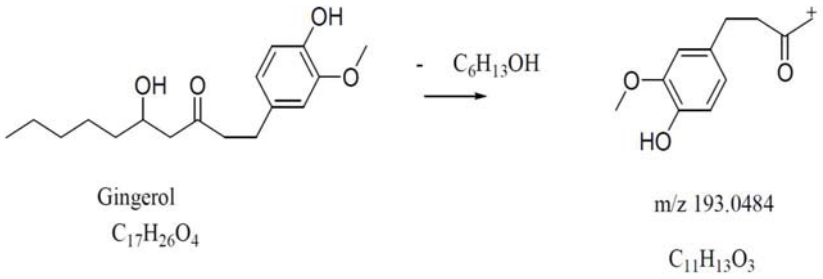


Figure 5.51. UPLC-ESI-QTOF- Mass Spectrum of 6-Gingerol



Scheme 5.8 Mass fragments of 6-Gingerol

Compound with retention time 4.573 produces a molecular ion ($M\text{-}H^+$) peak at m/z 293. Secondary fragments at m/z 193.0484 probably corresponds to the elimination of hexanal. The elemental analysis and the information given above confirmed this compound as 6-Gingerol with the molecular formula $C_{17}H_{26}O_4$.6-Gingerol or (5*S*) -1-(3,4-dimethoxyphenyl) -5-hydroxy decan-3-one, is a phenolic compound identified from the plant *Zingiber officinale* Rosc. It is the further confirmation of HRMS identification.

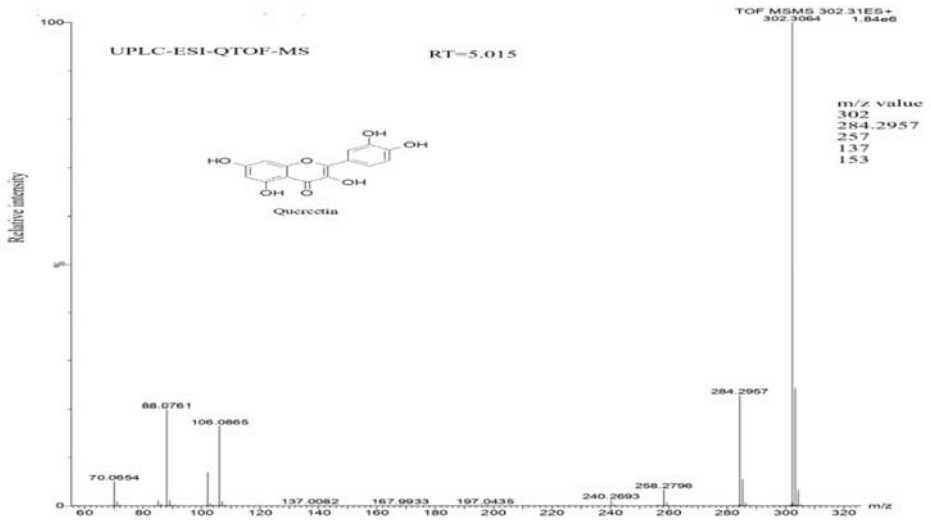
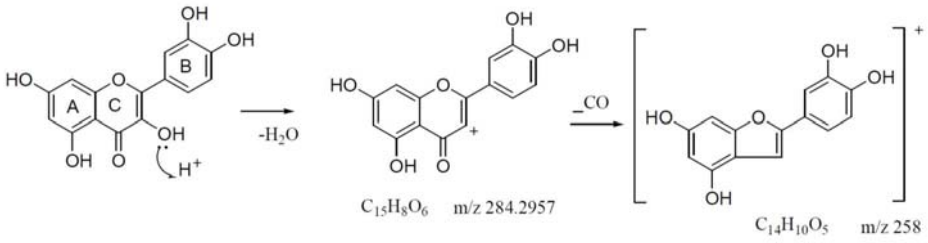
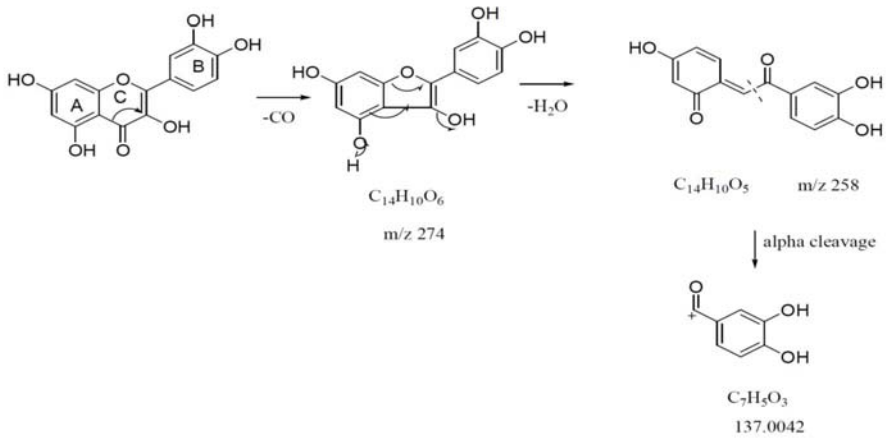


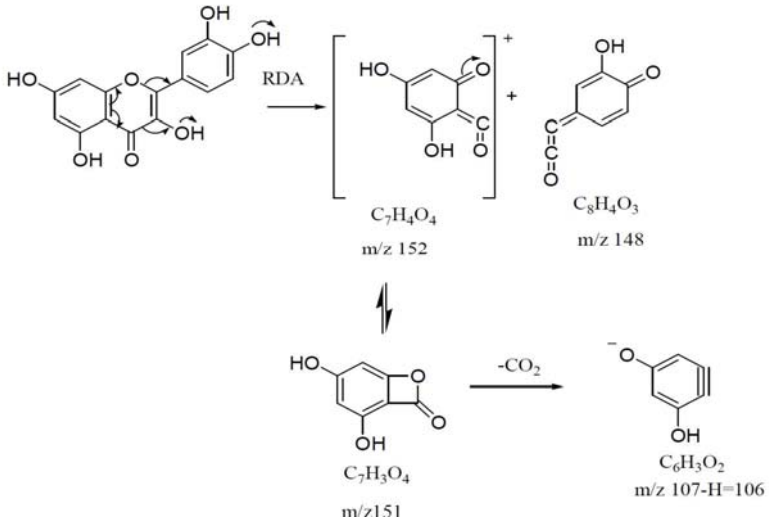
Figure 5.52. UPLC-ESI-QTOF-Mass Spectrum of Quercetin



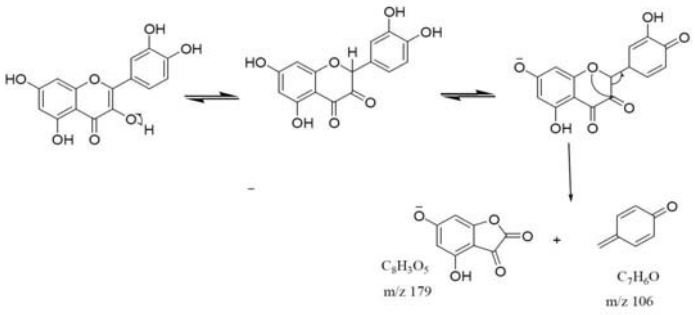
Scheme a



Scheme b



Scheme c



Scheme d

Scheme 5.9. Mass fragments of Quercetin

Compound with retention time 5.015 produces a molecular ion (M^+) peak at m/z 302.3064 (100%). Secondary fragments formed at m/z 284.2996 (21%), 258, 240 and 137.0082. Pseudo purpurine, ellagic acid and quercetin are compounds considered for the molecular mass 302.The elimination of OH group as water molecule from the ring C results in the fragment at m/z 284. 2996. The peak at m/z 258 could be explained by two different fragmentation reactions. One method is from m/z 284 with

the loss of a CO molecule. The second path way is the loss of CO from the C ring of quercetin and ring opening produces an ion at m/z 258.This subsequently undergoes fragmentation by α-cleavage at the C=O functional group to produce an ion at m/z 137. 0042.As shown in the scheme, the major peak at m/z 106 could be explained by two different mechanisms. One method is the RDA reaction in the molecular ion and subsequent loss of CO_2 from the fragment with the m/z 152.Fragments obtained are similar to that of quercetin reported by Dimitrios et al [50]. The elemental analysis and the mass fragments given above confirmed this compound as Quercetin with the molecular formula $C_{15}H_{10}O_7$. Quercetin or 2-(3, 4-dihydroxyphenyl)-3, 5, 7-trihydroxychromen-4-one, is a flavonoid identified from *Alpinia calcarata* Roscoe and *Vigna mungo* (L.) Hepper. It is also identified with HRMS analysis.

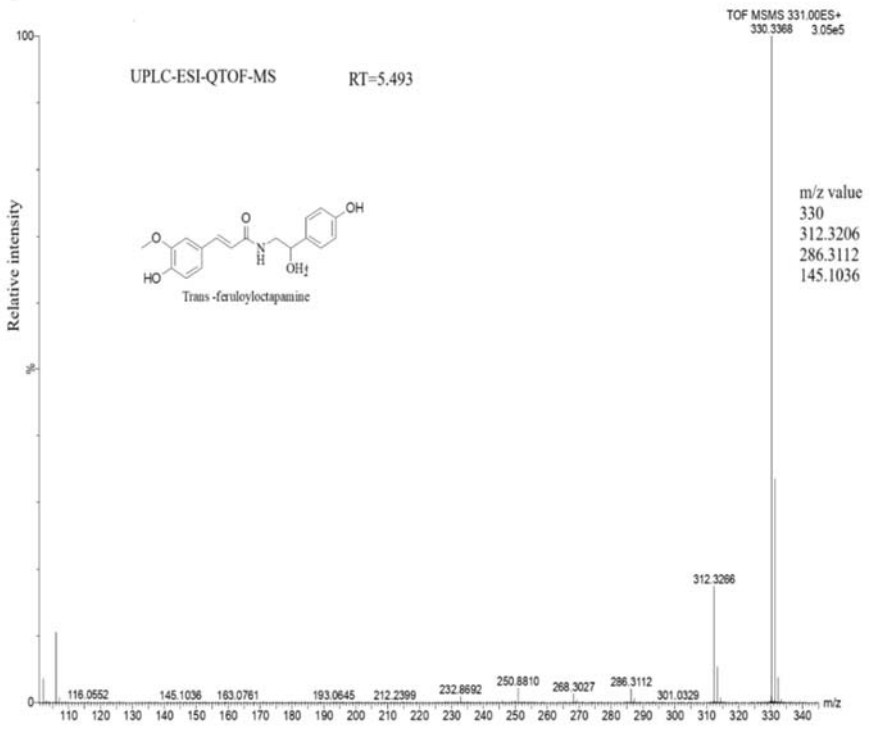
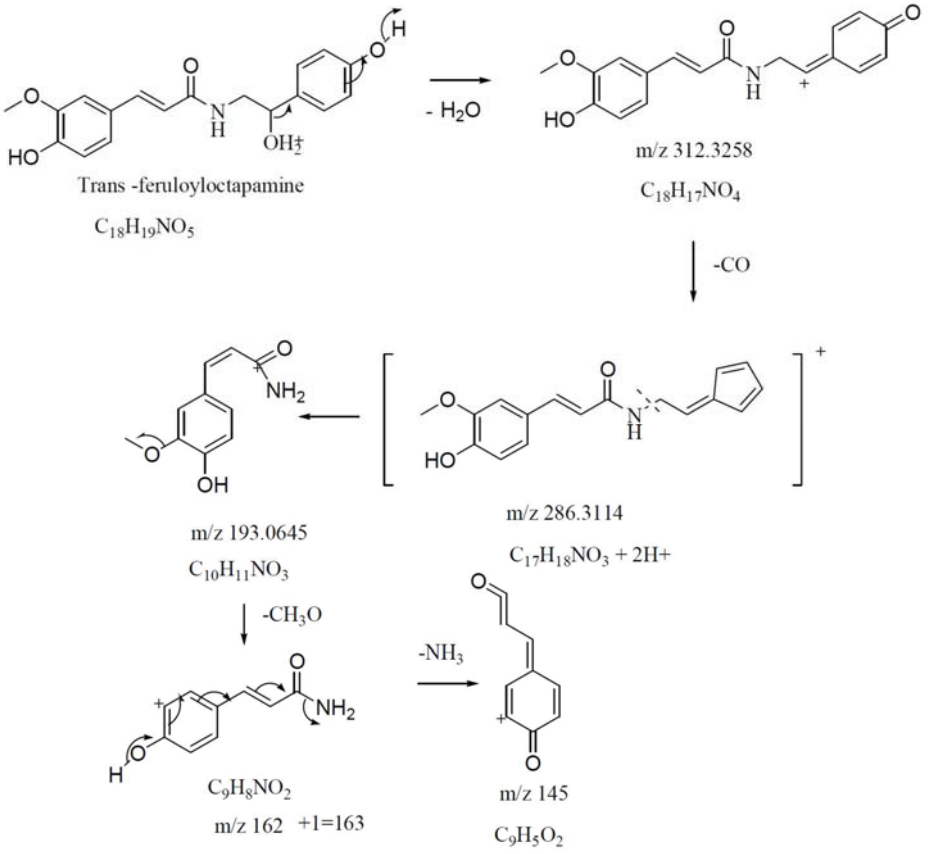


Figure 5.53. UPLC-ESI-QTOF-Mass Spectrum of Trans feruloyl octopamine



Scheme 5.10 Mass fragments of Trans feruloylctopamine

Compound with retention time 5.493 form a molecular ion, $M+H^+$ at m/z 330.3368(100%) and other fragments at m/z 312.3266 (20%) and 286.3112(2%). The peak at m/z 312.3266 due to the removal of water molecules from precursor ions. The loss of -CO moiety from this produced the fragment at m/z 286.3112. The cleavage of the carbon-nitrogen bond of the amide linkage gave the fragment ion at m/z 193 and further a molecule of $-CH_3O$ is lost by hydrogen capture of the methoxyl function gave the ion at m/z 163 and that gives off an NH_3 loose of the amide linkage giving an ion at m/z 145. Based on this reaction pathway the presence of Trans feruloyl octopamine, (*E*)-*N*-[2-hydroxy-2-(4-

hydroxyphenyl) ethyl]-3-(4-hydroxy-3-methoxyphenyl) prop-2-enamide, with the molecular formula $C_{18}H_{19}NO_5$ is ascertained. This molecule is a constituent of *Allium sativum* L. used in the kashayam.

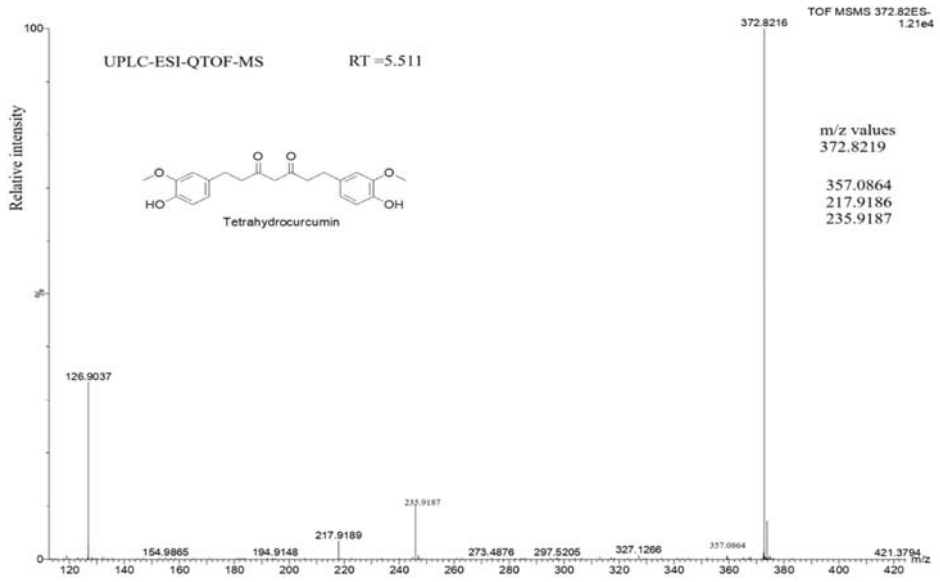
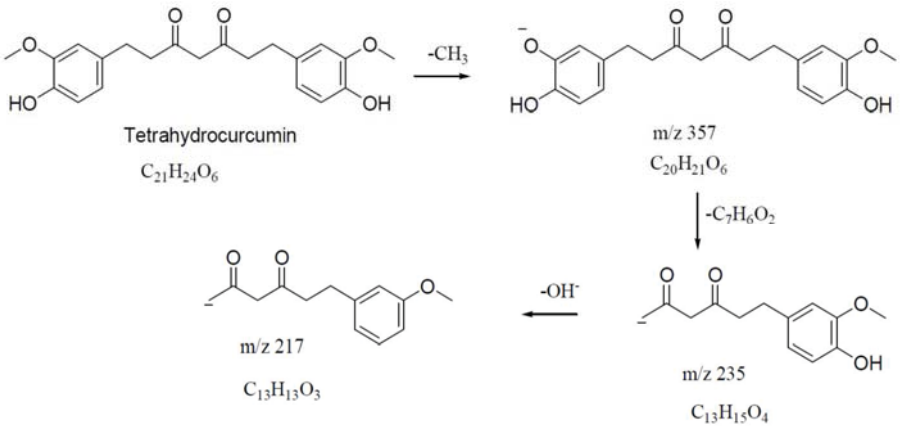


Figure 5.54. UPLC-ESI-QTOF- Mass Spectrum of Tetrahydrocurcumin



Scheme 5.11 Mass fragments of Tetrahydrocurcumin

Compound with retention time 5.511 gave a molecular ion M^+ peak at m/z 372.8219(100%) and the most prominent fragments at m/z 357.0864 and 217.9250(2%). The fragment at m/z 357.0864 formed by the removal of methyl group from the molecular ion and the fragment at m/z 235 by the cleavage of C1-C2 bond, that is alpha cleavage of ketone functional group, which then eliminate a hydroxyl function as water which leads to the fragment at m/z 217.9250. This fragmentation pattern confirms the compound as Tetrahydrocurcumin with the molecular formula $C_{21}H_{24}O_6$. Tetrahydrocurcumin or 1,7-bis(4-hydroxy-3-methoxyphenyl)-3,5-heptanedione is a beta-diketone present in *Zingiber officinale* Rosc. This is the confirmation of HRMS identification of Tetrahydrocurcumin.

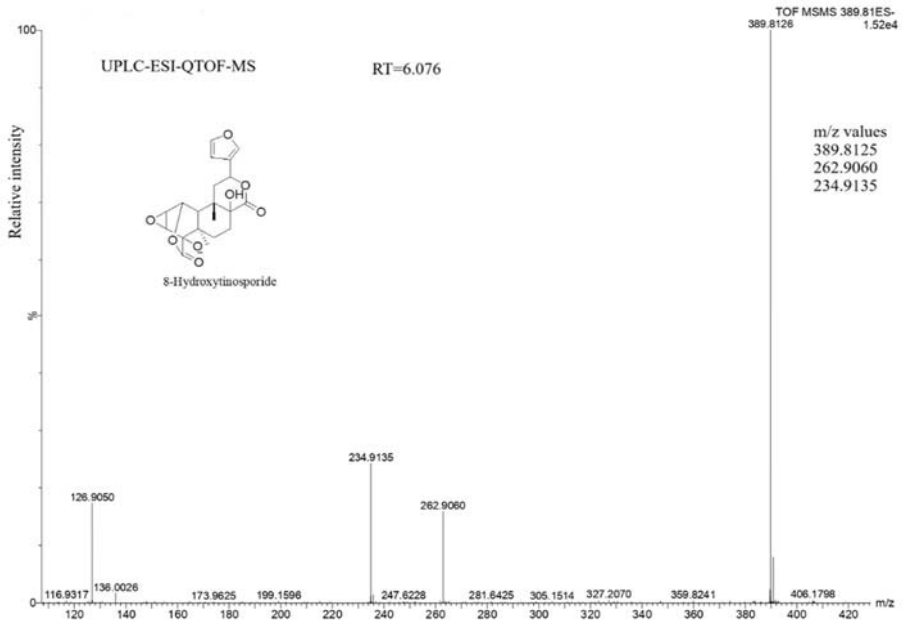
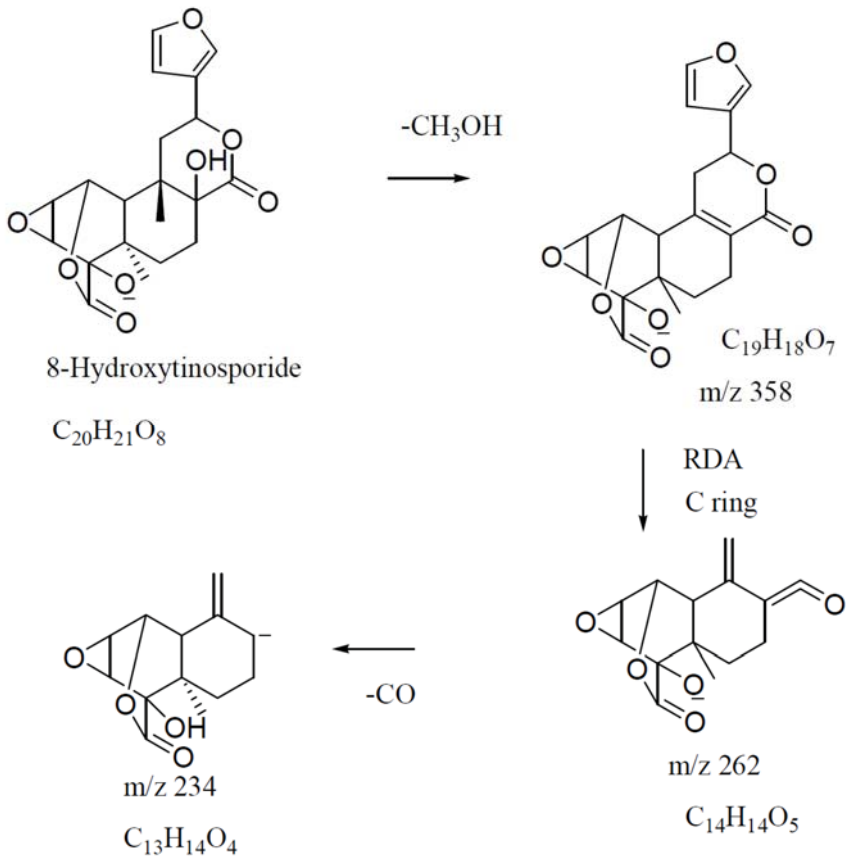


Figure 5.55. UPLC-ESI-QTOF-Mass Spectrum of 8-Hydroxytinosporide.



Scheme 5.12 Mass fragments of 8-Hydroxytinosporide

Compound with retention time 6.076 produces a molecular ion peak at m/z 389.8125, M-H^+(100%). Other fragments are at m/z 262.9072(16%) and m/z 234.9126 (28%). The fragment at m/z 262.9072 produced by the loss of CH_3OH (the angular CH_3 and OH groups from the C ring) and subsequent RDA reaction in the C ring. The fragment at m/z 234.9126 formed from 262.9072 by the removal of carbon monoxide molecule. The mass fragmentation pattern and its elemental analysis confirmed the compound as 8-hydroxy tinosporide, (1S,2S,3R,4R,5R, 8S,10R,12S)-4-hydroxy-2,3:15,16-diepoxycleroda-13(H),14-dieno-17,12:18,1-biscarbolactone-8-ol, with the molecular formula $C_{20}H_{22}O_8$. It is the confirmation of HRLCMS identification.

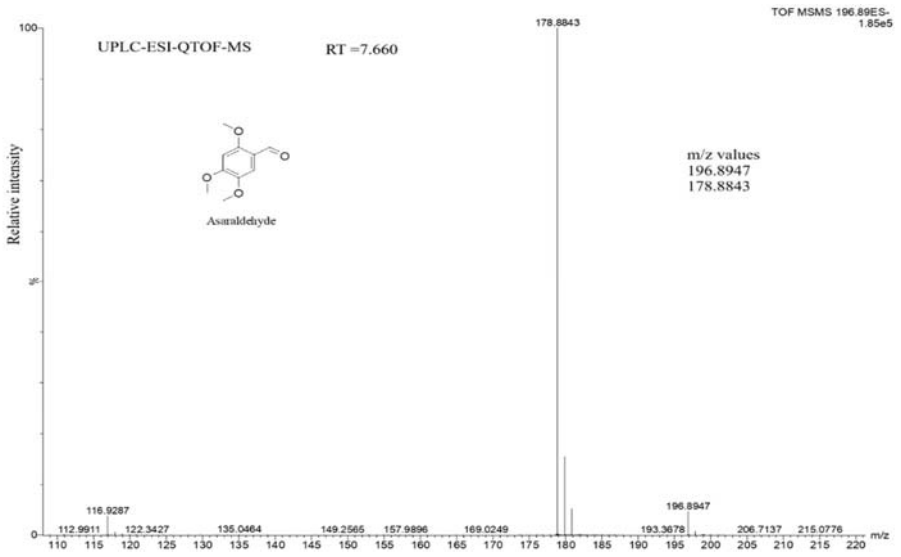
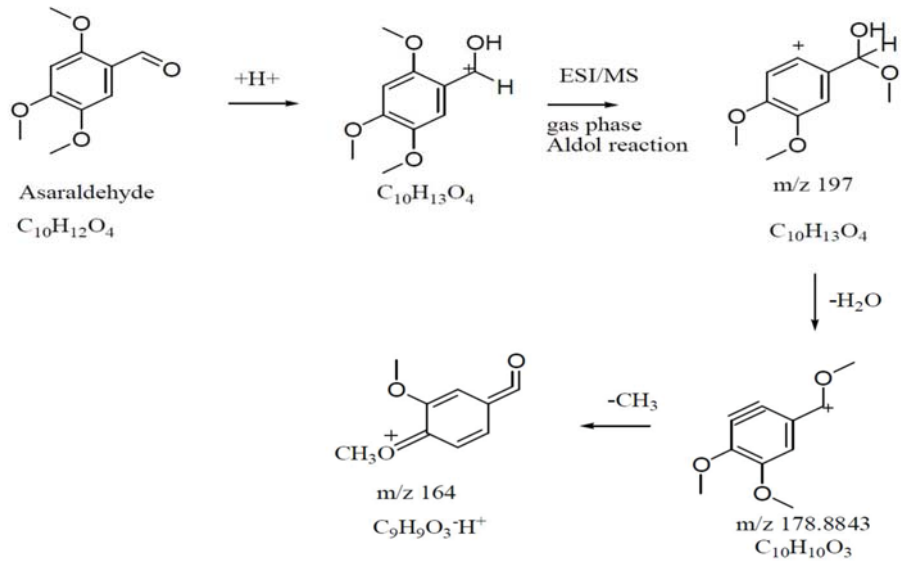


Figure 5.56. UPLC-ESI-QTOF-Mass Spectrum of Asaraldehyde



Scheme 5.13 Mass fragments of Asaraldehyde

The compound with retention time 7.660 forms a molecular ion peak at m/z 196.8947 (2%) and base peak at m/z 178.8843(100%). Asaraldehyde, Fenchyl acetate and Linalyl acetate are the candidates for the molecular mass 196.The parent compound forms a gas phase Aldol reaction to produce an ion at m/z 197 which subsequently loses a molecule of water to form the base peak at m/z 178. 8843.The peak at m/z 164 is due to the loss of methyl molecules from the para methoxy function. From the elemental analysis the molecular formula of the compound was found to be $C_{10}H_{12}O_4$. The mass fragmentation patterns confirmed the compound as Asaraldehyde or 2, 4, 5-trimethoxy benzaldehyde. Asaraldehyde is the constituent of *Zingiber officinale* Rosc. UPLC QTOF analysis validated the presence of Asaraldehyde and confirmed the identification by HR LCMS.

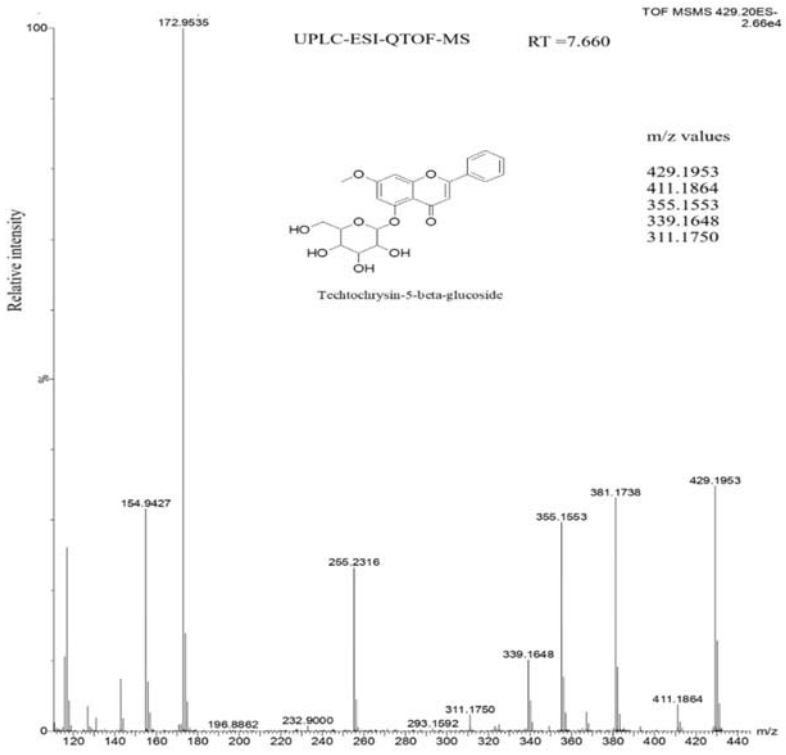
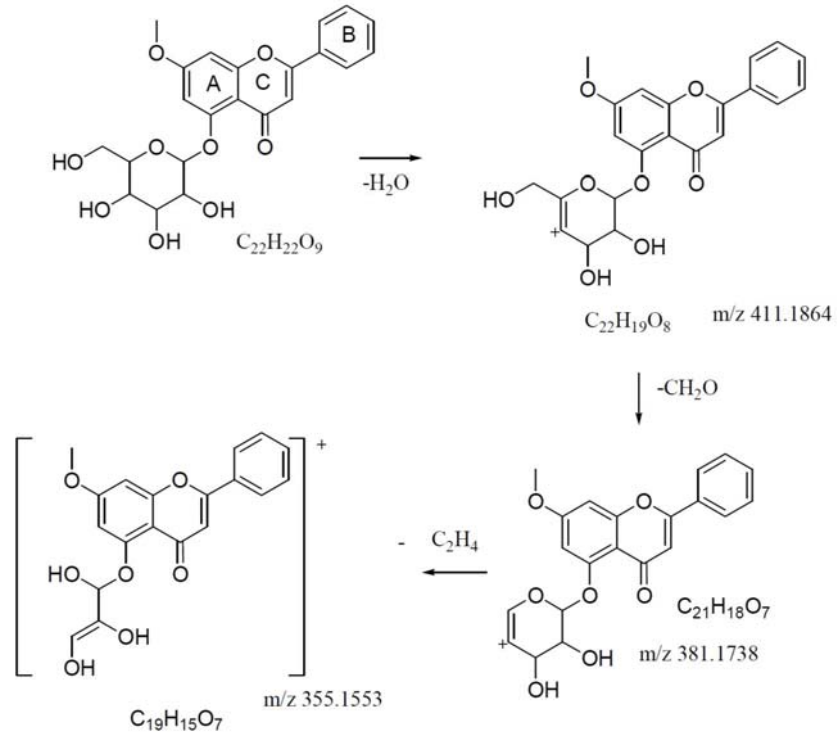
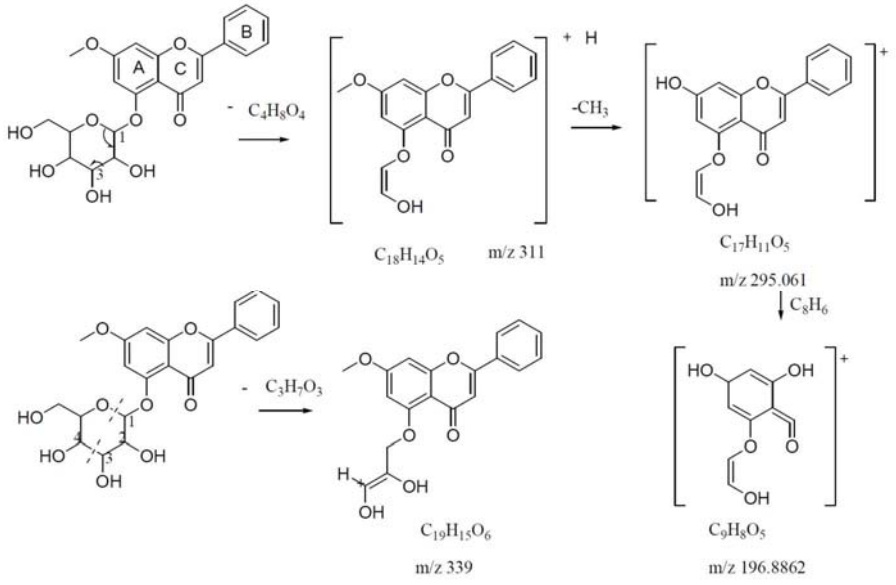


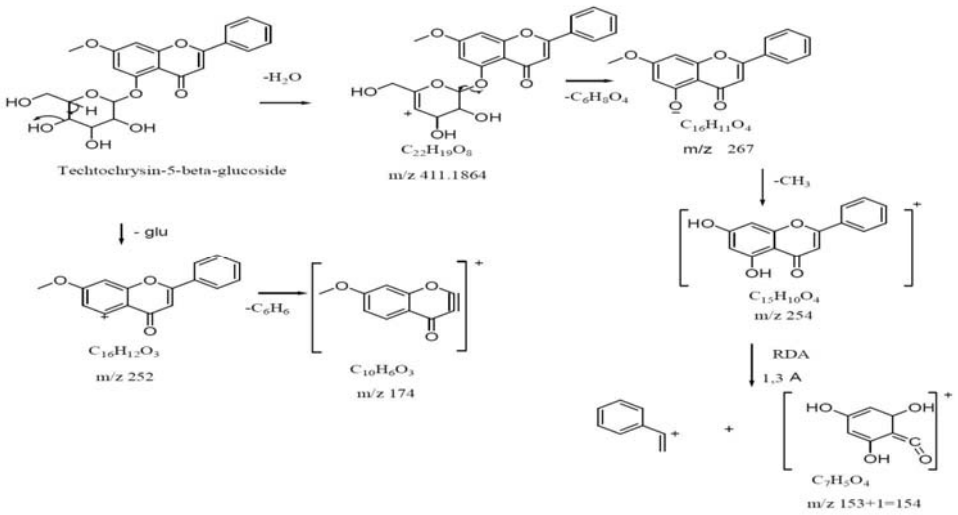
Figure 5.57. UPLC-ESI-QTOF-Mass Spectrum of Techtochrysin -5-β-glc



Scheme a



Scheme b



Scheme c

Scheme 5.14 Mass fragments of Techtochrysin-5-glucoside

The compound eluted with the retention time 7.660 produces the molecular ion peak at m/z 429(32%) and base peak at m/z 172.9535(100%). The peak at m/z 411 was formed by the loss of water molecules in the sugar moiety . The peak at m/z 381 formed by the loss of CH_2O from the peak at m/z 411.The secondary fragments at m/z 355,339 and 311 are formed by the ring opening of sugar moiety. The formation of the peak at m/z 154 can be explained in the reaction sequence(scheme c) . The mass peak at m/z 174 was formed as given above(scheme c) [51]. The elemental analysis and the information given above confirmed this compound as Techtochrysin- 5-β-glucoside with the molecular formula $C_{22}H_{22}O_9$.Techtochryin- 5-β-glucoside(7-Methoxy-2-phenyl-4H-1-benzopyran-4-one-5-β-glucoside) is a flavonoid reported in the plant *Alpinia calcarata* Roscoe. It is the further confirmation of HRMS identification.

In the ESI MS spectrum of flavonoid-O-glycosides the aglycone moiety should form a prominent peak at 267. In the present case, the peak

at m/z 267 is not obtained. Only the RDA fragmentation product of the C-ring appears as a prominent peak. The pattern of fragmentation resembles that of a C-glycoside. The peaks at m/z 411,381 and 355 are usual with a C-glycoside pattern where these peaks are formed by fragmentation of the glucose moiety attached to the aglycone via C-glycosidic linkage. Hence the confirmation whether it is a C-glycoside or O-glycoside is not stated conclusively.

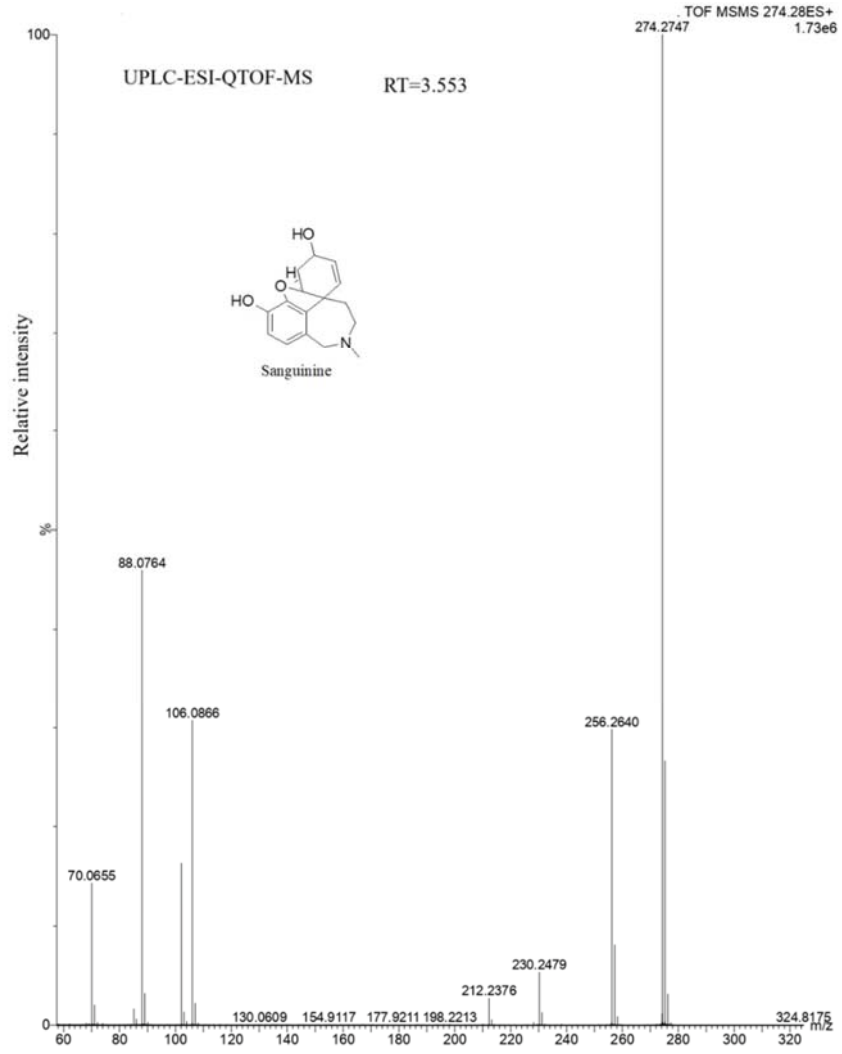


Figure 5.58. UPLC-ESI-QTOF-Spectrum of Sanguinine

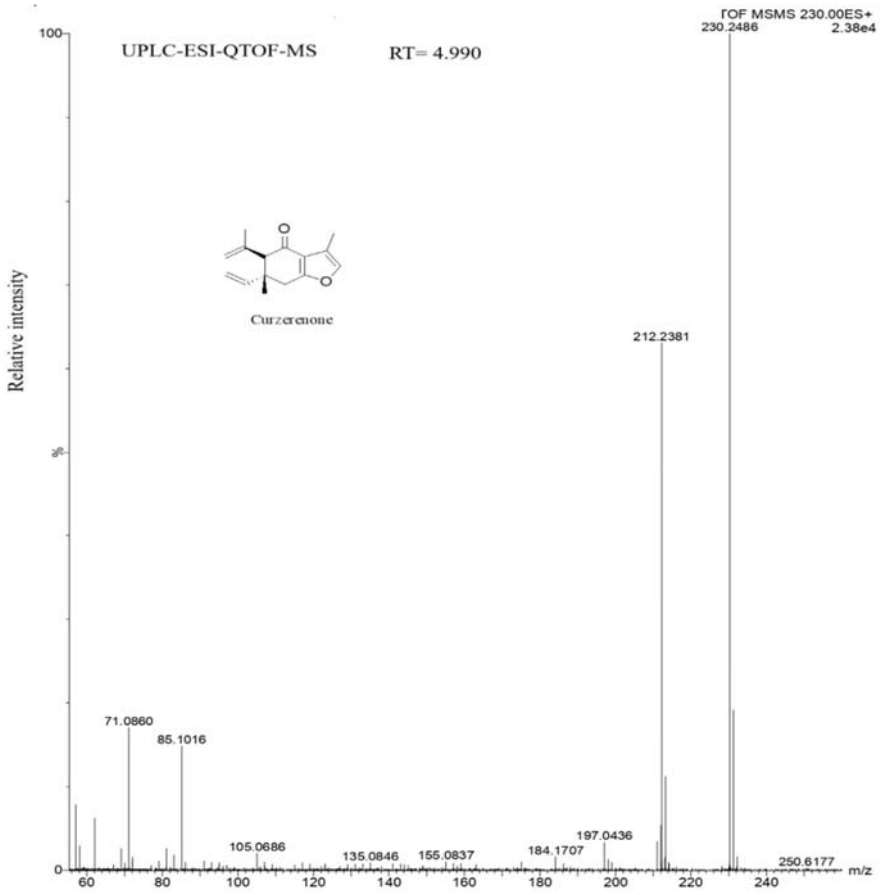


Figure 5.59. UPLC-ESI-QTOF-Spectrum of Curzerenone

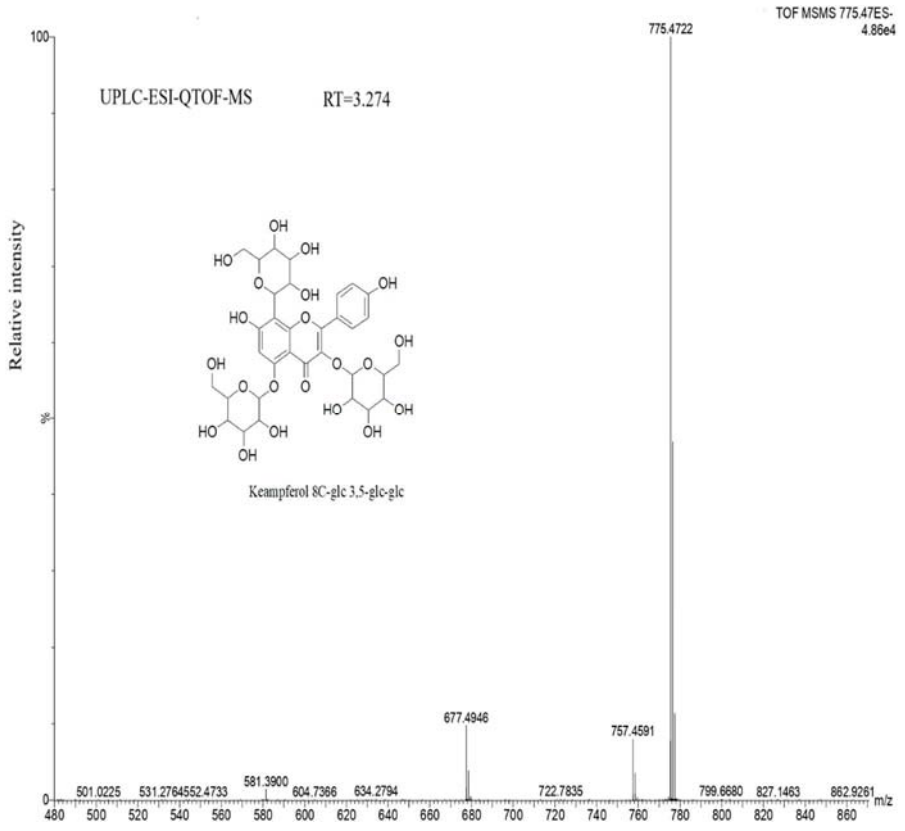


Figure 5.60. UPLC-ESI-QTOF-Spectrum of kaempferol 8C-glc-3, 5-glc-glc

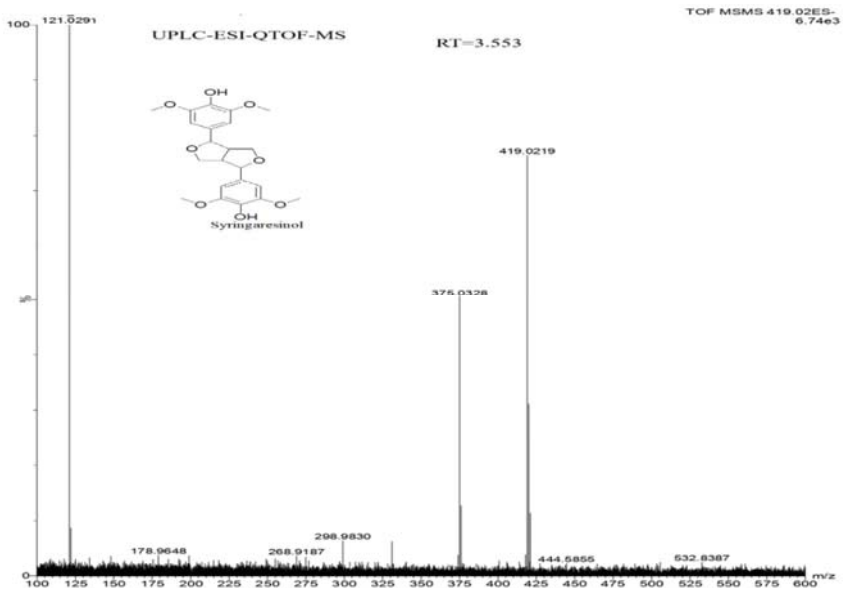


Figure 5.61. UPLC-ESI-QTOF-Spectrum of Syringaresinol

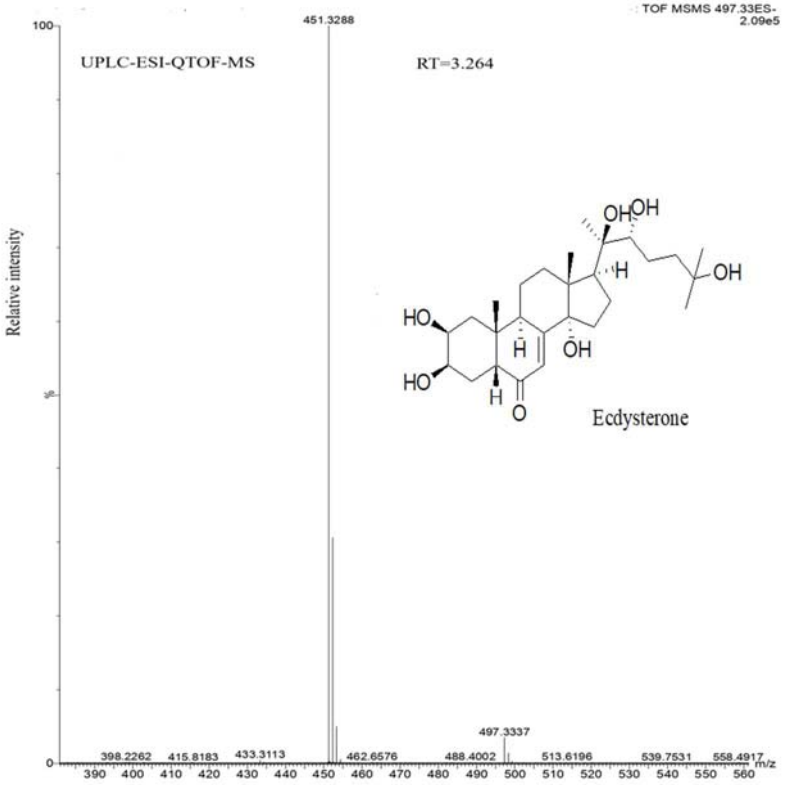


Figure 5.62. UPLC-ESI-QTOF-Spectrum of Ecdysterone

5.5. Experimental

LC-MS analysis

The chemical fingerprint of the extracts was determined using LC-MS analysis. The LC-MS was performed on a SHIMADZU-LC-MS machine (Model: 2010A) using the solvent system of formic acid – acetonitrile gradient and RP-C18 analytical column (240 mm × 2 cm) with a flow rate of 0.5 mL/min. samples were nebulized with nitrogen gas, and the ion mass (electrospray ionization [ESI]) was generated in both positive mode and negative mode.

High Resolution Liquid Chromatography –Mass Spectrometry (HRLCMS) study

HR-LCMS analysis of samples was carried out in Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay, Pawai, Mumbai. HRLCMS study conducted on methanol extract of both the kashayam. Preparation of methanol extract explained in chapter 2. Chromatographic separation was performed on Hypersil GOLD C18 column (100 x 2.1mm) and 3-micron particle size. Applying the following gradient at a pressure 1200.00 bar; solvent A (0.1%Formic acid in water) and solvent B (90% ACN +10%H_2O+ 0.1% Formic acid) ;1.00 min 95%A and 5%B at a flow rate 0.300 mL/min; 20.00 min 0.00%A and 100%B at a flow rate 0.300 mL/min;25.00min 0.00%A and 100%B at a flow rate 0.300 mL/min;26.00min 95%A and 5%B at a flow rate 0.200 mL/min; 30.00 min 95%A and 5%B at a flow rate 0.300 mL/min; Injection Volume 5.00 µL.

UPLC-Q-TOF-MS

300 ml of the well shaken Kashayam is centrifuged at 4000 rpm (3000g) in a REMI 8C centrifuge for 30 minutes. The Clear supernatant was carefully decanted (220ml). This was analyzed in QTOF MSMS.

UPLC-Q-TOF-MS analyses were carried out at MG University Kottayam. The chromatographic separation and detection of analytes was carried out with ultra-performance liquid chromatography coupled to quadrupole time of flight mass spectrometry(UPLC-Q-TOF-MS).The acquity UPLC system (Waters)consists of a TUV detector(JI2TUV750A), a column chamber(JI2 CHA730G), a quaternary solvent manager(HI2 QSM632A), and a sample manager FTN (KI2 SDI069G).A reversed - phase BEH C18 column (of dimension 50mm X 2.1mm X 1.7 μm) with a flow rate of 0.3mL min -1 was used for chromatographic separation(Waters)The mobile phase was a mixture of 0.1% Formic acid in water (A) and acetonitrile (B) in a gradient elution as follows initial 95% A, 0.1min 95%A, 6.00 min 5%A, 6.5 min 5%A, 9min 95%A and 10 min 95%A.The UPLC system was connected to the quadrupole time of flight mass spectrometer (Waters Xevo G2 QTOF) with electrospray ionization(ESI)interface working in positive and negative ionization modes .The injection volume was 10μL**.**The scanning m/z range was between 50 and 1000 .The desolvation gas flow and the temperature were 900L/h and 350 ^{0}C, respectively. The mass spectra were obtained using collision energy ranging from 5 to 30eV.The instrument control and data acquisition was done using Mass Lynx software (v 4.1).

5.4. Conclusion

Total ion chromatogram obtained from the LCMS study revealed the presence of a large number of compounds in the Prasarinyadi kashayam. The HRLCMS study identified 42 compounds tentatively. Out of identified compounds phenolics, alkaloids and terpenes are in larger numbers. UPLC Q-TOF analysis conducted on the supernatant of the kashayam also showed the presence of 10 phenolics, 5 alkaloids and 4 terpenes. The alkaloids, phenolic and terpenes are most characterized phytochemicals for antioxidant and anti-inflammatory activities. The kashayam possessing these phytochemicals have served as sources of potent anti-oxidant and anti-inflammatory agents. From the constituent molecules identified and molecular characteristics depicted in this study, gives an insight into the pharmacologically active molecules that are responsible for the therapeutic validity of the anti-inflammatory property of Prasarinyadi kashayam.

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Chapter 6
LCMS Study on Gulguluthikthakam Kashayam

6.1 Introduction

6.2 Result and Discussion

6.2.1. LCMS study on methanol extract

6.2.2. HRLCMS study

6.2.3. UPLC-QTOFMS analysis

6.3. Experimental

6.6. Conclusion

References

Abstract

Gulguluthikthakam kashayam is one of Ayurveda formulations commonly used as anti-inflammatory. It is the decoction of 29 plants. Hence Gulguluthikthakam kashayam is a complex mixture of different phytochemicals. The identification of phytochemicals from such a complex mixture is possible with LC MS only. It is a highly sophisticated instrument combining chromatography and mass spectrometry. In this instrument each compound is isolated by liquid chromatography separation and then the isolated compound identified with the help of mass spectrometry. This chapter describes the identification of molecules responsible for the anti-inflammatory activity of Gulguluthikthakam kashayam using HRLCMS and UPLC-QTOF

6.1. Introduction

Ayurveda medicines are very effective in certain diseases where there is no satisfactory result using modern medicine. Single plant or a combination of several plants may possess anti-inflammatory and antioxidant properties that could have therapeutic efficacy to ameliorate /cure diseases such as diabetes, atherosclerosis, neurodegenerative, or cancer [1]. In addition, plant extracts could regulate the composition of the gut microbiota and contain a large number of phytochemicals responsible for their medicinal properties. In certain ailments whose etiology involves immune dysfunction or persistent inflammation can be protected by plants consumption. The plant extract contains the chemicals capable of pro-inflammatory cytokines and reduce the translocation of NF-kB to the nucleus [2]. Also, bioactive principles of plants can regulate the oxidative stress caused by an imbalance in the production of reactive oxygen species (ROS) and the reduction in the antioxidant capability of the cell enzymes. The plant extract can also be used to alleviate the harmful side effects of drugs. Plant extracts have been used as natural therapeutic agents against inflammation. The biggest disadvantage of recently available potent synthetic drugs is concerning their toxicity and the reappearance of symptoms after discontinuation of the medicine. Therefore, the screening and development of drugs with anti-inflammatory activity are necessary and there are many efforts to find anti-inflammatory drugs from medicinal plants. Inflammation is a huge challenge for humans. Plants have many phyto-constituents helpful in reducing inflammation with fewer side effects. When a decoction of different plant parts(kashayam) is used, there is a good chance of synergism between active components. There are many Ayurveda formulations with anti-inflammatory therapeutic effects with low or no side effects. The resolution of inflammation is influenced by several anti-

inflammatory mediators [3]. Since the plants are rich in secondary metabolites the kashayam constitutes a complex mixture of alkaloids, tannins, saponins, phenolic and flavonoids that possibly explain their potential anti-inflammatory and antioxidant activities. Currently, phytochemical and ethnobotanical studies are being carried out in order to identify the mechanism of action of a wide variety of Ayurveda formulations.

Gulguluthikthakam kashayam is one of the Ayurveda formulations commonly used as anti-inflammatory. It is the decoction of 29 plants. Hence Gulguluthikthakam kashayam is a complex mixture of different phytochemicals. The identification of phytochemicals from such a complex mixture is possible with LC MS analytical techniques. It is a highly sophisticated instrument combining chromatography and mass spectrometry. In this instrument each compound is isolated by liquid chromatography separation and then the isolated compound identified with the help of mass spectrometry. This chapter describes the identification of chemicals responsible for the anti-inflammatory activity of Gulguluthikthakam kashayam using HRLCMS and UPLC-QTOF.

6.2. Result and Discussion

6.2.1. LCMS study on methanol extracts

LCMS study conducted in C18 column with the linear gradient elution with an eluent system of A (0.1%formic acid) and B (acetonitrile). The gradient has been used in the following order, 0-15min 20%B, 15-35 min 80%B, 35-37 min 20%B, 35-37 min 20%B. The mobile phase system used was filtered through a 0.45µm membrane filter. The solvent flow rate was 0.4ml/min.

The LC MS spectrum of Gulguluthikthakam kashayam thickly packed mass peaks were observed (Figure 6.1). The relative intensity varies from 5 to 100.Number of ions per peak is 5 to 6.

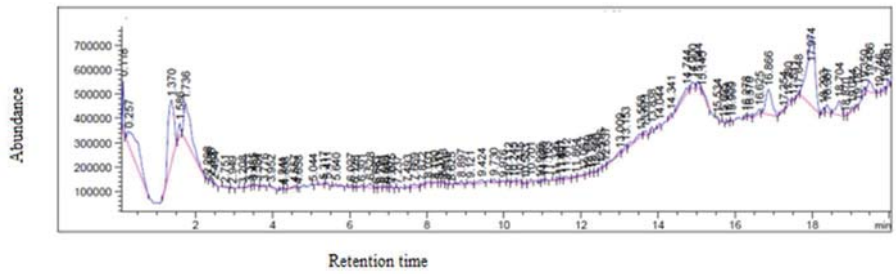


Figure 6.1. TIC of methanol extract of Gulguluthikthakam kashayam

6.2.2. HRLCMS study on methanol extract.

More than 100 peaks are observed in the HR LCMS spectrum of methanol extract of Gulguluthikthakam kashayam. 73 mass peaks have been tentatively identified in Table 6.1. This includes different classes of biologically active compounds such as alkaloids, terpenoids and phenolics. Out of 73 compounds identified 30 compounds are terpenoids, 22 are phenolic, 21 are alkaloids. Mass spectrum of all the identified compounds is given.

Table 6.1. List of identified compounds from methanol extract of Gulguluthikthakam kashayam using HRLCMS.

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
1	3.734	$C_{21}H_{30}O_3$	330.46	20-Hydroxyprogenene-3, 16-dione	20-Hydroxyprogenene-3, 16-dione	Steroids	328 M-2H$^+$	*Commiphora mukul*(Hook.ex Stocks) Engl. [4]
2	3.819	$C_{20}H_{24}O_6$	360.208	Dibenzylbutyrolactol	3,4-bis[(4-hydroxy-3-methoxyphenyl) methyl] oxolan-2-ol	Lignan	360.2160 M$^+$	*Cedrus deodara* (Roxb.ex D.Don)G.Don [5]
3	6.308	$C_{20}H_{24}NO_4$	342.4	Magnoflorin	(6aS)-1,11-dihydroxy-2,10-dimethoxy -6,6-dimethyl-5,6,6a,7-tetrahydro-4H-dibenzo[de,g]quinolinium	Alkaloid	344.2198 M+2H$^+$	*Celastrus paniculate* Willd [10], *Tinospora cordifolia* (Willd) Miers ex Hook.f.&Thoms [26]
4	4.352	$C_{20}H_{22}O_7$	374.4	Wikstromal	(3*R*,4*R*)-3-hydroxy-3,4-bis[(4-hydroxy-3-methoxyphenyl) methyl] oxolan-2-one	Lignan	356.1846 M+H$^+$-H_2O	*Cedrus deodara*(Roxb.ex D.Don)G.Don [5]
5	4.41	$C_{21}H_{29}NO_3$	343.5	Piperolein B	(8E)-9-(1,3-Benzodioxol-5-yl)-1-(1-piperidinyl)-8-nonen-1-one	Alkaloid	344.1844M+H$^+$	*Piper longum* L. [6]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
6	4.807	$C_{19}H_{15}NO_4$	321.33	CepharadioneB	1,2-dimethoxy-6-methyl-4H-dibenzo[de,g]quinolin-4,5(6H)-dione	Alkaloid	344.25M+Na^+	*Piper longum* L. [6]
7	10.958	$C_{17}H_{23}NO_3$	289.4	Tetrahydropiperine	5-(1,3-benzodioxol-5-yl)-1-piperidin-1-ylpentan-1-one	Alkaloid	288.1585 M+H^+	*Piper longum* L. [6]
8	12.146	$C_{21}H_{30}O_3$	330.46	16-Alpha – hydroxyprogesterone	(1*R*,2*R*,3a*S*,3b*S*,9a*R*,9b*S*,11a*S*)-1-Acetyl-2-hydroxy-9a,11a-dimethyl-1,2,3,3a,3b,4,5,8,9,9a,9b,10,11,11a-tetradecahydro-7*H*-cyclopenta[*a*]phenanthren-7-one	Steroids	331.46M+H^+	*Commiphora mukul* (Hook.ex Stocks) Engl. [4]
9	13.219	$C_{27}H_{44}O_3$	416.6	Guggulsterole III	16R,20R-Dihydroxycholest-4en-3-one	Steroids	437.2346 M+Na-2H^+	*Commiphora mukul* (Hook.ex Stocks) Engl. [4]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
10	14.385	$C_{26}H_{34}O_6$	442.552	Nimbidinin	6-(furan-3-yl)-11,16,18-trihydroxy-1,5,10,15-tetramethyl-13-oxapentacyclo [10.6.1.02,10.05,9.015,19] nonadec-8-en-4-one	Terpenes	425.3579 M-H_2O+H^+	*Azadirachta indica*(L.)A.Juss. [9]
11	12.414	$C_{30}H_{48}O_3$	456.7	Ursolic acid	(1S,2R,4aS,6aS,6bR,8aR,10S,12aR,12bR,14bS)-10-Hydroxy-1,2,6a,6b,9,9,12a-heptamethyl-1,3,4,5,6,6a,6b,7,8,8a,9,10,11,12,12a,12b,13,14b-octadecahydropicene-4a(2H)-carboxylic acid	Terpenes	439.3562 M- H_2O	*Tinospora cordifolia*(Willd) Miers ex Hook.f.&Thoms [8]
12	14.428	$C_{30}H_{48}O$	424.7015	Limocinone	Tirucalla-8,24-dien-16-one	Terpenes	425.3661 M+H^+	*Azadirachta indica* (L.)A.Juss. [9]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
13	15.203	$C_{30}H_{48}O_2$	440.7	Kulinone	(13α,14β,16β,17α)-16-Hydroxylanosta-7,24-dien-3-one	Terpenes	423.377 M-H_2O	*Azadirachta indica*(L.)A.Juss. [9]
14	15.231	$C_{30}H_{48}O_4$	472.3346	Odoratone	23 R,24 S-dihydroxy-22 S,25-epoxytirucall-7-en-3-one	Terpenes	455.3570 M-H_2O	*Azadirachta indica*(L.)A.Juss. [9]
15	16.414	$C_{26}H_{32}O_7$	456.5	Nimbandiol	methyl 2-[(1*S*,2*R*,3*S*,4*R*,8*S*,9*S*,10*R*,13*R*,15*S*)-13-(furan-3-yl)-2,4-dihydroxy-4,8,10,12-tetramethyl-7-oxo-16-oxatetracyclo [8.6.0.$0^{3,8}$.$0^{11,15}$]hexadeca-5,11-dien-9-yl]acetate	Terpenes	437.3405 M-H^+-H_2O	*Azadirachta indica*(L.)A.Juss. [9]
16	26.458	$C_{30}H_{50}O_3$	458.7	Commipherol	(4*aS*,5*R*,6*R*,8*aR*)-6-hydroxy-5-[(3*E*,7*E*,11*E*)-13-hydroxy-4,8,12-trimethyltrideca-3,7,11-trienyl]-1,1,4*a*,6-tetramethyl-3,4,5,7,8,8*a*-hexahydronaphthalen-2-one	Terpenoids	481.2635 M+Na	*Commiphora mukul*(Hook.ex Stocks) Engl. [4]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
17	15.312	$C_{28}H_{36}O_4$	436.6	Azadirone	[(5*R*,7*R*,8*R*,9*R*,10*R*,13*S*,17*R*)-17-(furan-3-yl)-4,4,8,10,13-pentamethyl-3-oxo-5,6,7,9,11,12,16,17-octahydrocyclopenta[a]phenanthren-7-yl] acetate	Terpenes	437.2351 $M+H^+$	*Azadirachta indica*(L.)A.Juss. [9]
18	13.336	$C_{29}H_{38}O_4$	450.6	Celastrol	(2*R*,4*aS*,6*aR*,6*aS*,14*aS*,14*bR*)-10-hydroxy-2,4*a*,6*a*,6*a*,9,14*a*-hexamethyl-11-oxo-1,3,4,5,6,13,14,14*b*-octahydropicene-2-carboxylic acid	Terpenes	473.360M+Na	*Celastrus paniculate* Willd [10]
19	13.462	$C_{27}H_{44}O_4$	432.6	Guggulusterol	16-beta hydroxy-4,17(20)-Z-pregnadiene-3-one	Steroids	455.3506M+Na	*Commiphora mukul* (Hook.ex Stocks) Engl. [4]
20	13.531	$C_{25}H_{44}N_2O_2$	404.480	Regholarrhenine E	3-(N, N-Di-methylamino)-4-methyl-N-methylconanine-5,7-diol	Alkaloid	441.3716 M-2H+K	*Holarrhena antidysenterica* (Roth)DC [11]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
21	14.006	$C_{27}H_{44}O_7$	480.2367	Ecdysterone	2β,3β,14α,20β,22α,25 β-exahydroxycholest-7-en-6-one	Steroid	485.2153 M+Na-H_2O	*Tinospora cordifolia*(Willd) Miers ex Hook.f.&Thoms [8]
22	14.135	$C_{24}H_{22}O_{10}$	470.339996	Dillanoside	11-hydroxy-2-methoxy-9-[3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]oxybenzo[a]xanthen-12-one	Terpenes	471.34 $M+H^+$	*Anetham graveolens* L. [12]
23	14.422	$C_{30}H_{48}O_3$	456.3595	Oleanolic acid	(4aS,6aR,6aS,6bR,8aR,10S,12aR,14bS)-10-hydroxy-2,2,6a,6b,9,9,12a-heptamethyl-1,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydropicene-4a-carboxylic acid	Terpenes	439.3560 $M+H^+$-H_2O	*Tinospora cordifolia*(Willd) Miers ex Hook.f.&Thoms [8]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
24	14.293	$C_{22}H_{22}O_8$	414.4	Beta –Peltatin	(5*aR*,8*aR*,9*R*)-4-hydroxy-9-(3,4,5-trimethoxyphenyl)-5*a*,6,8*a*,9-tetrahydro-5*H*-[2] benzofuro[5,6-f][1,3]benzodioxol-8-one	Phenolic	437.3404 $M+Na^+$	*Saussurea lappa*(Decne)Sch-Bip [13]
25	4.485	$C_{24}H_{40}N_2$	356.3178	Conessine	(1*R*,2*S*,5*S*,6*S*,9*R*,12*S*,13*R*,16*S*)-*N*,*N*,6,7,13-pentamethyl-7-azapentacyclo[10.8.0.$0^{2,9}$.$0^{5,9}$.$0^{13,18}$]icos-18-en-16-amine	Alkaloid	357.3224 $M+H^+$	*Holarrhena antidysenterica* (Roth)DC [11]
26	4.653	$C_{20}H_{26}O_3$	314.1881	Nimosone	12-Hydroxy-8,11,13-abietatriene-3,7-dione	Terpenes	314.1741 M^+	*Azadirachta indica*(L.)A.Juss. [9]
27	5.231	$C_{37}H_{40}N_2O_6$	608.2901	Fangchinoline	(1*S*,14*S*)-9,20,25-trimethoxy-15,30-dimethyl-7,23-dioxa-15,30-diazaheptacyclo [22.6.2.2^{3,6}.1^{8,12}.1^{14,18}.$0^{27,31}$.$0^{22,33}$] hexatriaconta-3(36),4,6(35),8,10,12(34),18,20,22(33),24,26,31-dodecaen-21-ol	Alkaloid	609.2901 $M+H^+$	*Cyclea peltata* (Lam.) Hook.f.&Thoms [23]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
28	5.514	$C_{37}H_{40}N_2O_6$	608.2862	Berbamine	(1S,14R)-20,21,25-trimethoxy-15,30-dimethyl-7,23-dioxa-15,30-diazaheptacyclo [22.6.2.23,6.18,12.114,18.027,31.022,33] hexatriaconta-3(36),4,6(35),8,10,12(34),18,20,22(33),24,26,31-dodecaen-9-ol	Alkaloid	609.2934M+H^+	*Cyclea peltata*(Lam.) Hook.f.&Thoms [23]
29	10.199	$C_{29}H_{40}O_7$	500.6	Isonimolide	7-(acetyloxy)-21-hydroxy-6-methoxy-4,4,8-trimethyl-3-oxocarda-1,14,20(22)-trienolide	Terpenes	523.4237 M+Na	*Azadirachta indica* (L.)A.Juss. [9]
30	12.318	$C_{19}H_{22}O_5$	330.38	Cedrusinin	(2S,3R)-2-(4-hydroxy-3-methoxyphenyl)-3-(hydroxymethyl)-5-(3-hydroxypropyl)-2,3-dihydro-1-benzofuran	Flavanol	313.2150 M+H^+-H_2O	*Cedrus deodara*(Roxb.ex D.Don)G.Don [5]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
31	12.953	$C_{21}H_{28}O_2$	312.453	Guggulusterone E	(8*R*,9*S*,10*R*,13*S*,14*S*,17*E*)-17-ethylidene-10,13-dimethyl-1,2,6,7,8,9,11,12,14,15-decahydrocyclopenta[a]phenanthrene-3,16-dione	Steroids	313.2151M+H^+	*Commiphora mukul*(Hook.ex Stocks) Engl. [4]
32	13.17	$C_{26}H_{30}O_8$	470.6	Limonine	(1R,2R,7S,10R,13R,14R,16S,19S,20S)-19-(furan-3-yl)-9,9,13,20-tetramethyl-4,8,15,18-tetraoxahexacyclo [11.9.0.02,7.02,10.014,16.014,20] docosane-5,12,17-trione	Terpenes	471M+H^+	*Azadirachta indica*(L.)A.Juss. [9]
33	14.722	$C_{21}H_{20}O_8$	400.4	Alpha Peltatin	(5*aR*,8*aR*,9*R*)-4-hydroxy-9-(4-hydroxy-3,5-dimethoxyphenyl)-5*a*,6,8*a*,9-tetrahydro-5*H*-[2] benzofuro[5,6-f][1,3]benzodioxol-8-one	Phenolic	423.3606 M+Na	*Saussurea lappa* (Decne)Sch-Bip [13]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
34	17.116	$C_{26}H_{32}O_6$	440.52	7-Deacetylgedunin	(1*S*,2*R*,4*S*,7*S*,8*S*,11*R*,12*R*,17*R*,19*R*)-7-(furan-3-yl)-19-hydroxy-1,8,12,16,16-pentamethyl-3,6-dioxapentacyclo [9.8.0.$0^{2,4}$.$0^{2,8}$.$0^{12,17}$] nonadec-13-ene-5,15-dione	Terpenes	423.3614 M-H_2O	*Azadirachta indica* (L.)A.Juss. [9]
35	0.774	$C_{17}H_{24}O_3$	276.376	6-Shogaol	1-(4-hydroxy-3-methoxyphenyl) dec-4-en-3-one	Phenolic	299 M+Na^+	*Zingiber officinale* Rosc. [14]
36	3.42	$C_{18}H_{19}NO_5$	329.7	Transferuloyloctapamine	(*E*)-*N*-[2-hydroxy-2-(4-hydroxyphenyl) ethyl]-3-(4-hydroxy-3-methoxyphenyl) prop-2-enamide	Alkaloid	330.2053 M+H^+	*Tinospora cordifolia*(Willd) Miers ex Hook.f.&Thoms [18]
37	3.655	$C_{20}H_{21}N_3O_2$	335.4	Adhatodine	methyl 2-(methylamino)-5-(1,2,3,9-tetrahydropyrrolo[2,1-b] quinazolin-3-yl) benzoate	Alkaloid	358 M+Na^+	*Adhatoda vasica* Nees [15]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
38	3.79	$C_{18}H_{19}NO_4$	313.3	Moupinamide	(*E*)-3-(4-hydroxy-3-methoxyphenyl)-*N*-[2-(4-hydroxyphenyl) ethyl] prop-2-enamide	Alkaloid	314.1743 $M+H^+$	*Piper longum* L. [22]
39	7.353	$C_{21}H_{26}O_6$	374.43	Hexahydrocurcumin	(RS)-5-Hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-3-heptanone	Phenolic	373.1298 $M-H^+$	*Curcuma longa* L [17]
40	15.236	$C_{30}H_{48}O_4$	472.70	Rubifolic acid	10-hydroxy-2-(hydroxymethyl)-1,6a,6b,9,9,12a-hexamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid	Terpenes	471.3503 $M-H^+$	*Rubia cordifolia* L. [18]
41	26.949	$C_{18}H_{30}O_4$	310.43	3-Methylgingerdiol	(E)-1-(3,4-dimethoxyphenyl) dec-3,5-diol	Phenolic	309.1715 $M-H^+$	*Zingiber officinale* Rosc. [14]
42	4.485	$C_{20}H_{22}O_6$	358.38	Matairesinol	(3*R*,4*R*)-3,4-bis[(4-hydroxy-3-methoxyphenyl) methyl] oxolan-2-one	Phenolic	357.3264 $M-H^+$	*Cedrus deodara* (Roxb.ex D.Don)G.Don [25]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
43	3.966	$C_{17}H_{19}NO_3$	285.34	Piperine	(2*E*,4*E*)-5-(2*H*-1,3-Benzodioxol-5-yl)-1-(piperidin-1-yl)penta-2,4-dien-1-one	Alkaloid	286.1424	*Piper longum* L. [6], *Piper nigrum* L. [16]
44	5.643	$C_{14}H_{14}O_4$	246.26	Marmesin	(2*S*)-2-(2-hydroxypropan-2-yl)-2, 3-dihydrofuro [3, 2-g] chromen-7-one	Phenolic	246.1440	*Zingiber officinale* Rosc. [14]
45	1.687	$C_7H_6O_5$	170.12	Gallic acid	3,4,5-trihydroxybenzoic acid	Phenolic	169.0119 $M\text{-}H^+$	*Cuminum cyminum* L. [19]
46	5.231	$C_{27}H_{30}O_{16}$	610.517	Rutin	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-[[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl] oxymethyl] oxan-2-yl] oxychromen-4-one	Phenolic	609.2934 $M\text{-}H^+$	*Azadirachta indica*(L.)A.Juss. [9]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
47	1.578	$C_{11}H_{12}N_2O_2$	204.22	Vasicinol	(3*S*)-1,2,3,9-tetrahydropyrrolo[2,1-b] quinazoline-3,7-diol	Alkaloid	205.0940 $M+H^+$	*Adhatoda vasica* Nees [15]
48	2.057	$C_{11}H_6O_3$	186.16	Angelicine	Furo[2,3-h] chromen-2-one	Phenolic	186.1205	*Trachyspermum roxburghianum* (DC.) Wolff [20]
49	6.561	$C_{21}H_{32}O_2$	316.48	Guggulusterone VI	Cis 4(20) pregnene-3-one-16-ol	Terpene	316.1487	*Commiphora mukul* (Hook.ex Stocks) Engl. [27]
50	1.445	$C_{15}H_{16}O_2$	228.29	Rubiasin C	(4aS,9aR,10S)-10-hydroxy-2-methyl-4,4a,9a,10-tetrahydro-1H-anthracen-9-one	Phenolic	229.1507 $M-H^+$	*Rubia cordifolia* L. [18]
51	2.116	$C_{11}H_{12}N_2O$	188.23	Vasicine	(3*S*)-1,2,3,9-Tetrahydropyrrolo[2,1-*b*] quinazolin-3-ol	Alkaloid	189.1012 $M+H^+$	*Adhatoda vasica* Nees [15]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
52	8.918	$C_{30}H_{48}O_5$	486.7	Chinovic acid	(1*S*,2*R*,4*aS*,6*aR*,6*aR*,6*bR*,8*aR*,10*S*,12*aR*,14*bS*)-10-hydroxy-1,2,6*b*,9,9,12*a*-hexamethyl-2,3,4,5,6,6*a*,7,8,8*a*,10,11,12,13,14*b*-tetradecahydro-1*H*-picene-4*a*,6*a*-dicarboxylic acid	Terpenes	485.2827 $M-H^+$	
53	4.815	$C_{18}H_{22}O_4$	302.4	Nimbionone	(4*aS*,10*aR*)-6-hydroxy-7-methoxy-1,1,4*a*-trimethyl-3,4,10,10*a*-tetrahydrophenanthrene-2,9-dione	Terpenes	302.3000 M^+	*Azadirachta indica* (L.)A.Juss. [9]
54	4.501	$C_{14}H_8O_4$	240.21	1, 4-Dihydroxyanthraquinone	1,4-Dihydroxyanthracene-9,10-dione	Phenolic	241.1069 $M+H^+$	*Rubia cordifolia* L. [18]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
55	5.825	$C_{15}H_8O_5$	268.22	Nordamnacanthal	1,3-dihydroxy-9,10-dioxoanthracene-2-carbaldehyde	Phenolic	267.05 $M-H^+$	*Rubia cordifolia* L. [18]
56	3.656	$C_{11}H_{10}N_2O_3$	218.21	Vasicinolone	3,7-dihydroxy-1H,2H,3H,9H-pyrrolo[2,1-b] quinazolin-9-one	Phenolic	218.21 M^+	*Adhatoda vasica* Nees [15]
57	7.218	$C_{14}H_8O_5$	256.21	Purpurin	1,2,4-trihydroxyanthracene-9,10-dione	Phenolic	256.2199 M^+	*Rubia cordifolia* L. [18]
58	4.543	$C_{16}H_{19}NO_2$	257.83	Coumaperine	(2*E*,4*E*)-5-(4-hydroxyphenyl)-1-piperidin-1-ylpenta-2,4-dien-1-one	Alkaloid	258.2789 $M+H^+$	*Piper longum* L. [6], *Piper nigrum* L. [16]
59	3.273	$C_{15}H_{19}NO_3$	261.313	Trans feruloylpiperidine	(E)-3-(4-hydroxy-3-methoxyphenyl)-N-piperidin-1-ylprop-2-enamide.	Alkaloid	262.2332 $M+H^+$	*Piper longum* L. [6], *Piper nigrum* L.[16]
60	8.114	$C_{16}H_{12}O_5$	283.37	Galangin -3-methyl ether	5,7-dihydroxy-3-methoxy-2-phenylchromen-4-one	Phenolic	284.3 $M+H^+$	*Alpinia calcarata* Roscoe [21]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
61	4.501	$C_{20}H_{30}O_3$	318.4	Calcaratarin D	(3Z,4S)-3-[2[(1S,4aS,8aS)-5.5.8a-trimethyl-2-methylidene-3,4,4a,6,7,8-hexahydro-1H-naphthalene-1-yl] ethylidene]-4-hydroxyoxolan-2-one	Terpene	318 M^+	*Alpinia calcarata* Roscoe [21]
62	5.233	$C_{14}H_8O_4$	240.21	2, 4-Dihydroxyanthraquinone	2,4-Dihydroxyanthracene-9,10-dione	Phenolic	239.03 $M\text{-}H^+$	*Rubia cordifolia* L. [18]
63	4.874	$C_{17}H_{26}O_4$	294.4	6-Gingerol	(5S)-5-Hydroxy-1-(4-hydroxy-3-methoxyphenyl) decan-3-one	Phenolic	293.175 $M\text{-}H^+$	*Zingiber officinale* Rosc. [14]
64	2.831	$C_{28}H_{34}O_8$	498.6	6-Deacetyl nimbin	Methyl (1*S*,2*R*,3*R*,4*R*,8*R*,9*S*,10*R*,13*R*,15*R*)-13-(furan-3-yl)-2-hydroxy-9-(2-methoxy-2-oxoethyl)-4,8,10,12-tetramethyl-7-oxo-16-oxatetracyclo [8.6.0.$0^{3,8}$.$0^{11,15}$] hexadeca-5,11-diene-4-carboxylate	Terpenes	497.332	*Azadirachta indica*(L.)A.Juss. [9]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
65	2.977	$C_{41}H_{58}O_9$	694.9	Melianin A	[(1S,3R,5S,7R,8R,9R,10S,13S,17S)-1,7-diacetyloxy-17-[(3S,5R,6R)-5-hydroxy-6-(2-hydroxypropan-2-yl)oxan-3-yl]-4,4,8,10,13-pentamethyl-2,3,5,6,7,9,11,12,16,17-decahydro-1H-cyclopenta[a]phenanthren-3-yl] benzoate	Terpenes	713.47[$M+H_2O]^-$	*Azadirachta indica* (L.)A.Juss. [9]
66	5.388	$C_{15}H_{10}O_5$	270.24	Galangin	3,5,7-trihydroxy-2-phenylchromen-4-one	Phenolic	269.045 $M-H^+$	*Alpinia calcarata* Roscoe [21]
67	6.756	$C_{21}H_{30}O_2$	314.46	Z-Guggulusterol	Cis-4,17(20) -pregnadiene-3-one-16-ol	Terpenes	313.2131 $M-H^+$	*Commiphora mukul*(Hook.ex Stocks) Engl. [24]

Sl No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
68	3.274	$C_{40}H_{72}NO_{13}$	775.00	Solasodine(3->1)β-gal-(2->1)rhamnose	(2S,2′R,4aR,4bS,5′R,6aS,6bR,7S,9aS,10aS,10bS)-4a,5′,6a,7-tetramethyl-1,2,3,4,4a,4b,5,6,6a,6b,7,9a,10,10a,10b,11-hexadecahydrospiro[naphtho [2′,1′:4,5] indeno[2,1-b]furan-8,2′-piperidin]-2-ol)(3->1)β-gal-(2->1)rhamnose	Alkaloid	775.4722	*Solanum indica* L.[22]
69	3.994	$C_{15}H_{10}O_7$	302.236	Quercetin	2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one	Phenolic	302.1299 M^+	*Cuminum cyminum* L. [19]
70.	1.073	$C_{11}H_{10}N_2O_2$	202.21	Vasicinone	(3S)-3-Hydroxy-2, 3-dihydro-1H-pyrrolo [2, 1-b] quinazolin-9-one)	Alkaloid	203.0519 $M+H^+$	*Adhatoda vasica* Nees [15]

SI No.	Rt	Molecular formula	Molecular weight	Compound name	IUPAC name	Class of compound	Molecular ion	Plant source
71.	16.593	$C_{30}H_{47}NO_6$	517.70	Holarosin B	3-[(3S,5S,8R,9S,10S,13R,14S,17S)-3-[(2R,4R,5S,6S)-5-amino-4-methoxy-6-methyloxan-2-yl]oxy-14-hydroxy-10,13-dimethyl-1,2,3,4,5,6,7,8,9,11,12,15,16,17-tetradecahydrocyclopenta[a]phenanthren-17-yl]-2H-furan-5-one	Alkaloid	518.3314 $M+H^+$	*Holarrhena antidysenterica* (Roth)DC [11]
72	14.769	$C_{30}H_{48}O_5$	488.70	Arjunolic acid	(4*aS*,6*aR*,6*aS*,6*bR*,8*aR*,9*R*,10*R*,11*R*,12*aR*,14*bS*)-10,11-dihydroxy-9-(hydroxymethyl)-2,2,6*a*,6*b*,9,12*a*-hexamethyl-1,3,4,5,6,6*a*,7,8,8*a*,10,11,12,13,14*b*-tetradecahydropicene-4*a*-carboxylic acid	Terpenes	519.3350 $M-H^+$	

73	16.355	$C_{30}H_{48}O_7$	520.7	Platycodigenin	(4*aR*,5*R*,6*aR*,6*aS*,6*bR*, 8*aR*,10*R*,11*S*,12*aR*,14 *bS*)-5,10,11-trihydroxy-9,9-bis(hydroxymethyl)-2,2,6*a*,6*b*,12*a*-pentamethyl-1,3,4,5,6,6*a*,7,8,8*a*,10, 11,12,13,14*b*-tetradecahydropicene-4*a*-carboxylic acid	Terpenes	487.3438 $M-H^+$	

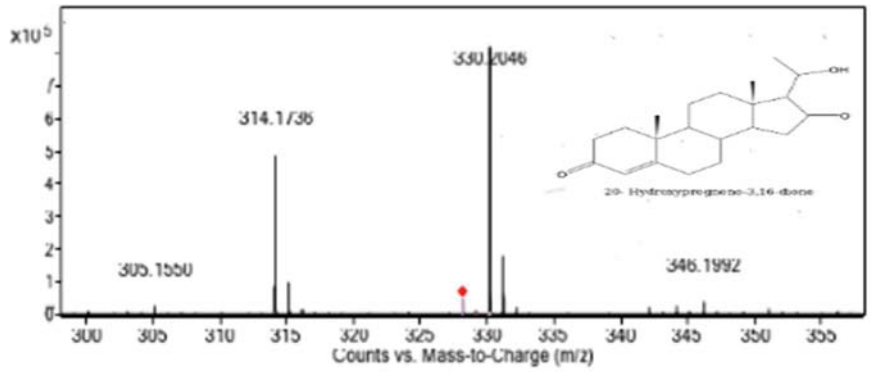


Figure 6.2. Mass spectrum of 20 –Hydroxyprogenene 3, 16-dione

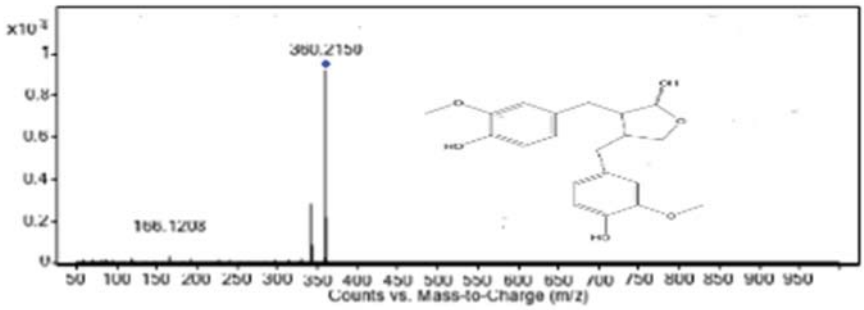


Figure 6.3. Mass spectrum of Dibenzylbutyrolactol

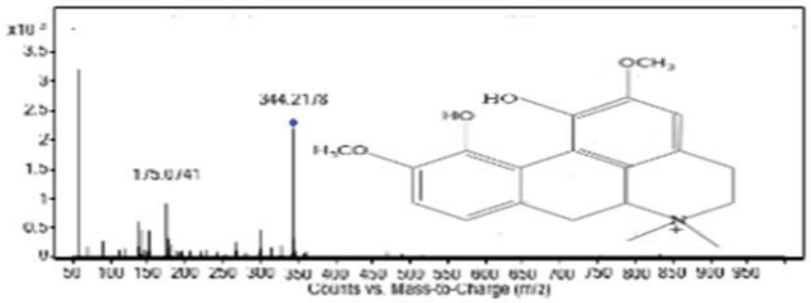


Figure 6.4. Mass spectrum of Magnoflorin

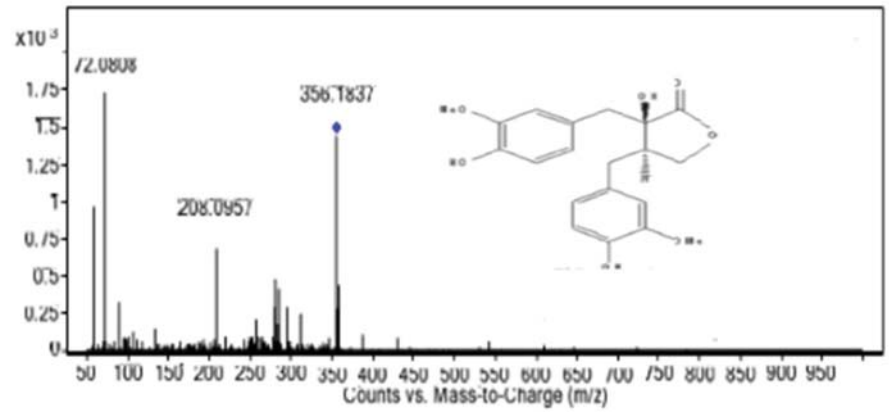


Figure 6.5. Mass spectrum of Wikstromal

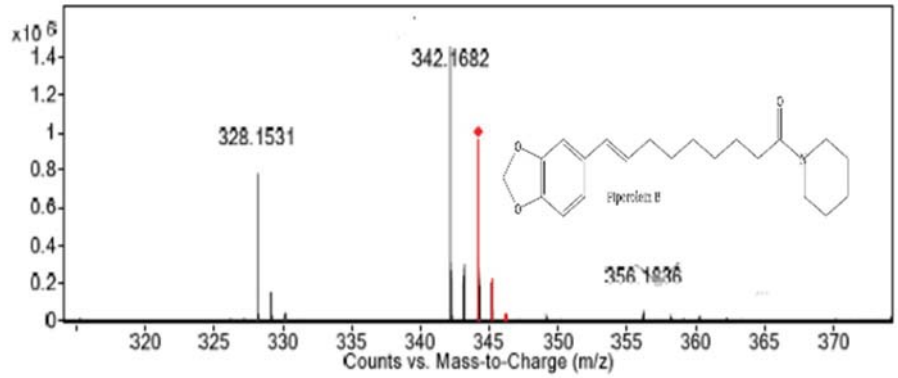


Figure 6.6. Mass spectrum of Piperolein B

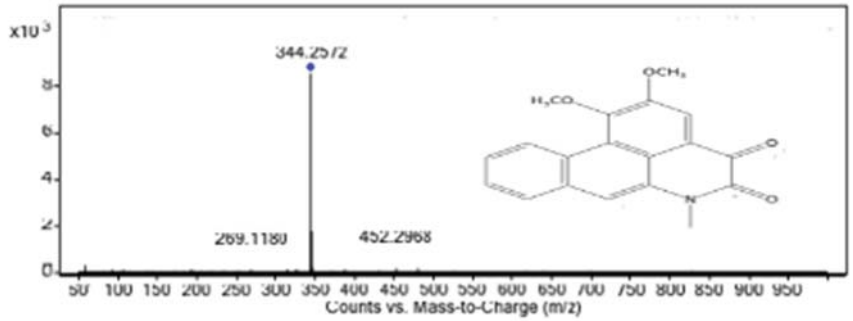


Figure 6.7. Mass spectrum of Cepharadione B

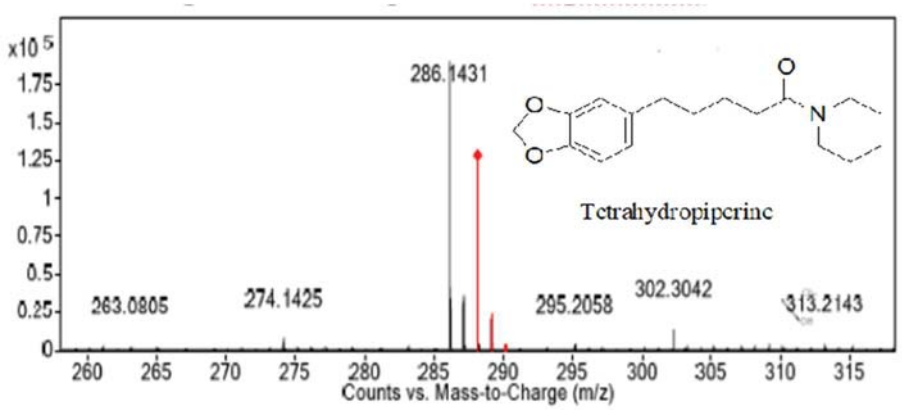


Figure 6.8. Mass spectrum of Tetrahydropiperine

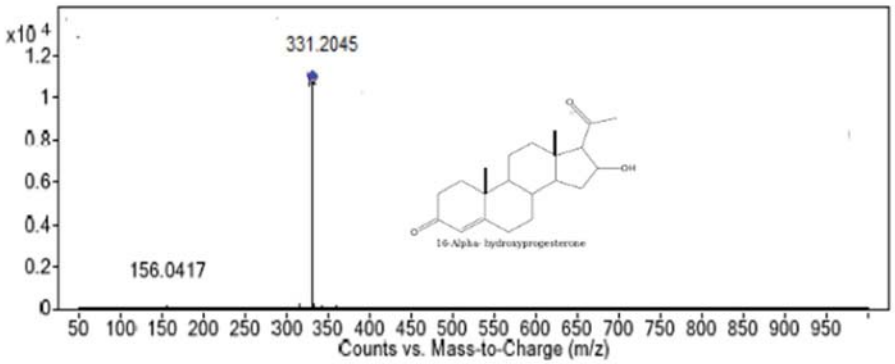


Figure 6.9. Mass spectrum of 16-Alpha-hydroxyprogesterone

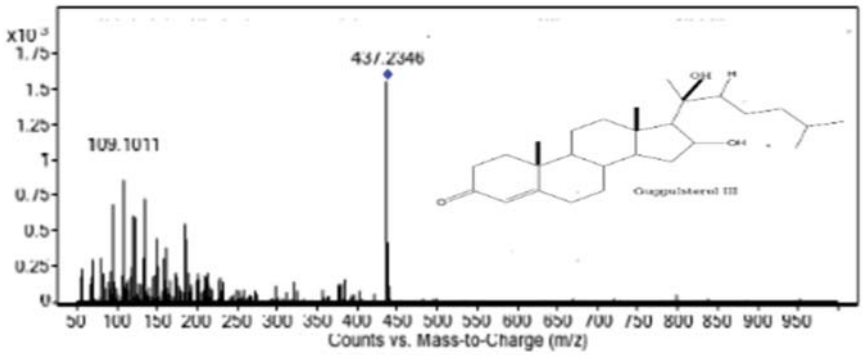


Figure 6.10. Mass spectrum of Guggulsterol III

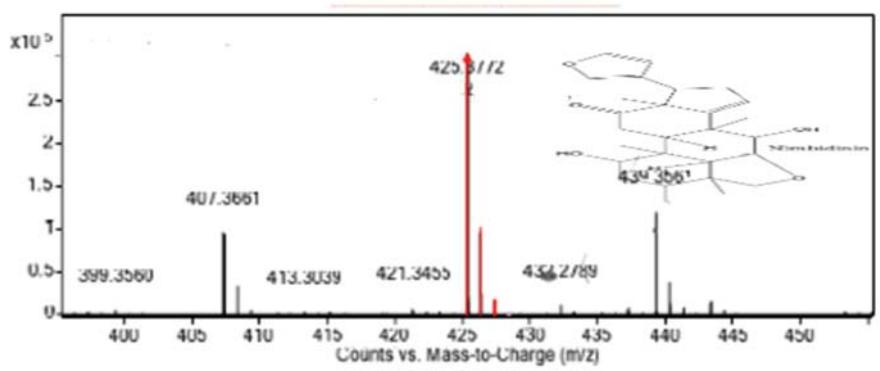


Figure 6.11. Mass spectrum of Nimbidinin

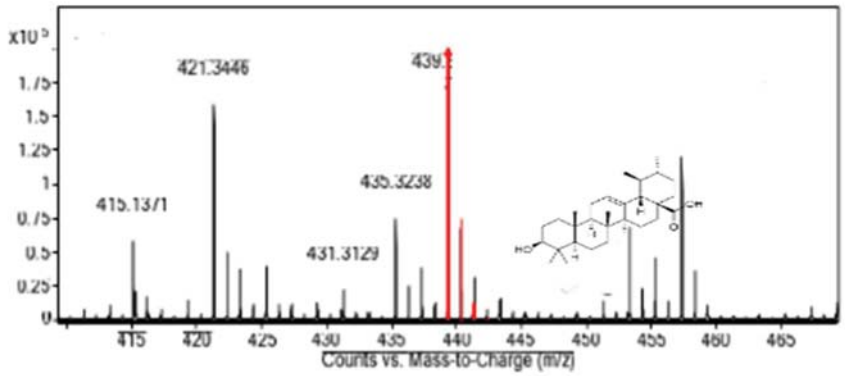


Figure 6.12. Mass spectrum of Ursolic acid

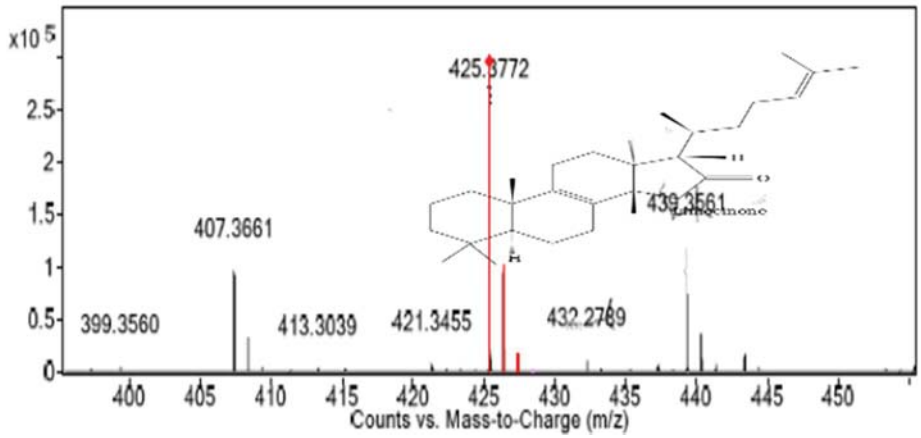


Figure 6.13. Mass spectrum of Limocinone

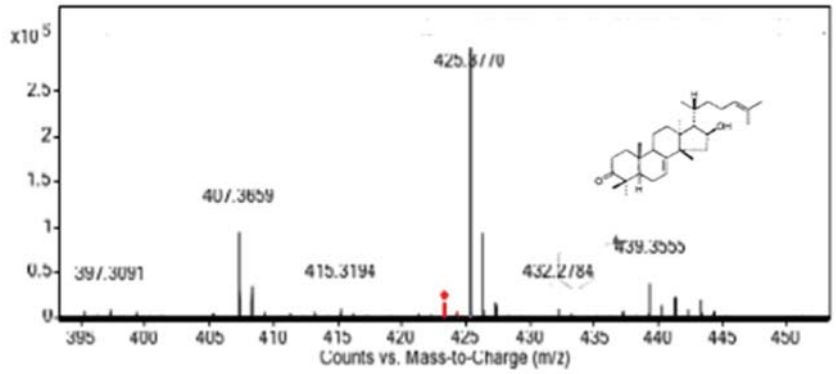


Figure 6.14. Mass spectrum of Kulinone

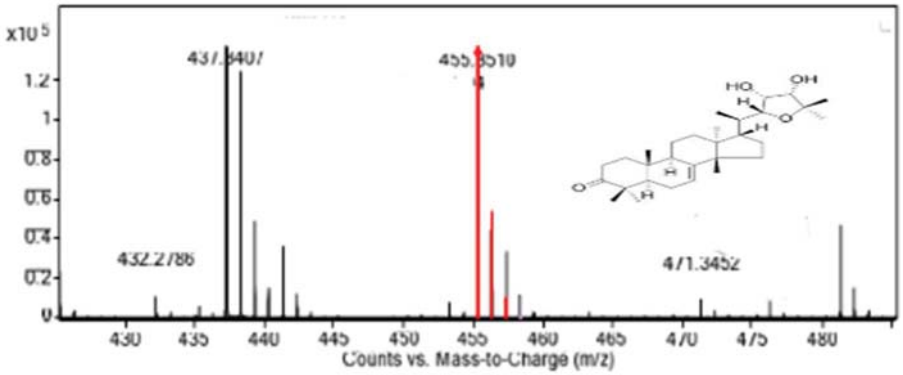


Figure 6.15. Mass spectrum of Odoratone

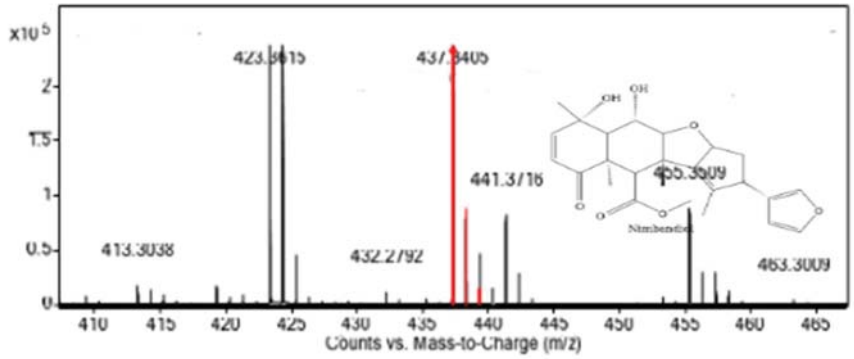


Figure 6.16. Mass spectrum of Nimbandiol

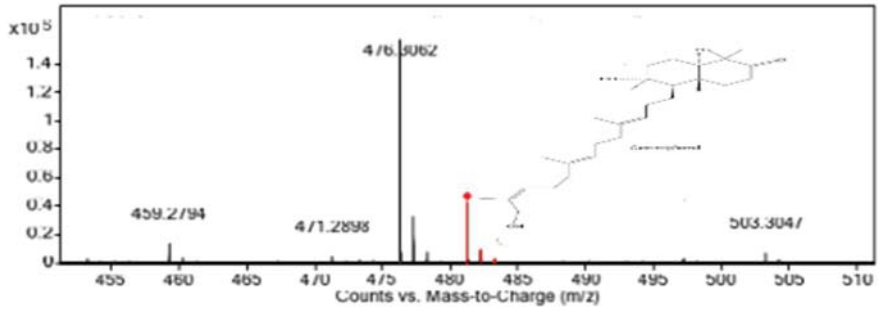


Figure 6.17. Mass spectrum of Commipherol

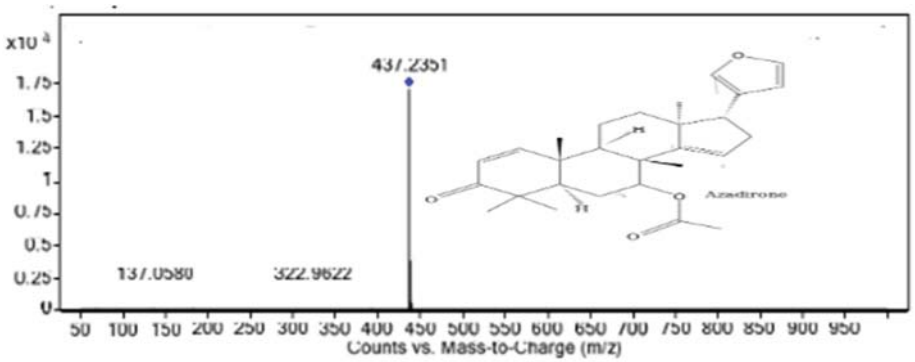


Figure 6.18. Mass spectrum of Azadirone

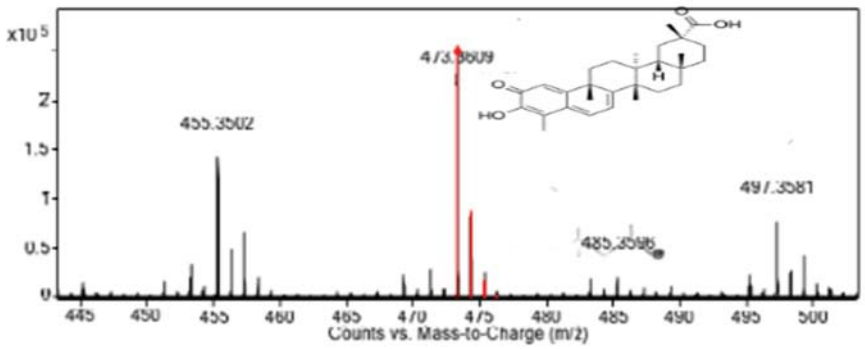


Figure 6.19. Mass spectrum of Celastrol

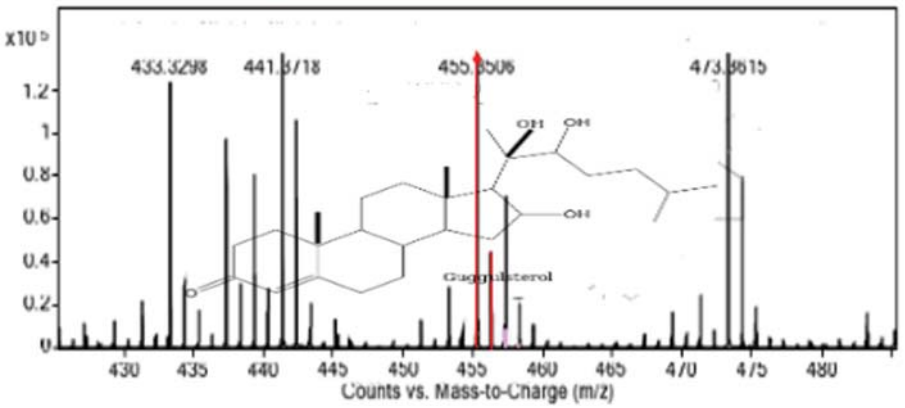


Figure 6.20. Mass spectrum of Guggulsterol

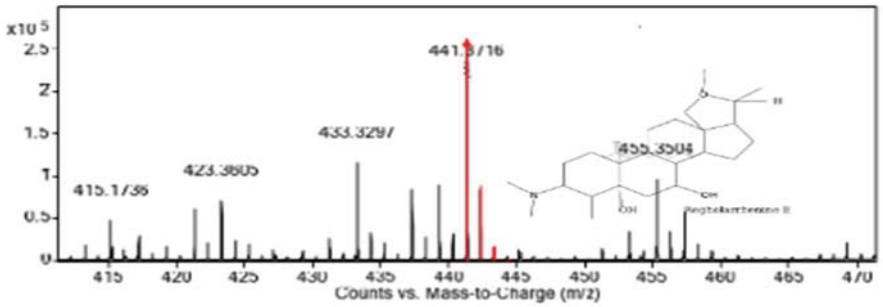


Figure 6.21. Mass spectrum of Regholarrhenine E

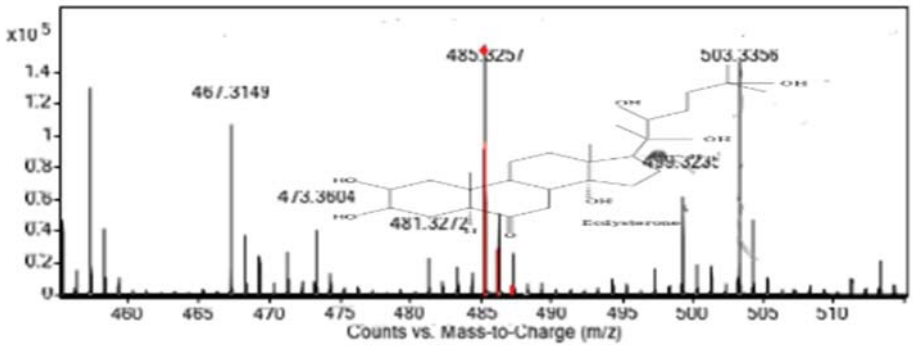


Figure 6.22. Mass spectrum of Ecdysterone

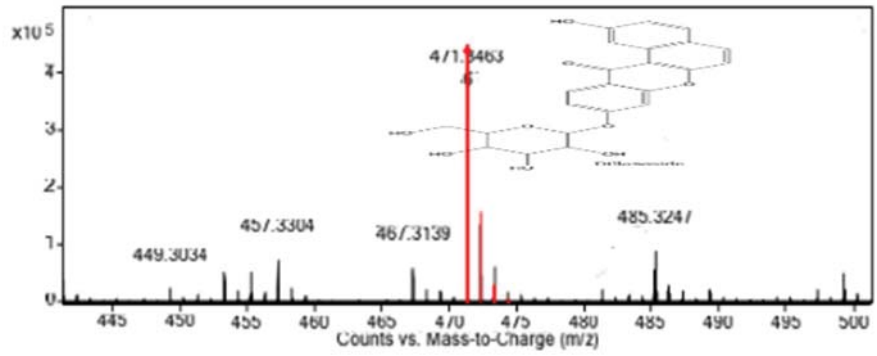


Figure 6.23. Mass spectrum of Dillanoside

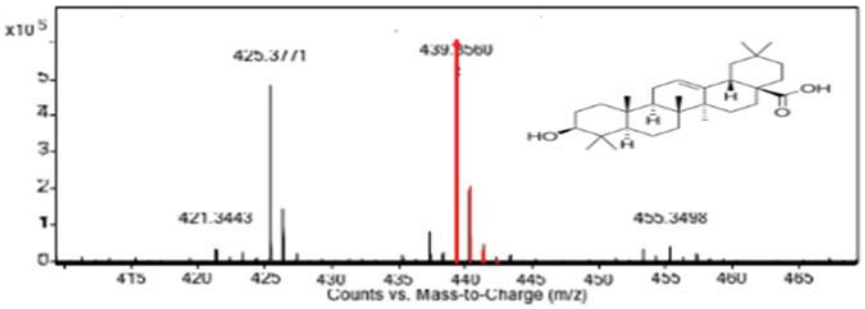


Figure 6.24. Mass spectrum of Oleanolic acid

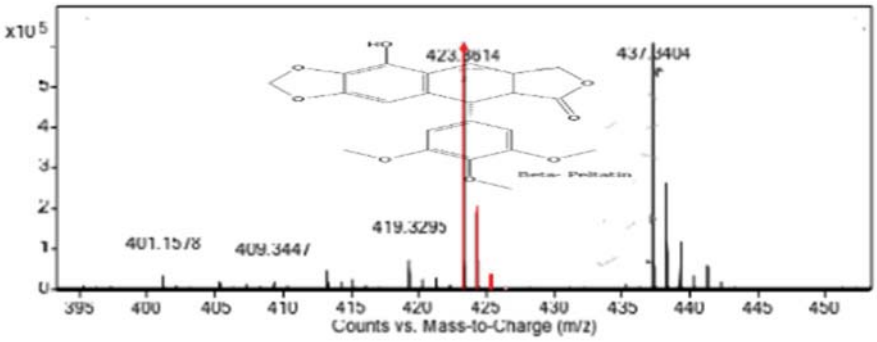


Figure 6.25. Mass spectrum of Beta -Peltatin

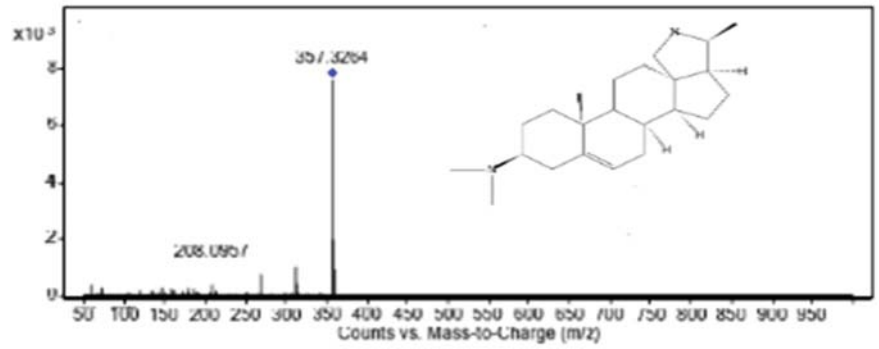


Figure 6.26. Mass spectrum of Conessine

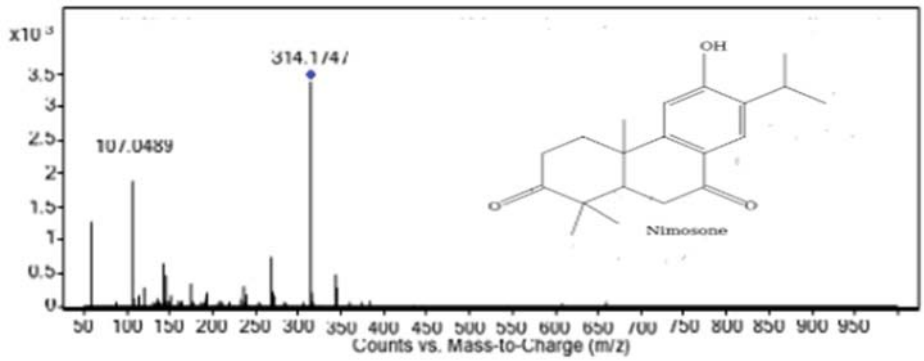


Figure 6.27. Mass spectrum of Nimosone

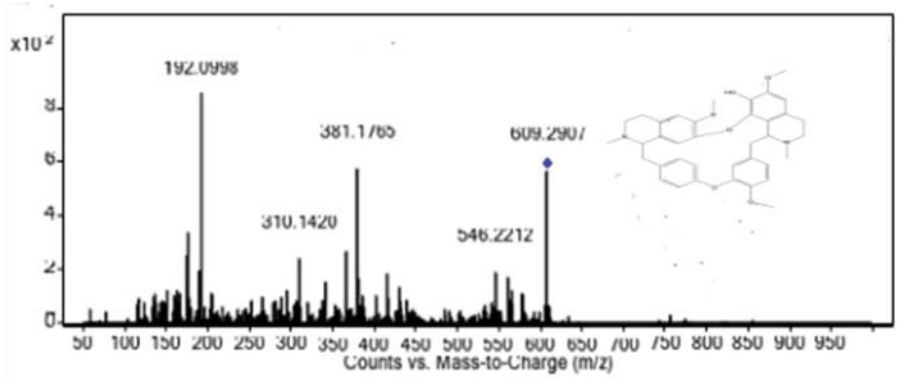


Figure 6.28. Mass spectrum of Fangchinoline

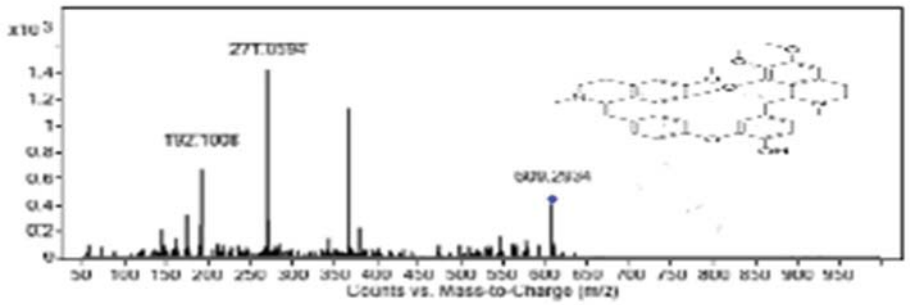


Figure 6.29. Mass spectrum of Berbamine

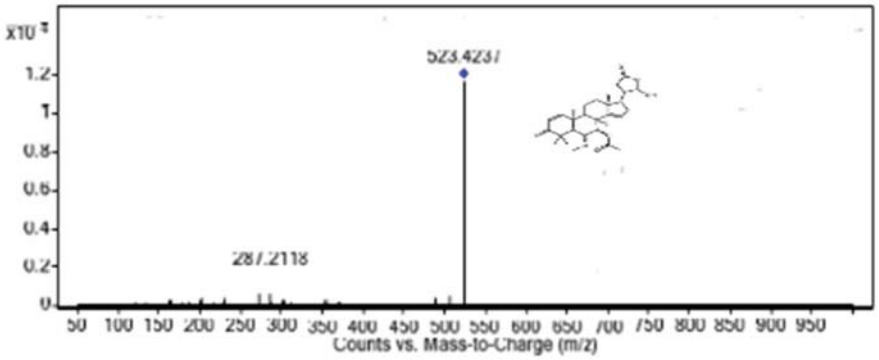


Figure 6.30. Mass spectrum of Isonimolide

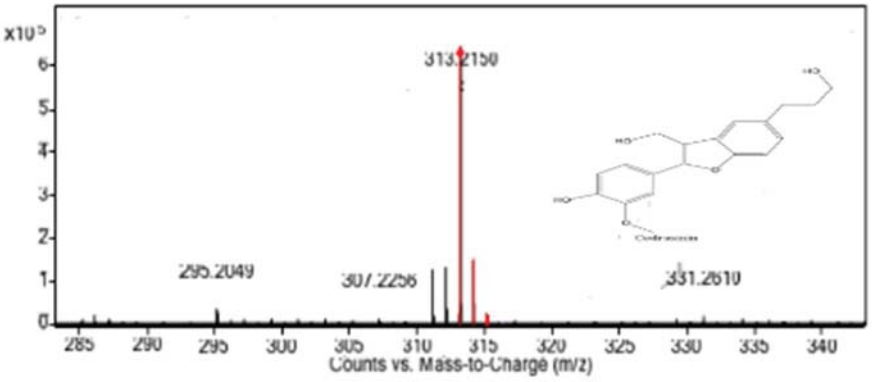


Figure 6.31. Mass spectrum of Cedrusinin

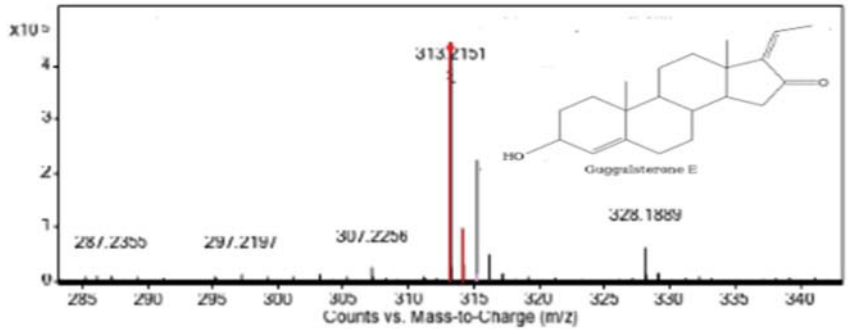


Figure 6.32. Mass spectrum of Guggulsterone E

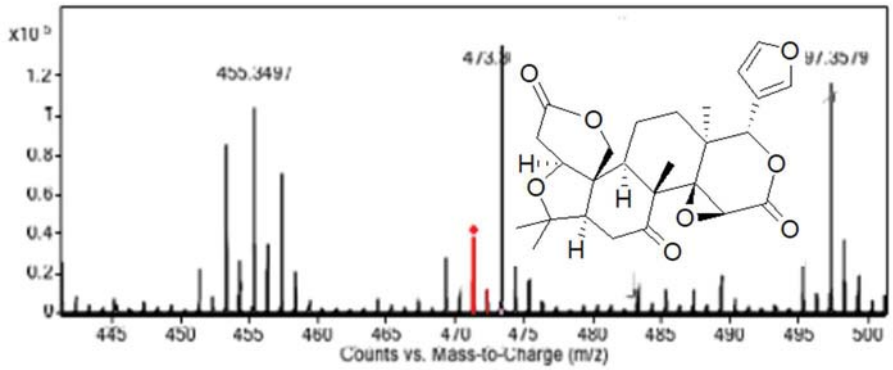


Figure 6.33. Mass spectrum of Limonin

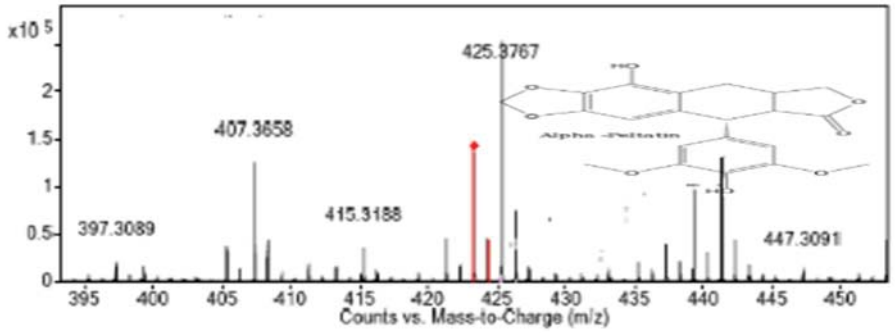


Figure 6.34. Mass spectrum of Alpha-Peltatin

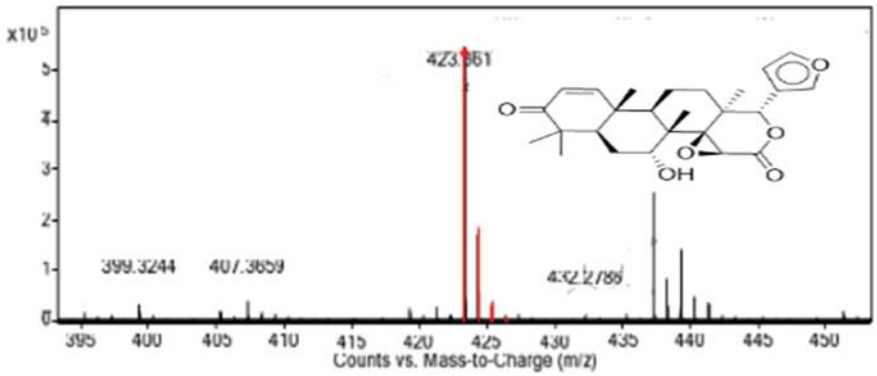


Figure 6.35. Mass spectrum of 7-Deacetylgedunin

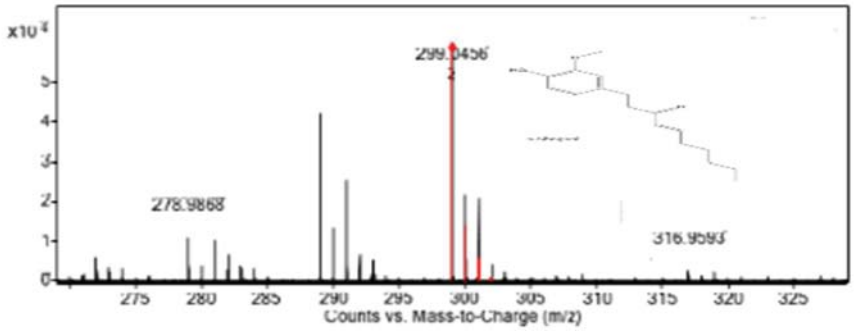


Figure 6.36. Mass spectrum of Shogaol

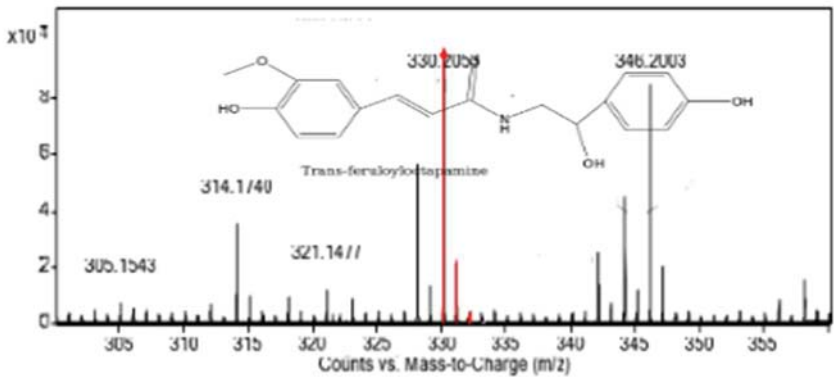


Figure 6.37. Mass spectrum of Trans feruloyl octopamine

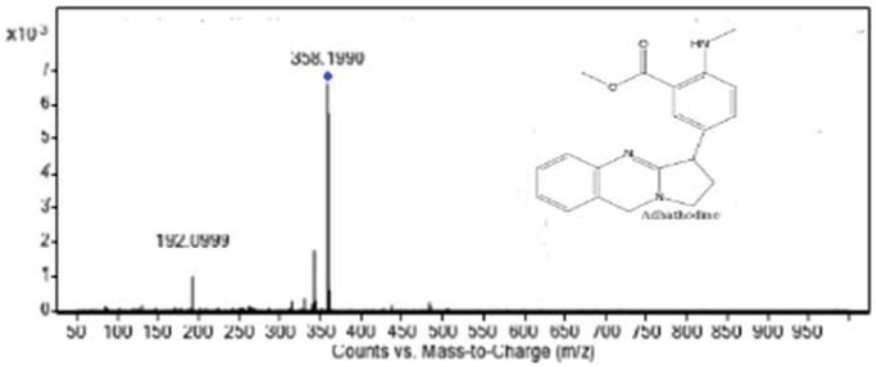


Figure 6.38. Mass spectrum of Adhatodine

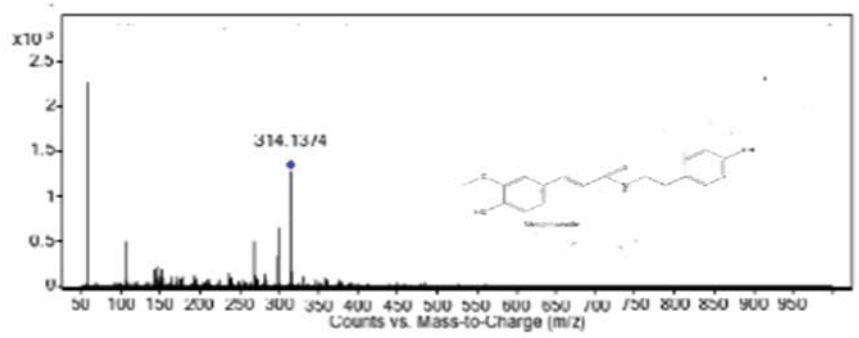


Figure 6.39. Mass spectrum of Moupinamide

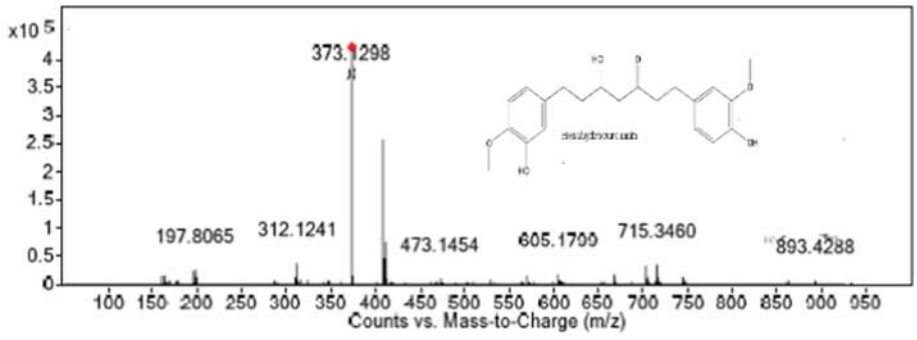


Figure 6.40. Mass spectrum of Hexahydrocurcumin

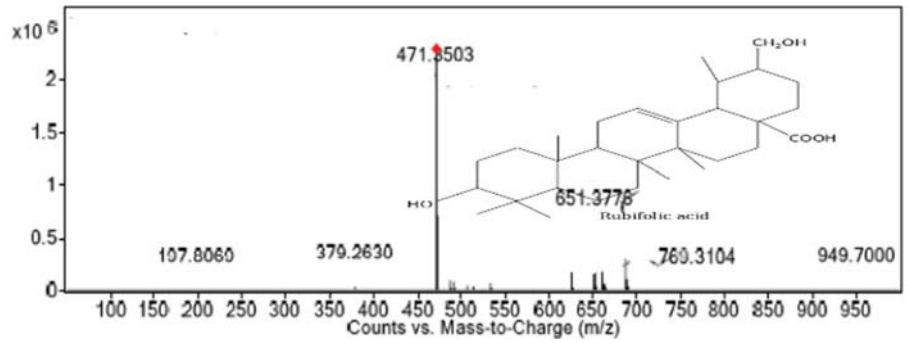


Figure 6.41. Mass spectrum of Rubifolic acid

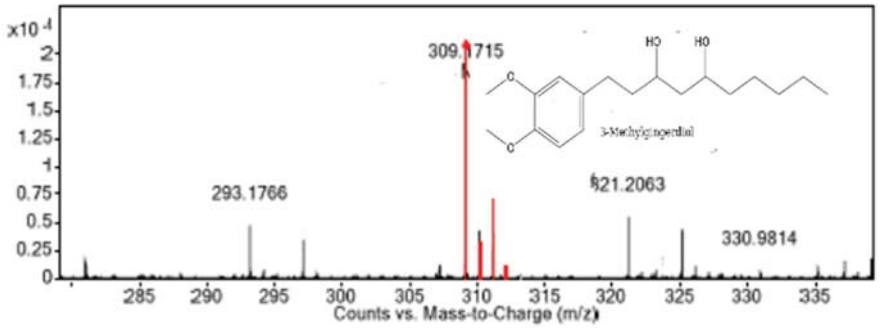


Figure 6.42. Mass spectrum of 3-Methylgingerdiol

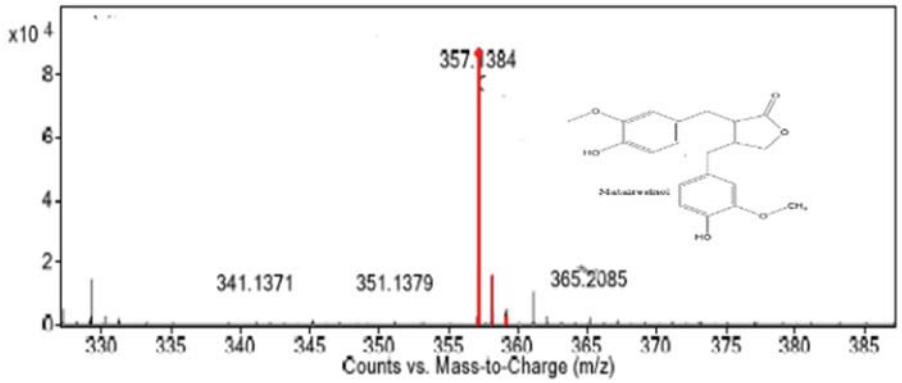


Figure 6.43. Mass spectrum of Matairesinol

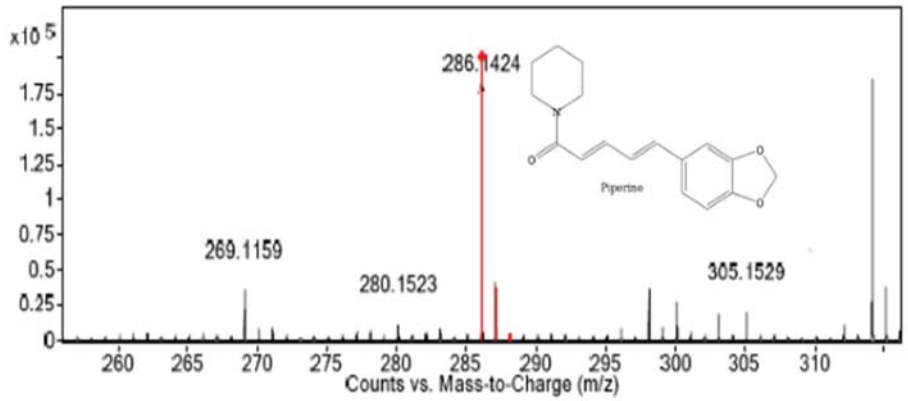


Figure 6.44. Mass spectrum of Piperine

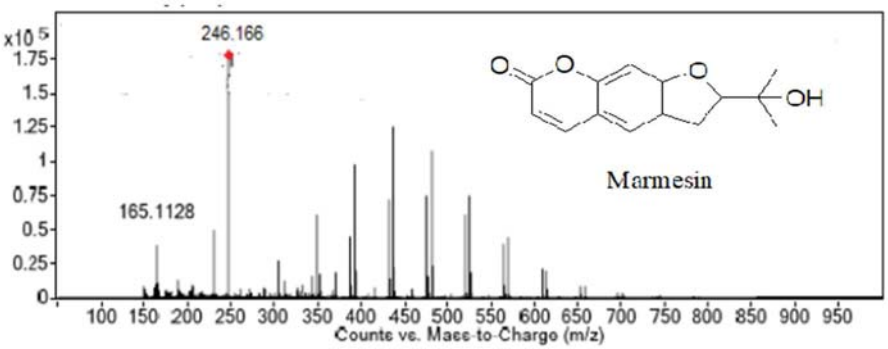


Figure 6.45. Mass spectrum of Marmesin

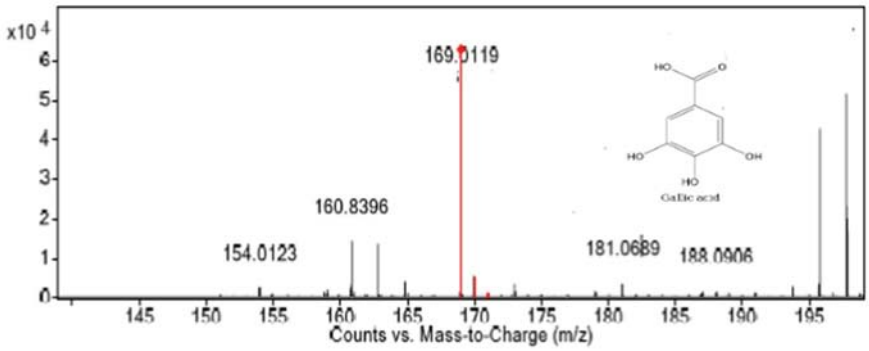


Figure 6.46. Mass spectrum of Gallic acid

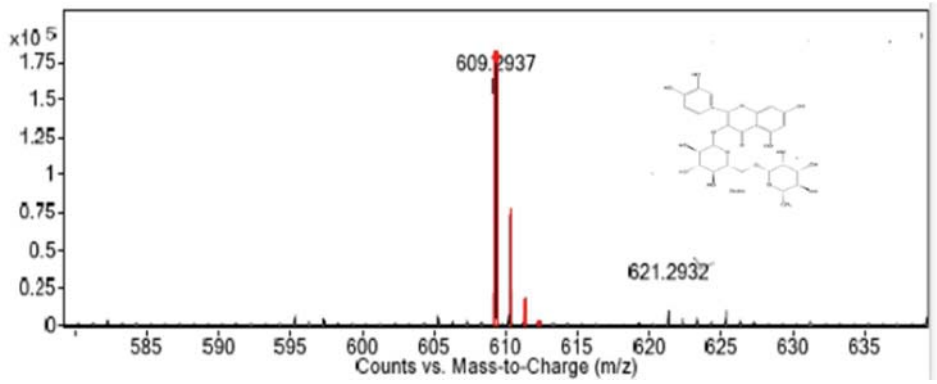


Figure 6.47. Mass spectrum of Rutin

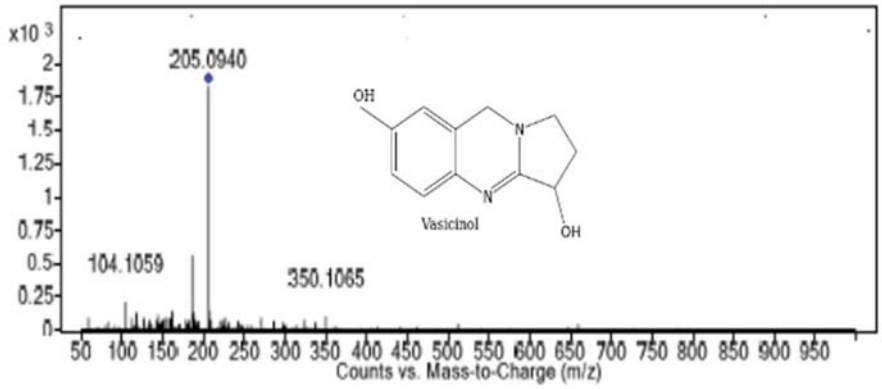


Figure 6.48. Mass spectrum of Vasicinol

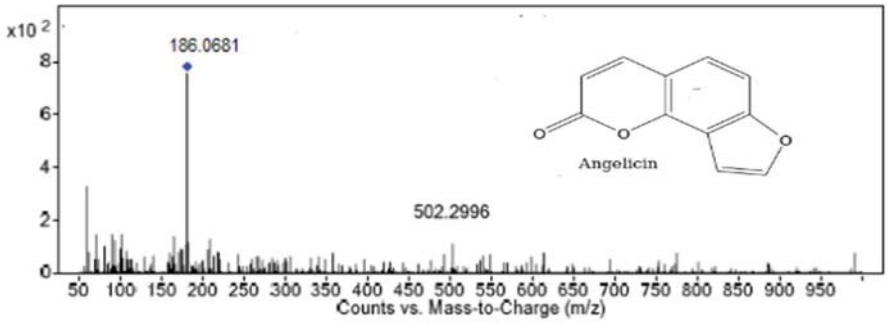


Figure 6.49. Mass spectrum of Angelicine

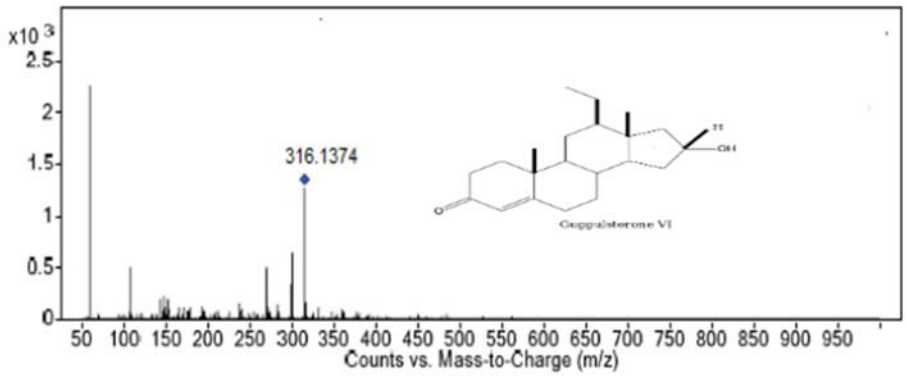


Figure 6.50. Mass spectrum of Guggulsterone VI

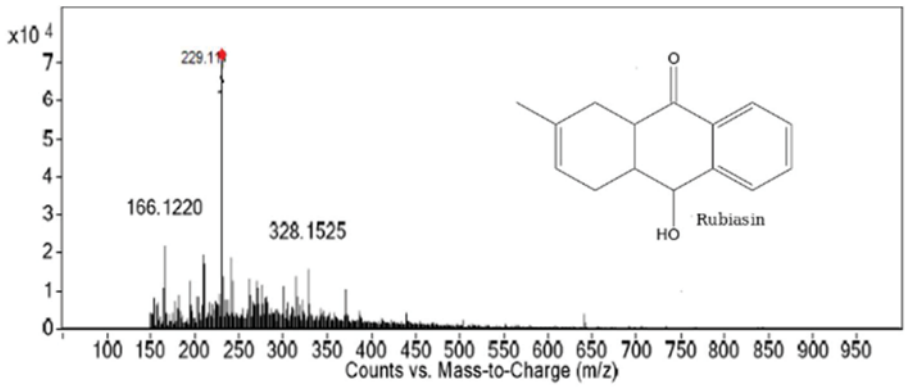


Figure 6.51. Mass spectrum of Rubiasin C

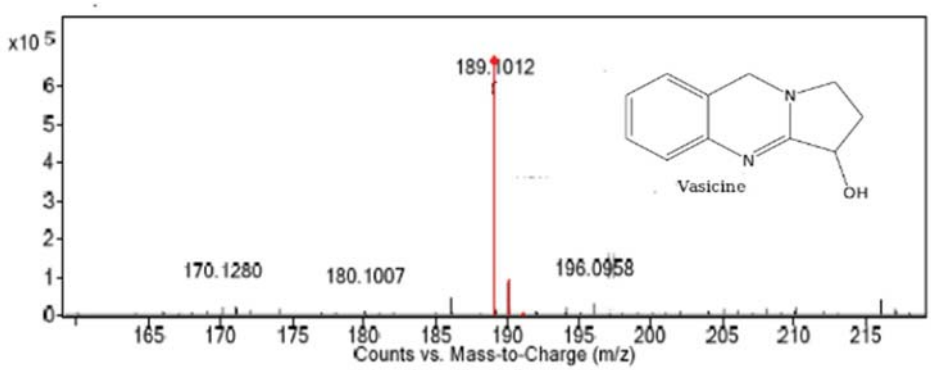


Figure 6.52. Mass spectrum of Vasicine

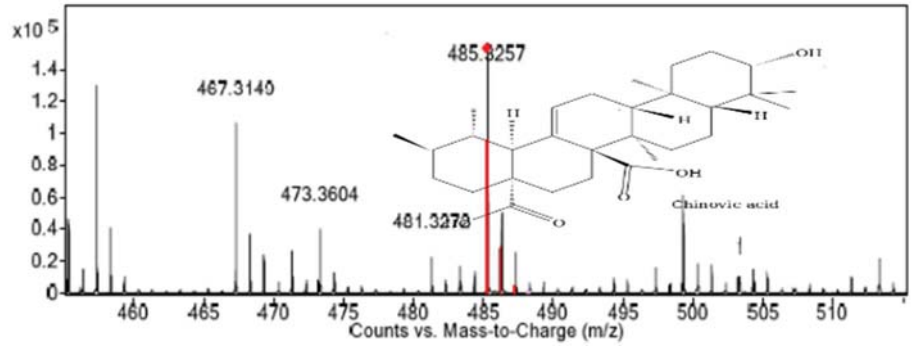


Figure 6.53. Mass spectrum of Chinovic acid

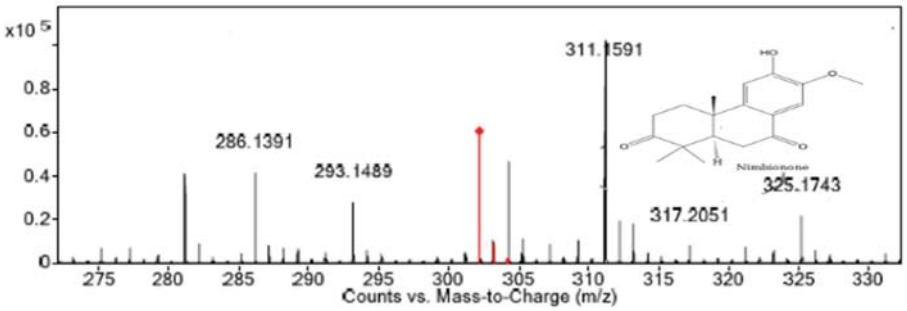


Figure 6.54. Mass spectrum of Nimbionone

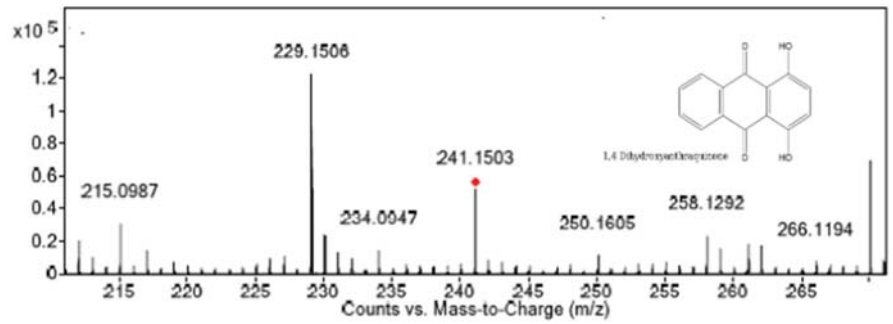


Figure 6.55. Mass spectrum of 1, 4-Dihydrxyanthraquinone

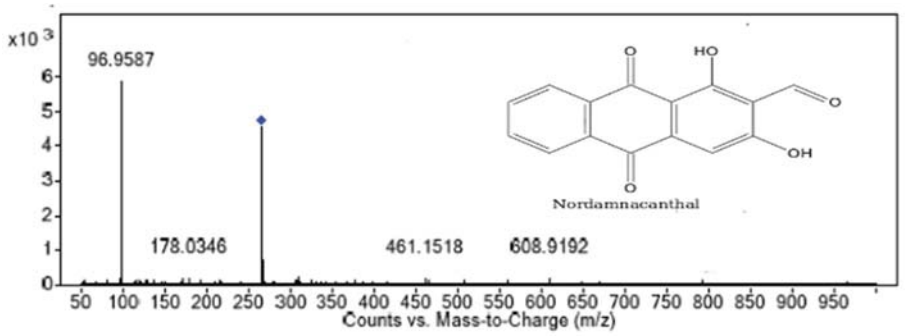


Figure 6.56. Mass spectrum of Nordamnacanthal

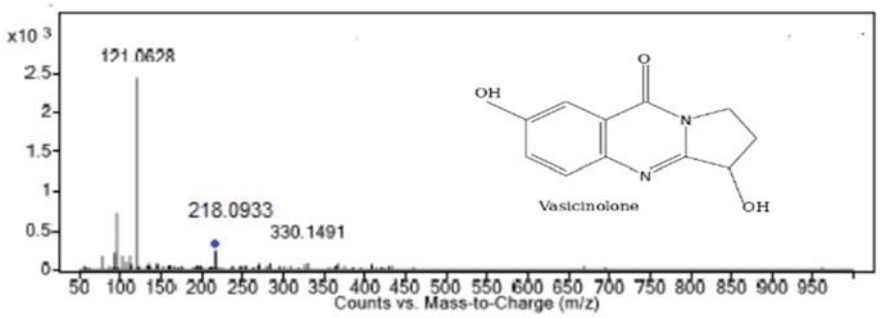


Figure 6.57. Mass spectrum of Vasicinolone

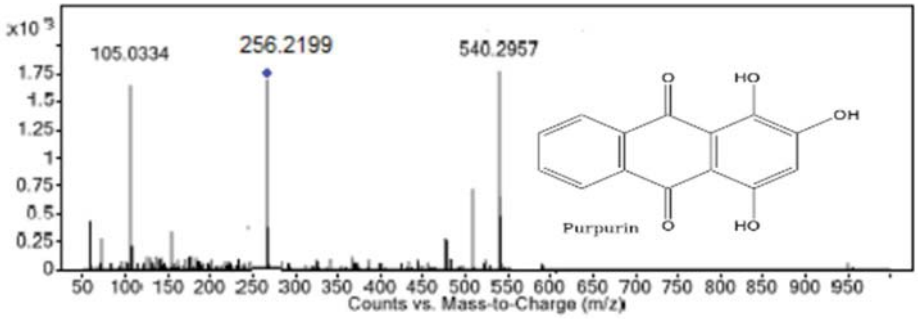


Figure 6.58. Mass spectrum of Purpurin

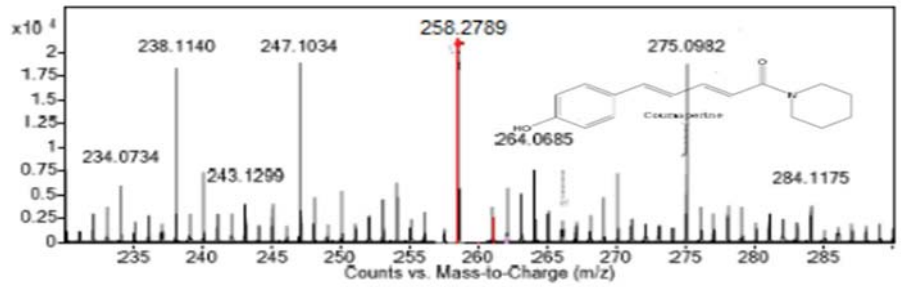


Figure 6.59. Mass spectrum of Coumaperine

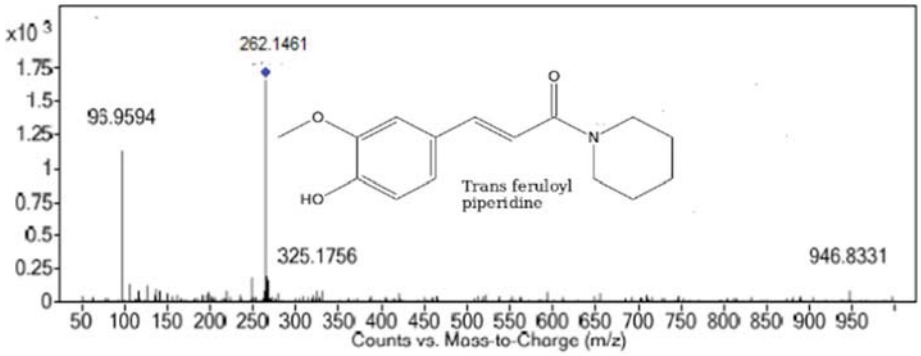


Figure 6.60. Mass spectrum of Trans feruloylpiperidine

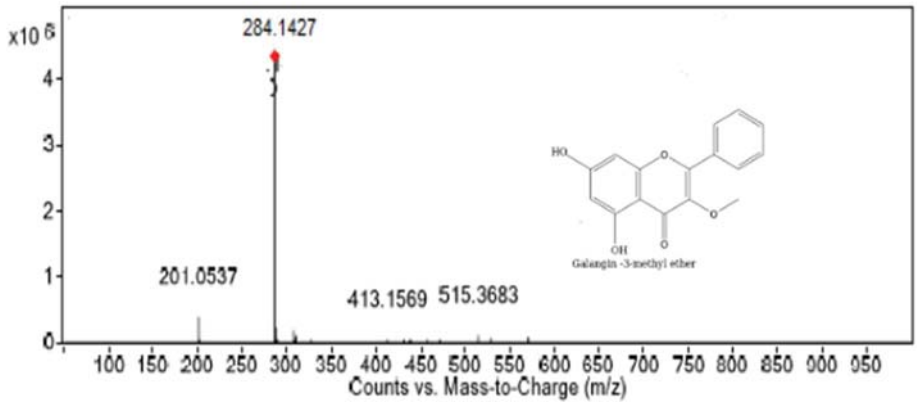


Figure 6.61. Mass spectrum of Galangin-3-methylether

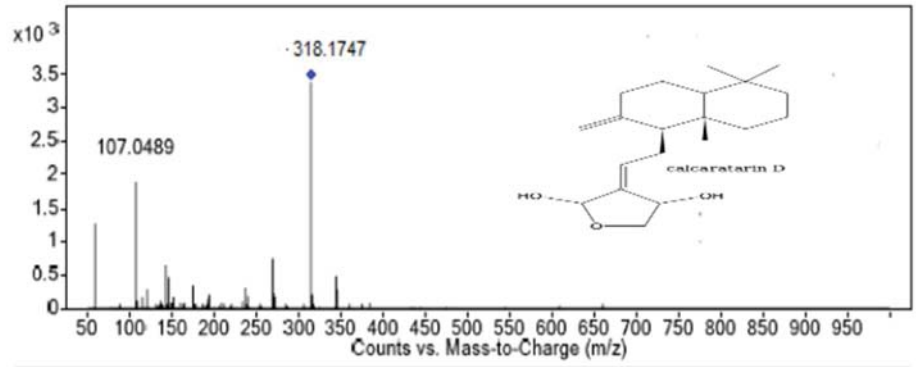


Figure 6.62. Mass spectrum of Calcaratarin D

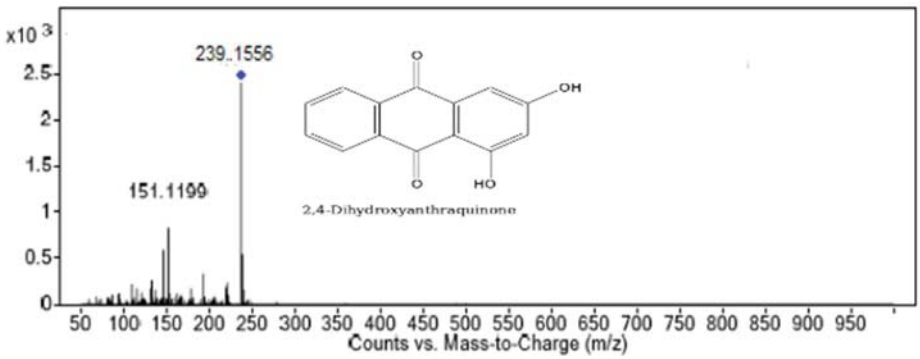


Figure 6.63. Mass spectrum of 2, 4 Dihydroxyanthraquinone

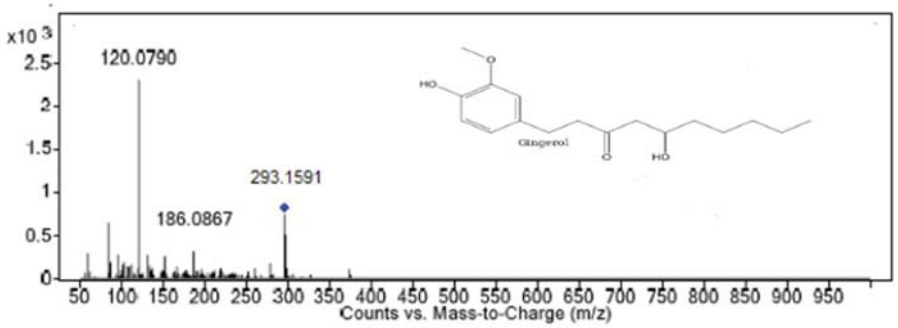


Figure 6.64. Mass spectrum of Gingerol

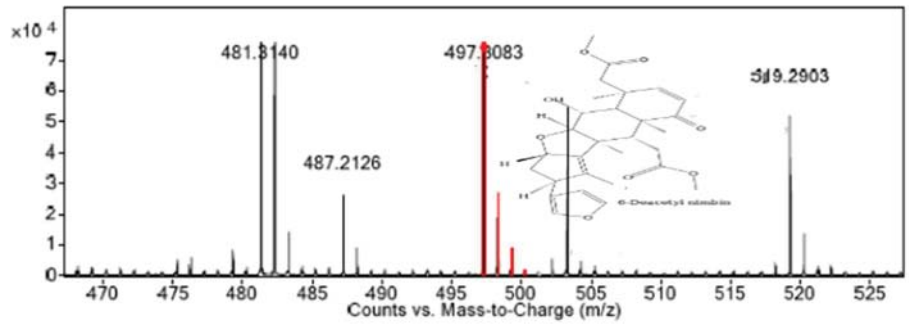


Figure 6.65. Mass spectrum of 6-Deacetylnimbin

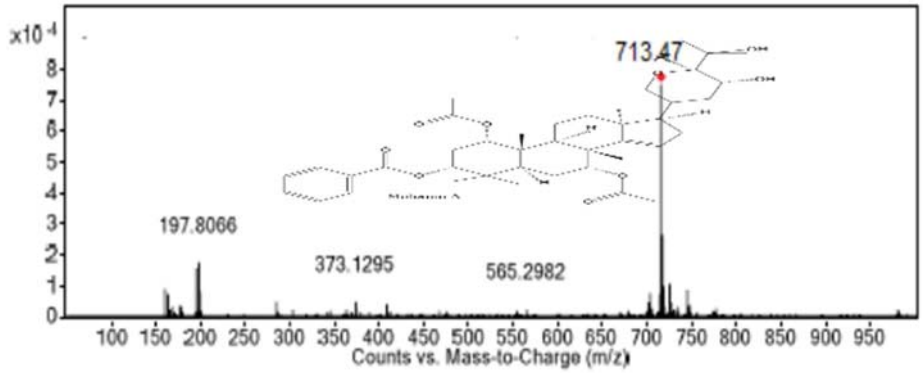


Figure 6.66. Mass spectrum of Melianin A

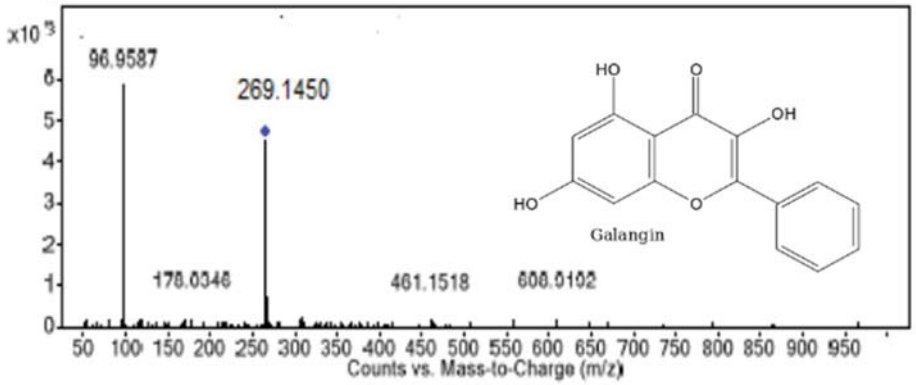


Figure 6.67. Mass spectrum of Galangin

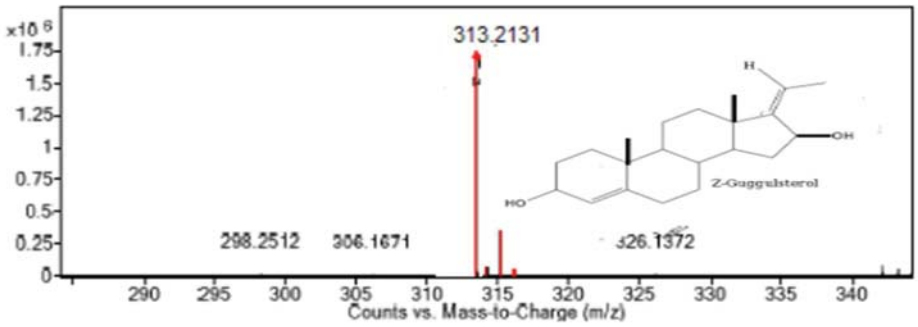


Figure 6.68. Mass spectrum of Z-Guggulsterol

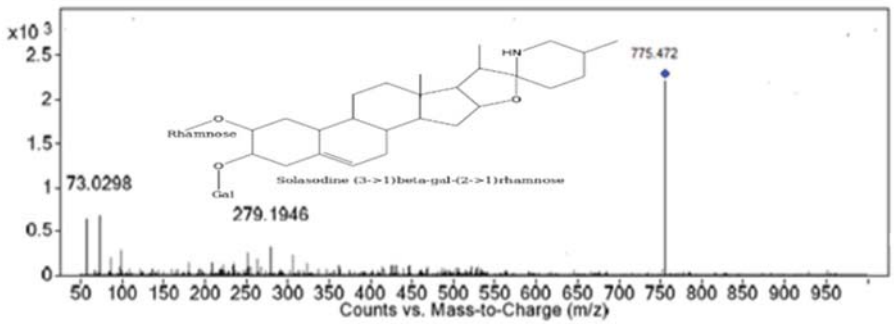


Figure 6.69. Mass spectrum of Solasodine (3->1) β-gal (2->1) rhamnose

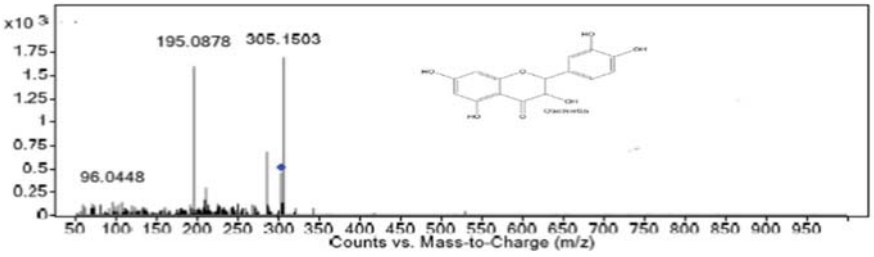


Figure 6.70. Mass spectrum of Quercetin

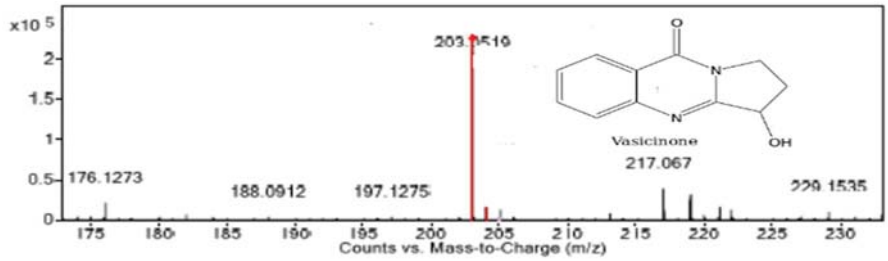


Figure 6.71. Mass spectrum of Vasicinone

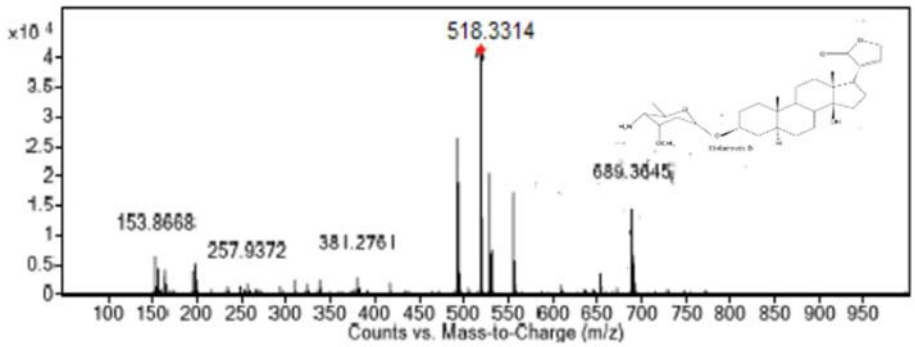


Figure 6.72. Mass spectrum of Holarosin B

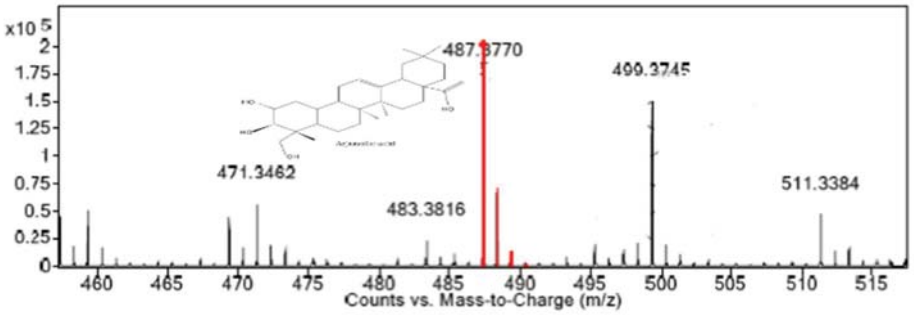


Figure 6.73. Mass spectrum of Arjunolic acid

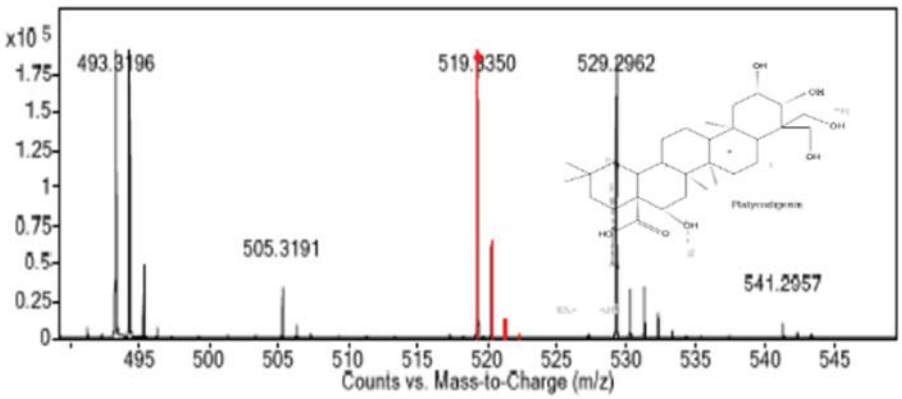


Figure 6.74. Mass spectrum of Platycodigenin

Identified compounds have different pharmacological activity including anti-inflammatory, antioxidant, anticancer etc. On literature survey it has been found that about 65% of the identified compounds exhibit very specific anti-inflammatory activity (see Table 6.2). Remaining compounds may increase the activity of the lead compound, stabilize the compound or help their absorption.

Table 6.2. List of identified compounds having anti -inflammatory activity

SI No.	Compound name	Biological activity	Plant source
1.	20-Hydroxyprogenene-3, 16-dione	Anti-inflammatory [24]	*Commiphora mukul*(Hook.ex Stocks) Engl.
2.	Magnoflorin	Anti-inflammatory [26]	*Celastrus paniculatus* Willd, *Tinospora cordifolia* (Willd) Miers ex Hook.f.&Thoms
3.	16-Alpha -hydroxyprogesterone	Anti-inflammatory activity [27]	*Commiphora mukul* (Hook.ex Stocks) Engl.
4.	Commipherol	Anti-inflammatory activity [27]	*Commiphora mukul* (Hook.ex Stocks) Engl.
5.	Z-Guggulusterol	Anti-inflammatory activity [27]	*Commiphora mukul* (Hook.ex Stocks) Engl.
6.	Guggulsterole III	Anti-inflammatory activity [27]	*Commiphora mukul* (Hook.ex Stocks) Engl.
7.	Isonimolide	Anti-inflammatory activity [28]	*Azadirachta indica* (L.)A.Juss.
8.	Nimbidinin	Anti-inflammatory activity [28]	*Azadirachta indica* (L.)A.Juss.
9.	Ursolic acid	Anti-inflammatory [29]	*Tinospora cordifolia* (Willd) Miers ex Hook.f.&Thoms
10	Oleanolic acid	Anti-inflammatory [30]	*Tinospora cordifolia* (Willd) Miers ex Hook.f.&Thoms
11	Conessine	Anti-inflammatory effect [31]	*Holarrhena antidysenterica*(Roth)DC
12	Fangchinoline	Anti-inflammatory [32]	*Cyclea peltata*(Lam.) Hook.f.&Thoms
13	Berbamine	Anti-inflammatory [33]	*Cyclea peltata*(Lam.) Hook.f.&Thoms

SI No.	Compound name	Biological activity	Plant source
14.	Guggulusterol	Anti-inflammatory activity [34]	*Commiphora mukul*(Hook.ex Stocks) Engl.
15.	Guggulusterone VI	Anti-inflammatory activity [34]	*Commiphora mukul*(Hook.ex Stocks) Engl.
16.	6-Shogaol	Anti-inflammatory [35]	*Zingiber officinale* Rosc.
17.	Transferuloyloctapamine	Anti-oxidant [36]	*Tinospora cordifolia*(Willd) Miers ex Hook.f.&Thoms
18.	Vasicinol	Anti-inflammatory [37]	*Adhatoda vasica* Nees
19	Moupinamide	Anti-inflammatory activity [38]	*Piper longum* L.
20.	7-Deacetylgedunin	Anti-inflammatory agent [39]	*Azadirachta indica*(L.)A.Juss.
21.	Hexahydrocurcumin	Anti-inflammatory activity [40]	*Zingiber officinale* Rosc.
22.	6-Gingerol	Anti-inflammatory [41]	*Zingiber officinale* Rosc.
23.	3-Methylgingerdiol	Anti-inflammatory [41]	*Zingiber officinale* Rosc.
24.	Matairesinol	Anti-inflammatory [42], Immunomodulatory effect,	*Cedrus deodara*(Roxb.ex D.Don)G.Don
25.	Piperine	Anti-inflammatory [43], Immunomodulatory	*Piper longum* L., *Piper nigrum* L.

SI No.	Compound name	Biological activity	Plant source
26.	Marmesin	Anti-inflammatory effects [44]	
27.	Gallic acid	Anti-inflammatory[45]	*Cuminum cyminum* L.
28.	Rutin	Anti-inflammatory [46] immunomodulatory	*Azadirachta indica* (L.)A.Juss.
29.	Angelicine	Anti-inflammatory [47]	*Trachyspermum roxburghianum* (DC.) Wolff
30	Rubiasin C	Anti-inflammation [48]	*Rubia cordifolia* L.
31.	Vasicine	Anti-inflammatory activity [49]	*Adhatoda vasica* Nees
32.	2, 4-Dihydroxyanthraquinone	Anti-inflammatory effects [50]	*Rubia cordifolia* L.
33.	1, 4-Dihydroxyanthraquinone	Anti-inflammatory effects [50]	*Rubia cordifolia* L.
34.	Nordamnacanthal	immunomodulatory effects[51]	*Rubia cordifolia* L.
35.	Purpurin	Anti-inflammatory [50]	*Rubia cordifolia* L.
36.	Coumaperine	Anti-inflammatory effects [52]	*Piper longum* L.
37.	Galangin -3-methyl ether	Anti-inflammatory [53]	*Alpinia calcarata* Roscoe
38.	Galangin	Anti-inflammatory[53]	*Alpinia calcarata* Roscoe
39.	Calcaratarin D	Anti-inflammatory [54]	*Alpinia calcarata* Roscoe
40.	Tetrahydropiperine	Anti-inflammatory[55]	*Piper longum* L.
41.	6-Deacetyl nimbin	Anti-inflammatory[56]	*Azadirachta indica*(L.)A.Juss.

SI No.	Compound name	Biological activity	Plant source
42.	Vasicinone	Anti-inflammatory[57]	*Adhatoda vasica* Nees
43.	Vasicinolone	Anti-inflammatory effects[57]	*Adhatoda vasica* Nees
44.	Nimbandiol	Anti-inflammatory activity [58]	*Azadirachta indica*(L.)A.Juss.
45.	Azadirone	Anti-inflammatory activity[59]	*Azadirachta indica*(L.)A.Juss.
46.	Quercetin	Anti-inflammatory [60], immune system regulation	*Cuminum cyminum* L.
47.	Chinovic acid	anti-inflammatory [61]	
48.	Arjunolic acid	Anti-inflammatory [62]	
49.	Platycodigenin	Anti-inflammatory [63]	
50.	Ecdysterone	Anti-oxidant	*Tinospora cordifolia* (Willd) Miers ex Hook.f.&Thoms
51.	Rubifolic acid	Anti-oxidant	*Rubia cordifolia* L.
52.	Trans feruloylpiperidine	Anti-oxidant	*Piper longum* L.

6.2.3. UPLC-Q-TOF-MS analysis

The HR LCMS analysis with the methanol extract of Gulguluthikthakam kashayam tentatively identified 73 compounds responsible for its medicinal use. The supernatant of the kashayam also has been subjected to UPLC-QTOF analysis. From the supernatant, thirty-four compounds have been identified. Identified compounds contain 8 alkaloids, 12 terpenes and 14 phenolic compounds. They are given in Table 6.3. Out of these twenty-four compounds have been characterized with the mass fragmentation pattern.

The compounds identified through QTOF analysis is the further confirmation of the HRLCMS identification.

Table 6.3. Compound identified with the UPLC-Q-TOF-MS

Sl No.	Retention Time	Compound name	Type of Compound	Molecular formula	IUPAC name of the Compound	Molecular weight	Molecular ion	Fragments
1.	6.148	Piperine	Alkaloid	$C_{17}H_{19}NO_3$	(2*E*,4*E*)-5-(2*H*-1,3-Benzodioxol-5-yl)-1-(piperidin-1-yl)penta-2,4-dien-1-one	285.34	286.1448(100%) $M+H^+$	201.0554(50%) and m/z 173.0600 (1%), 143
2.	5.451	Trans feruloylctopamine	Alkaloid	$C_{18}H_{19}NO_5$	(*E*)-*N*-[2-hydroxy-2-(4-hydroxyphenyl)ethyl]-3-(4-hydroxy-3-methoxyphenyl)prop-2-enamide	329.7	330.3372(100%) $M+H^+$	312.3265(33%), 286.3108(3%)
3.	4.058	Vasicinone	Alkaloid	$C_{11}H_{10}N_2O_2$	(3S)-3-Hydroxy-2, 3-dihydro-1H-pyrrolo [2, 1-b] quinazolin-9-one)	202.21	202.2168 (100%) M^+	185.2058(28%), 142.0722, 158. 1891
4.	4.883	Coumaperine	Alkaloid	$C_{16}H_{19}NO_2$	(2*E*,4*E*)-5-(4-hydroxyphenyl)-1-piperidin-1-ylpenta-2,4-dien-1-one	257.33	258.2788(100%) $M+H^+$	240.2677(38%) and 211.0569(2%)
5.	4.361	Tetrahydropiperine	Alkaloid	$C_{17}H_{23}NO_3$	5-(1,3-benzodioxol-5-yl)-1-piperidin-1-ylpentan-1-one.	289.4	290.2694(100%) $M+H^+$	272.2581
6.	3.499	Vasicinolone	Alkaloid	$C_{11}H_{10}N_2O_3$	3,7-dihydroxy-1H,2H,3H,9H-pyrrolo[2,1-b]quinazolin-9-one	218.21	218.2122 (100%) M^+	200.2018, 174.1859(100%), 156.1761
7.	3.927	Marmesin	Phenolics	$C_{14}H_{14}O4$	(2*S*)-2-(2-hydroxypropan-2-yl)-2, 3-dihydrofuro [3, 2-g] chromen-7-one	246.26	246.2444 (100%) M^+	228.2334,202.2173(1%), 184.2063
8.	3.673	Angelicin	Phenolics	$C_{11}H_6O_3$	Furo[2,3-h]chromen-2-one	186.16	184.1151(9%)$M-2H^+$	143.0861(100%), 133. 079

Sl No.	Retention Time	Compound name	Type of Compound	Molecular formula	IUPAC name of the Compound	Molecular weight	Molecular ion	Fragments
9.	2.143	Rubiasin C	Phenolics	$C_{15}H_{16}O_2$	(4aS,9aR,10S)-10-hydroxy-2-methyl-4,4a,9a,10-tetrahydro-1H-anthracen-9-one	228.29	227.1756(2%) $M\text{-}H^+$	209.1654, 182.1516
10.	5.825	Nordamnacanthal	Phenolics	$C_{15}H_8O_5$	1,3-dihydroxy-9,10-dioxoanthracene-2-carbaldehyde	268.22	267.1598(100%) $M\text{-}H^+$	205.1594 (8%), 249.1495(2%)
11.	6.459	2, 4 – Dihydroxyanthraquinone	Phenolics	$C_{14}H_8O_4$	2,4-Dihydroxyanthracene-9,10-dione	240.21	239.0338(70%) $M\text{-}H^+$	211.0391(100%), 195.0441(80%), 167.0492(10%), 183.0436(2%), 155.0490(3%)
12.	4.501	1, 4-Dihydroxy anthraquinone	Phenolics	$C_{14}H_8O$	1,4-Dihydroxyanthracene-9,10-dione	240.21	241.1069 (2%) $M\text{+}H^+$	181.0864 (100%), 223.0971 (15%), 195.1018 (2%),
13.	4.874	6-Gingerol	Phenolics	$C_{17}H_{26}O_4$	(5S)-5-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)decan-3-one	294.4	293.1755(100%) $M\text{-}H^+$	193.1592(72%), 136.0897(2%).
14.	8.114	Galangin -3-methyl ether	Phenolics	$C_{16}H_{12}O_5$	5,7-dihydroxy-3-methoxy-2-phenylchromen-4-one	283.37	284.2974(75%) $M\text{+}H^+$	242. 2491
15.	4.309	3-Methylgingerdiol	Phenolics	$C_{18}H_{30}O_4$	(E)-1-(3,4-dimethoxyphenyl)dec-3,5-diol	310.43	309.1704(100%) $M\text{-}H^+$	291.1613(1%), 209.1535(15%).

Sl No.	Retention Time	Compound name	Type of Compound	Molecular formula	IUPAC name of the Compound	Molecular weight	Molecular ion	Fragments
16.	4.543	Matairesinol	Phenolics	$C_{20}H_{22}O_6$	(3*R*,4*R*)-3,4-bis[(4-hydroxy-3-methoxyphenyl)methyl]oxolan-2-one	358.38	357.1345 (100%) $M\text{-}H^+$	342.1104(30%), 313.1444, 298.1215(2%), 221.0816(20%), 147.0433 (2%)
17.	5.388	Galangin	Phenolics	$C_{15}H_{10}O_5$	3,5,7-trihydroxy-2-phenylchromen-4-one	270.24	269.0450(46%) $M\text{-}H^+$	117.0338(100%), 241.0488, 107.0131, 151.0026(48%)
18.	7.218	Purpurin	Phenolics	$C_{14}H_8O_5$	1,2,4-trihydroxyanthracene-9,10-dione	256.21	256.2655(100%) M^+	238.2529, 200.2024, 172.1703
19.	4.266	Hexahydrocurcumin	Phenolics	$C_{21}H_{26}O_6$	(RS)-5-Hydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)-3-heptanone	374.43	373.1258(100%) $M\text{-}H^+$	358.1055 (15%), 312.1007(5%)
20.	4.509	Rutin	Phenolics	$C_{27}H_{30}O_{16}$	2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-[[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one	610.517	609.3403(38%) $M\text{-}H^+$	387.1806(3%), 237.1079(15%), 221.1536(56%)
21.	4.815	Nimbionone	Terpenes	$C_{18}H_{22}O_4$	(4*aS*,10*aR*)-6-hydroxy-7-methoxy-1,1,4*a*-trimethyl-3,4,10,10*a*-tetrahydrophenanthrene-2,9-dione	302.4	302.3058(100%) M^+	284.2951(21%), 137.0101 and 106.0861(18%)
22.	4.415	Calcaratarin D	Terpenes	$C_{20}H_{30}O_3$	(3Z,4S)-3-[2[(1S,4aS,8aS)-5.5.8a-trimethyl-2-methylidene-3,4,4a,6,7,8-hexahydro-1H-naphthalene-1-yl]ethylidene]-4-hydroxyoxolan-2-one	318.4	318.3016(100%) M^+	300.2919(2%), 256.2654(38%)

SI No.	Retention Time	Compound name	Type of Compound	Molecular formula	IUPAC name of the Compound	Molecular weight	Molecular ion	Fragments
23.	6.633	Guggulusterone	Terpenes	$C_{21}H_{28}O_2$	(8*R*,9*S*,10*R*,13*S*,14*S*,17*E*)-17-ethylidene-10,13-dimethyl-1,2,6,7,8,9,11,12,14,15-decahydrocyclopenta[a]phenanthrene-3,16-dione	312.453	313.2177 (75%) $M+H^+$	109.0655 (89%), 189.1278, 297.2213(15%).
24.	5.224	Guggulusterone VI	Terpenes	$C_{21}H_{32}O_2$	16-alpha hydroxy pregna 4-en-3-one	316.48	317.1736(76%) $M+H^+$	261.1097(100%)
25.	7.503	Odaratone	Terpenes	$C_{30}H_{48}O_4$	23 R,24 S-dihydroxy-22 S,25-epoxytirucall-7-en-3-one	472.3346	471.3466 (100%) $M-H^+$	341.2850(28%), 137.0966(50%), 313.2539(5%), 176.0829(10%)
26.	7.791	Rubifolic acid	Terpenes	$C_{30}H_{48}O_4$	10-hydroxy-2-(hydroxymethyl)-1,6a,6b,9,9,12a-hexamethyl-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a-carboxylic acid	472.70	471.3478 (100%) $M-H^+$	411.3272(21%), 207.1378(41%), 219.1386(21%)
27.	6.434	Z-Guggulsterol	Terpenes	$C_{21}H_{30}O_2$	16-beta hydroxy-4,17(20)-Z-pregnadiene-3-one	314.46	313.2171(100%)$M-H^+$	109.0655(72%), 217.1600(12%)
28.	7.089	Arjunolic acid	Terpenes	$C_{30}H_{48}O_5$	(4*aS*,6*aR*,6*aS*,6*bR*,8*aR*,9*R*,10*R*,11*R*,12*aR*,14*bS*)-10,11-dihydroxy-9-(hydroxymethyl)-2,2,6*a*,6*b*,9,12*a*-hexamethyl-1,3,4,5,6,6*a*,7,8,8*a*,10,11,12,13,14*b*-tetradecahydropicene-4*a*-carboxylic acid	488.70	487.3441(35%)$M-H^+$	469.3322, (34%)425.3471(8%), 207.1379(48%)

Sl No.	Retention Time	Compound name	Type of Compound	Molecular formula	IUPAC name of the Compound	Molecular weight	Molecular ion	Fragments
29.	6.955	Platycodigenin	Terpenes	$C_{30}H_{48}O_7$	(4*aR*,5*R*,6*aR*,6*aS*,6*bR*,8*aR*,10*R*,11*S*,12*aR*,14*bS*)-5,10,11-trihydroxy-9,9-bis(hydroxymethyl)-2,2,6*a*,6*b*,12*a*-pentamethyl-1,3,4,5,6,6*a*,7,8,8*a*,10,11,12,13,14*b*-tetradecahydropicene-4*a*-carboxylic acid	520.70	519.3701(100%) $M\text{-}H^+$	487.3437(10%), 425.3436(15%)
30.	3.048	Solasodine (3->1) β-gal (2->1) rhamnose	Alkaloids	$C_{38}H_{60}NO_{12}$	(2S,2′R,4aR,4bS,5′R,6aS,6bR,7S,9aS,10aS,10bS)-4a,5′,6a,7-tetramethyl-1,2,3,4,4a,4b,5,6,6a,6b,7,9a,10,10a,10b,11-hexadecahydrospiro[naphtho[2′,1′:4,5]indeno[2,1-b]furan-8,2′-piperidin]-2-ol)(3->1)β-gal-(2->1)rhamnose	722.88	723.5018(100%) $M\text{+}H^+$	677.4964(48%), 151.6474, 241.2106, 405.6148, 451.3324, 511.6498
31.	2.768	6-Deacetylnimbin	Terpenes	$C_{28}H_{34}O_8$	methyl (1*S*,2*R*,3*R*,4*R*,8*R*,9*S*,10*R*,13*R*,15*R*)-13-(furan-3-yl)-2-hydroxy-9-(2-methoxy-2-oxoethyl)-4,8,10,12-tetramethyl-7-oxo-16-oxatetracyclo[8.6.0.0^{3,8}.0^{11,15}]hexadeca-5,11-diene-4-carboxylate	498.6	497.3345(48%) $M\text{-}H^+$	451.3297(100%), 225.1524
32.	7.358	Chinovic acid	Terpenes	$C_{30}H_{46}O_5$	(1*S*,2*R*,4*aS*,6*aR*,6*aR*,6*bR*,8*aR*,10*S*,12*aR*,14*bS*)-10-hydroxy-1,2,6*b*,9,9,12*a*-hexamethyl-2,3,4,5,6,6*a*,7,8,8*a*,10,11,12,13,14*b*-tetradecahydro-1*H*-picene-4*a*,6*a*-dicarboxylic acid	486.68	485.3281(22%)$M\text{-}H^+$	261.1497(32%), 441.3414(2%), 327.2686(3%), 279.1587(3%)
33.	4.484	Trans-feruloyl piperidine	Alkaloids	$C_{15}H_{19}NO_3$	(E)-3-(4-hydroxy-3-methoxyphenyl)-N-piperidin-1-ylprop-2-enamide.	261.32	262.1440(100%) $M\text{+}H^+$	247.1190(8%), 206.0798(3%), 161.0956(8%)
34	2.977	Melianin A	Terpenes	$C_{41}H_{58}O_9$	[(1S,3R,5S,7R,8R,9R,10S,13S,17S)-1,7-diacetyloxy-17-[(3S,5R,6R)-5-hydroxy-6-(2-hydroxypropan-2-yl)oxan-3-yl]-4,4,8,10,13-pentamethyl-2,3,5,6,7,9,11,12,16,17-decahydro-1H-cyclopenta[a]phenanthren-3-yl] benzoate	713.4	713.4714(100%) M^+	677.4968(8%), 143.1472, 223.0881, 335.1784, 452.3284, 530.9963

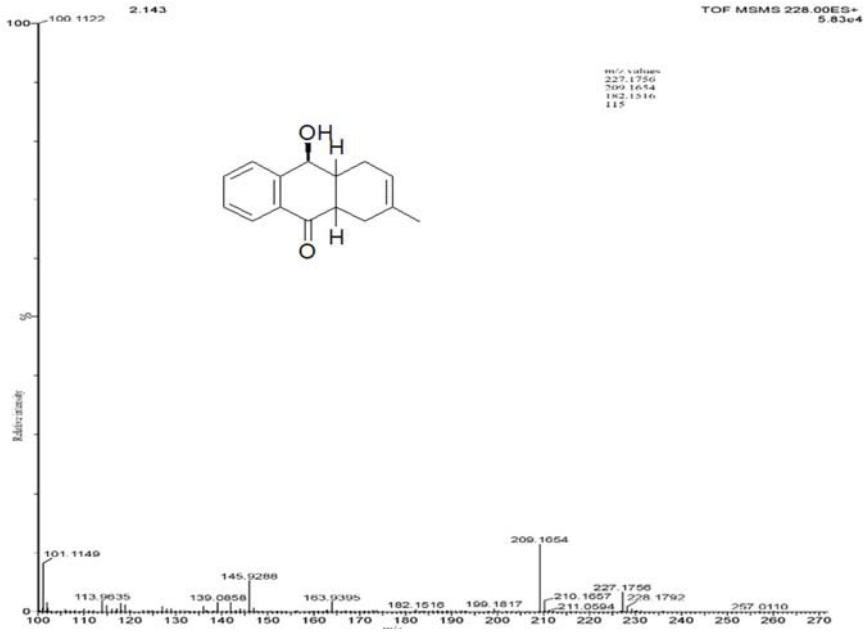
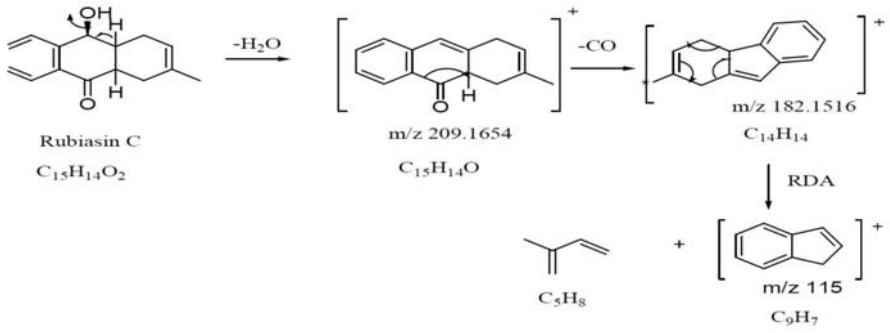


Figure 6.75. UPLC-ESI-QTOF- Mass spectrum of Rubiasin C



Scheme 6.1: Mass fragments of Rubiasin C

The compound 1 with retention time 2.143 produces a precursor ion at m/z 227.1756(2%) and characteristic fragments at m/z 209.1654(11%), m/z 182. 1516 and 115.The fragment at m/z 209.1654 formed by the loss of water molecules from the ring B. The peak at m/z 182 is due to CO elimination with ring contraction. The secondary ion at m/z 182 by an RDA reaction produces the product ion at m/z 115 and

substituted alkene indicate the presence of cyclohexene moiety. From the mass fragmentation it is identified as Rubiasin C, (4aS, 9aR, 10S)-10-hydroxy-2-methyl-4, 4a, 9a, 10-tetrahydro-1H-anthracen-9-one with the molecular mass $C_{15}H_{16}O_2$.It is the compound reported as a major constituent of *Rubia cordifolia* L.

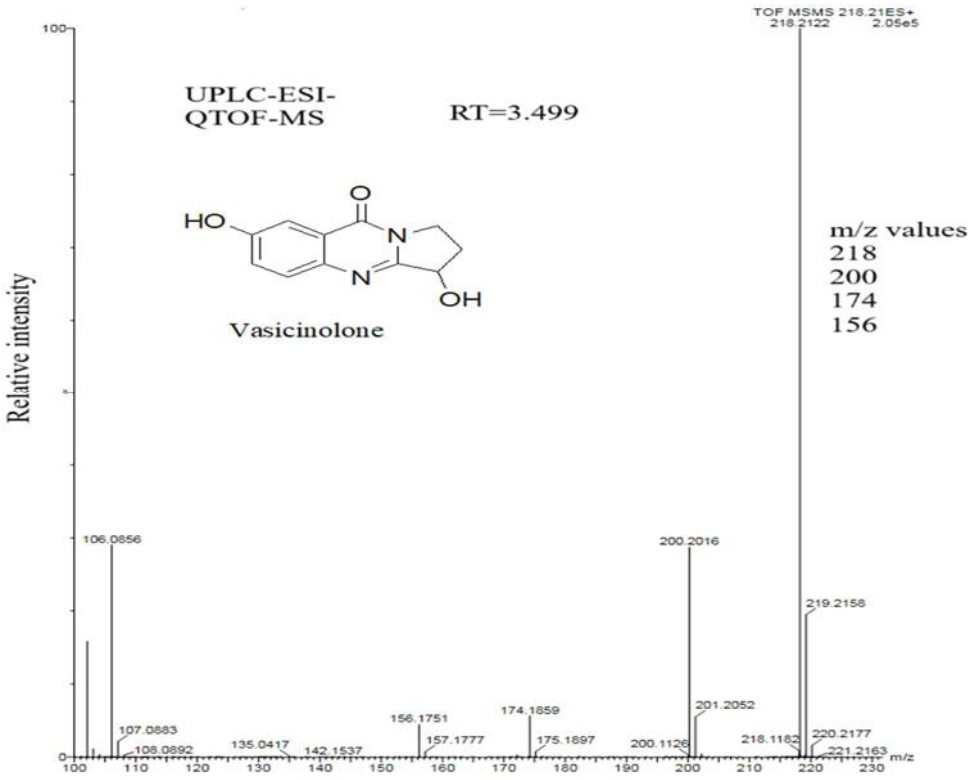
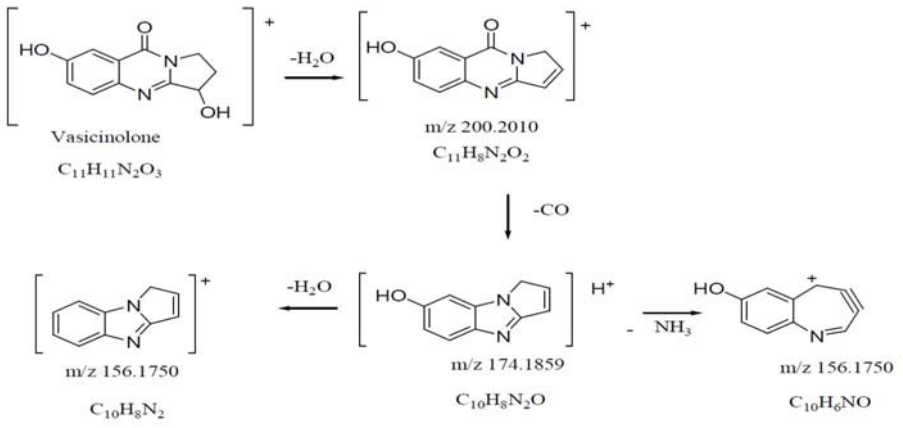


Figure 6.76. UPLC-ESI-QTOF- Mass spectrum of Vasicinolone



Scheme 6.2. Mass fragments of Vasicinolone

The compound 2 with retention time 3.499 forms a molecular ion peak at m/z 218.2122 (100%). Important fragment formed at m/z 200.2018 by the elimination of water molecules from the protonated molecule. The product ion at m/z 174.1859 formed by the CO elimination with ring contraction from the fragment at m/z 200.2018. The elimination of NH_3 from this produces the ion with m/z 156. 1751.The fragmentation pattern and the elemental analysis of the molecular formula of compound 2 was found to be $C_{11}H_{10}N_2O_3$. This compound is identified as Vasicinolone. Vasicinolone, (3S)3, 7-dihydroxy-2, 3-dihydro-1*H*-pyrrolo [2, 1-b] quinazolin-9-one, is the constituent of *Adhatoda vasica* Nees. UPLC QTOF analysis validated the presence of Vasicinolone and confirmed the identification by HR LCMS.

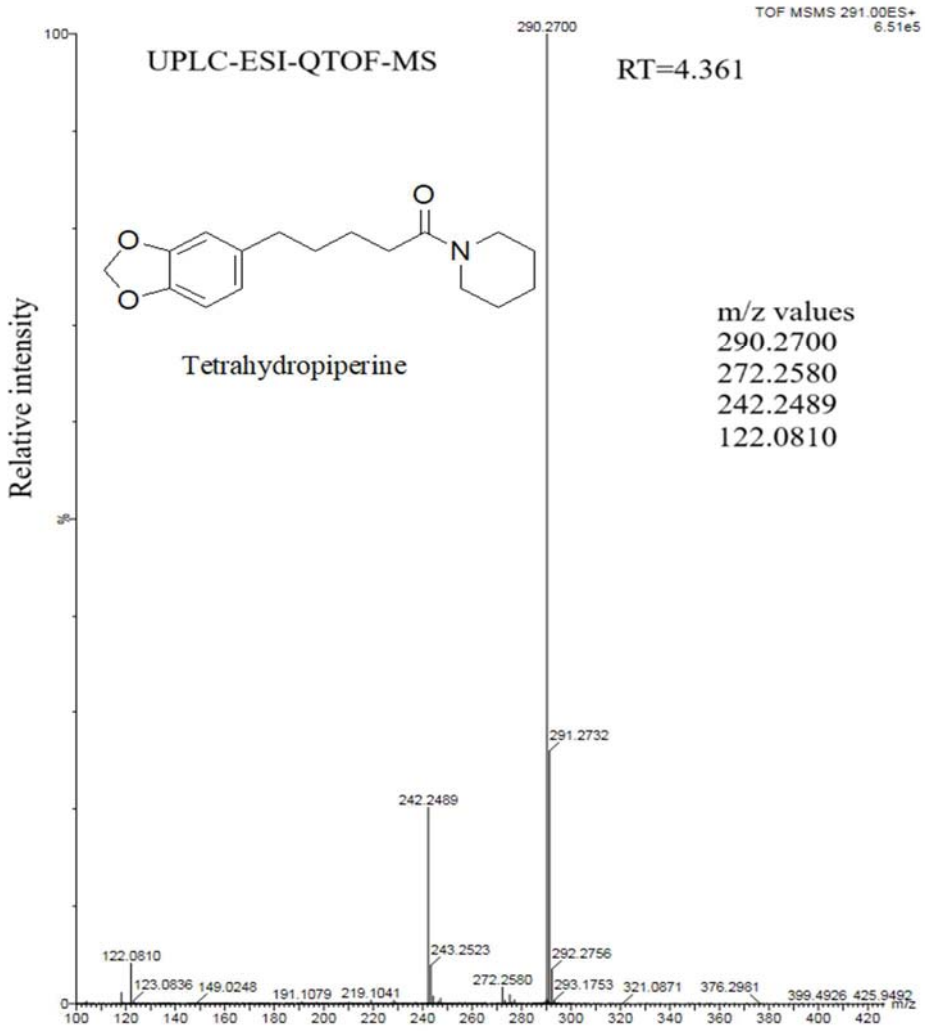
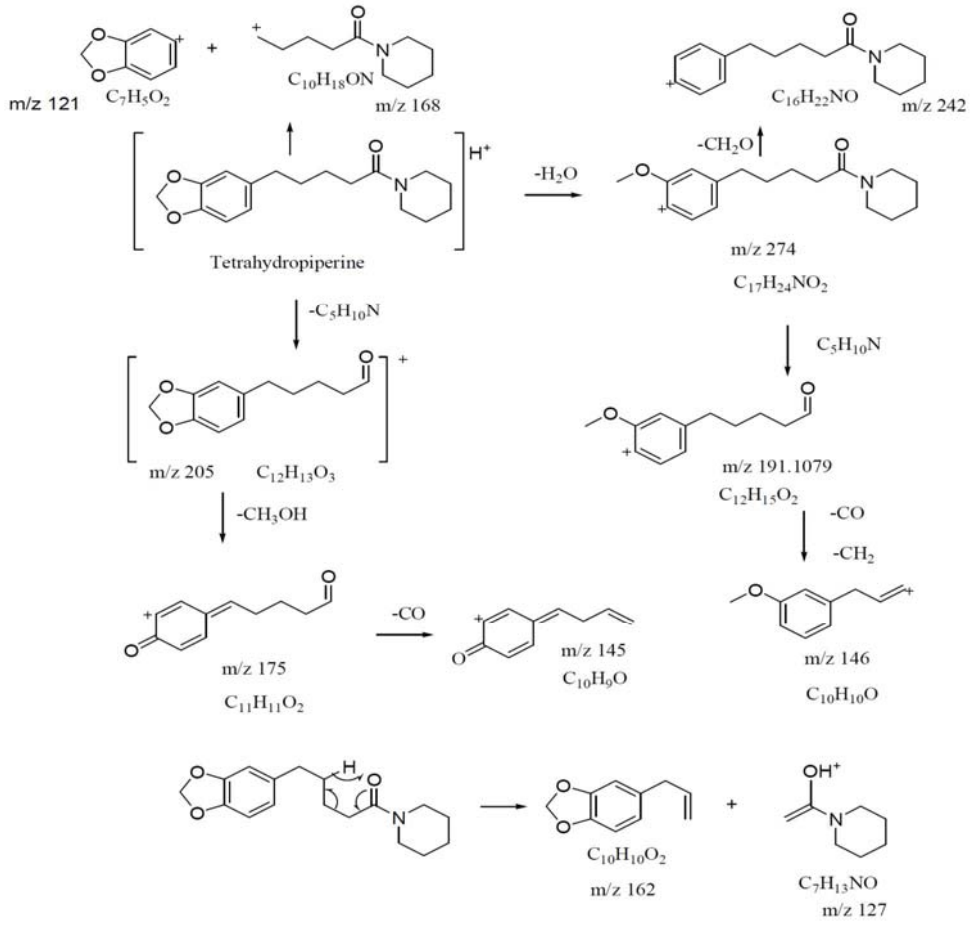


Figure 6.77. UPLC-ESI-QTOF- Mass spectrum of Tetrahydropiperine



Scheme 6.3. Mass fragments of Tetrahydropiperine

The compound 3 with retention time 4.361 forms a molecular ion peak at m/z 290.2694(100%). The peak at m/z 274 formed by the removal of water molecule from the protonated molecular ion. The cleavage of the carboxy amide moiety produces the fragment with mass m/z 205. The loss of methanol from the ion at m/z 205 followed by the loss of CO produces the peak at m/z 145. The elimination of $C_5H_{10}N$ molecule from the mass peak at m//z 274 produces a peak at m/z 191.The peak at m/z 242 is formed by the loss of CH_2O from the ion at m/z 274. Mc-Lafferty rearrangement produces the peak at m/z 162 and 127 as shown in the

scheme. From the elemental analysis and the mass fragmentation the compound 4 was found to be Tetrahydropiperine, 5-(1, 3-benzodioxol-5-yl)-1-piperidin-1-ylpentan-1-one, with the molecular formula $C_{17}H_{23}NO_3$[66]. It is an alkaloid found in Piper species.

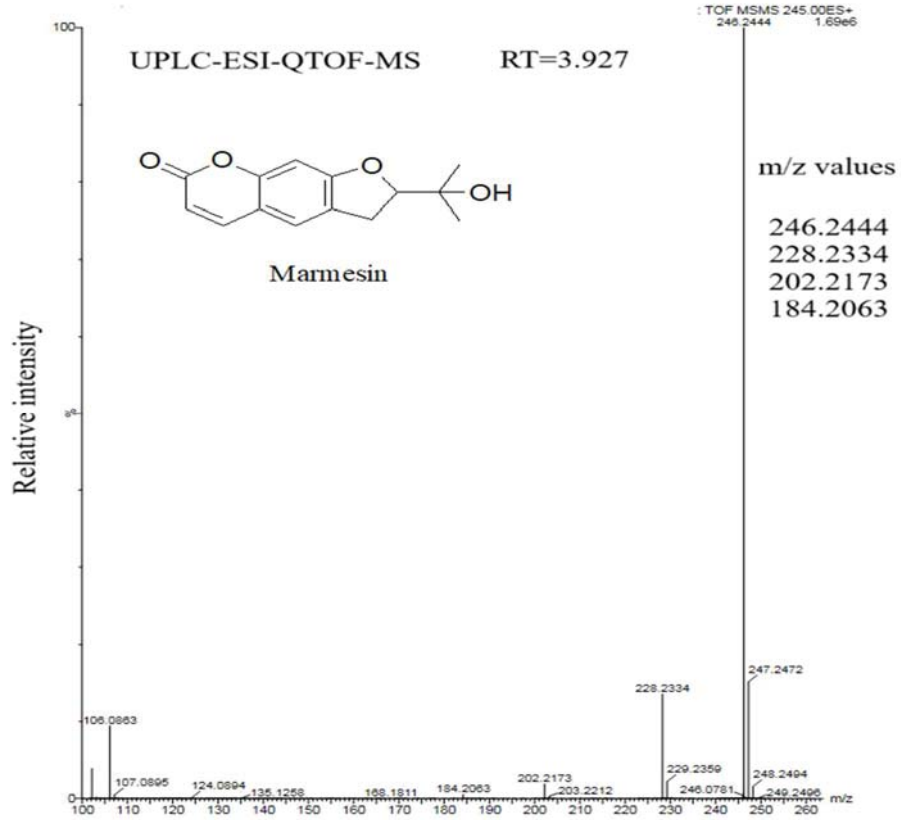
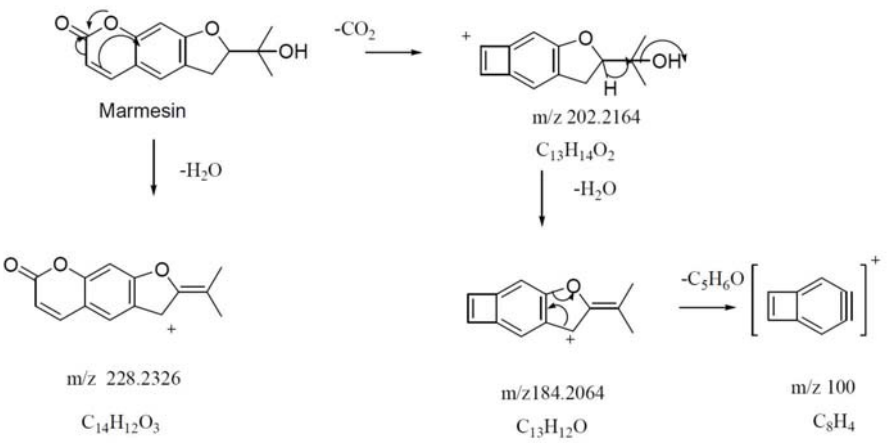


Figure 6.78. UPLC-ESI-QTOF- Mass spectrum of Marmesin



Scheme 6.4. Mass fragments of Marmesin

The compound 4 with retention time 3.927 forms a molecular ion peak at m/z 246.2444 (100%). Characteristic fragments formed at m/z 202.2173(1%) and 184.2063. The molecular ion losses a carbon dioxide moiety to form the product ion at m/z 202.2164 which subsequently loses a molecule of water to give the ion at m/z 184. The CO_2 loss is a characteristic reaction of benzopyrene-2-one derivatives in ESI spectra. This ion undergoes a reaction to fragment a 2-Methylbut-1-ene-one moiety to get an ion at m/z 100. The mass peak at 228 is formed by the loss of a water molecule from the molecular ion peak. The elemental analysis and the reactions given above confirmed this compound as Marmesin, (2*S*)-2-(2-hydroxypropan-2-yl)-2, 3-dihydrofuro [3, 2-g] chromen-7-one, with the molecular formula $C_{14}H_{14}O_4$. It is the additional confirmation of HRMS identification of Marmesin.

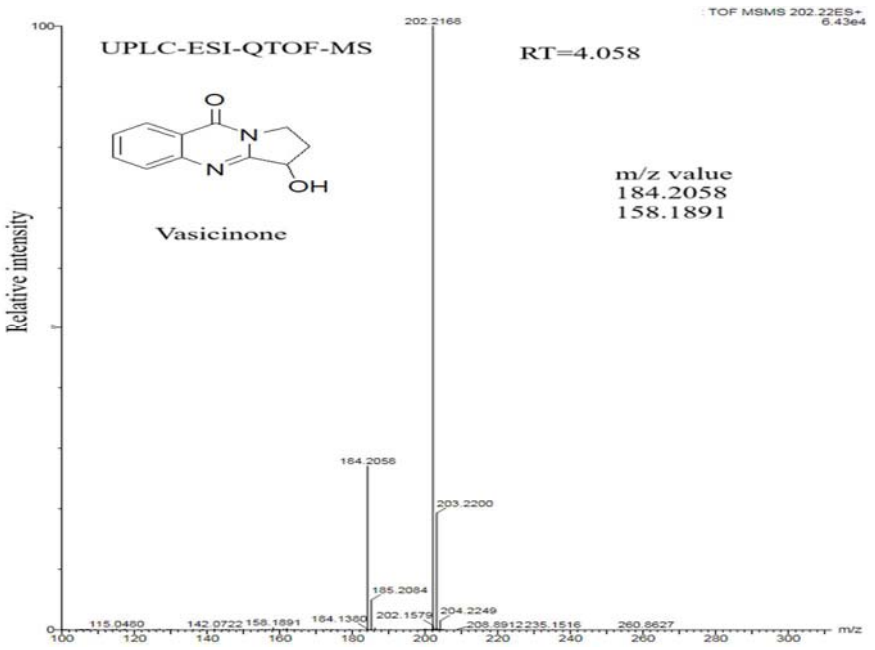
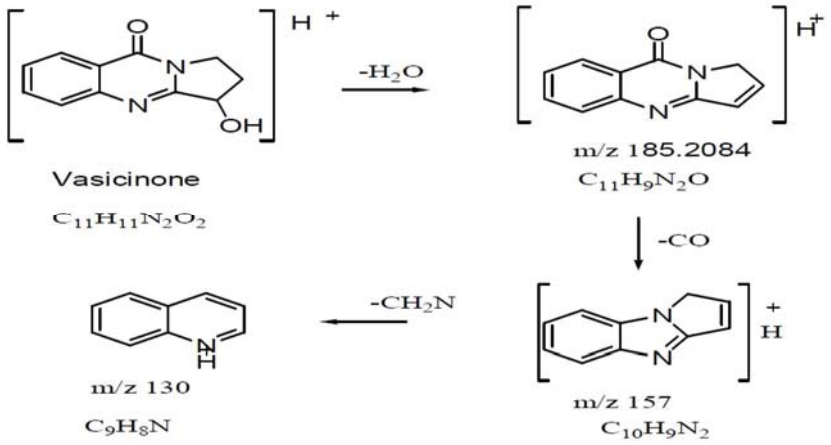


Figure 6.79. UPLC-ESI-QTOF- Mass spectrum of Vasicinone



Scheme 6.5. Mass fragments of Vasicinone

The compound 5 (retention time 4.058) forms a molecular ion peak at m/z 202.2168 (100%) in the positive ionization mode. The fragment ions formed at m/z 185.2084(28%), 140.0722 and 158. 1891. The mass peak at m/z 185.2084 formed by the loss of water molecules due to hydroxyl group at C-3. This moiety has extensive resonance stabilization with other rings. The product ions formed at m/z 158. 1891 and m/z 130.0722 by the successive loss of CO with ring contraction and CH_2N molecule respectively. The mass fragmentation patterns confirmed the compound 6 as Vasicinone, (1*S*)-1-Hydroxy-2, 3-dihydropyrrolo [2, 1-*b*] quinazolin-5(1*H*)-one. Vasicinone (3.5%), $C_{11}H_{10}N_2O_2$ has been reported in the plant *Adhatoda vasica* Nees . Tentatively identified Vasicinone through HRLCMS is validated through UPLC QTOF.

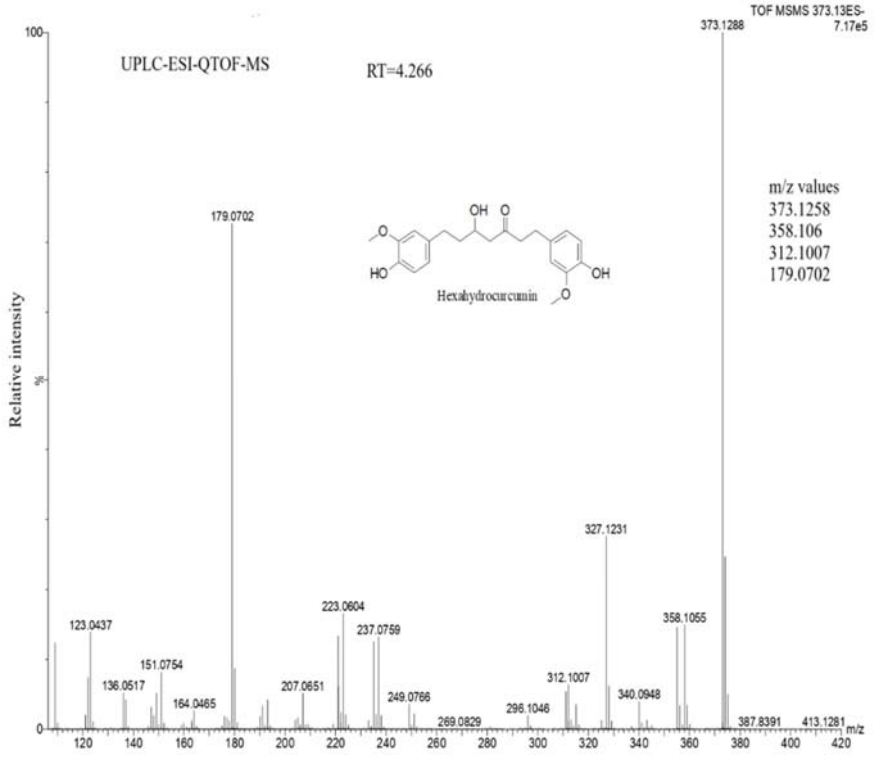
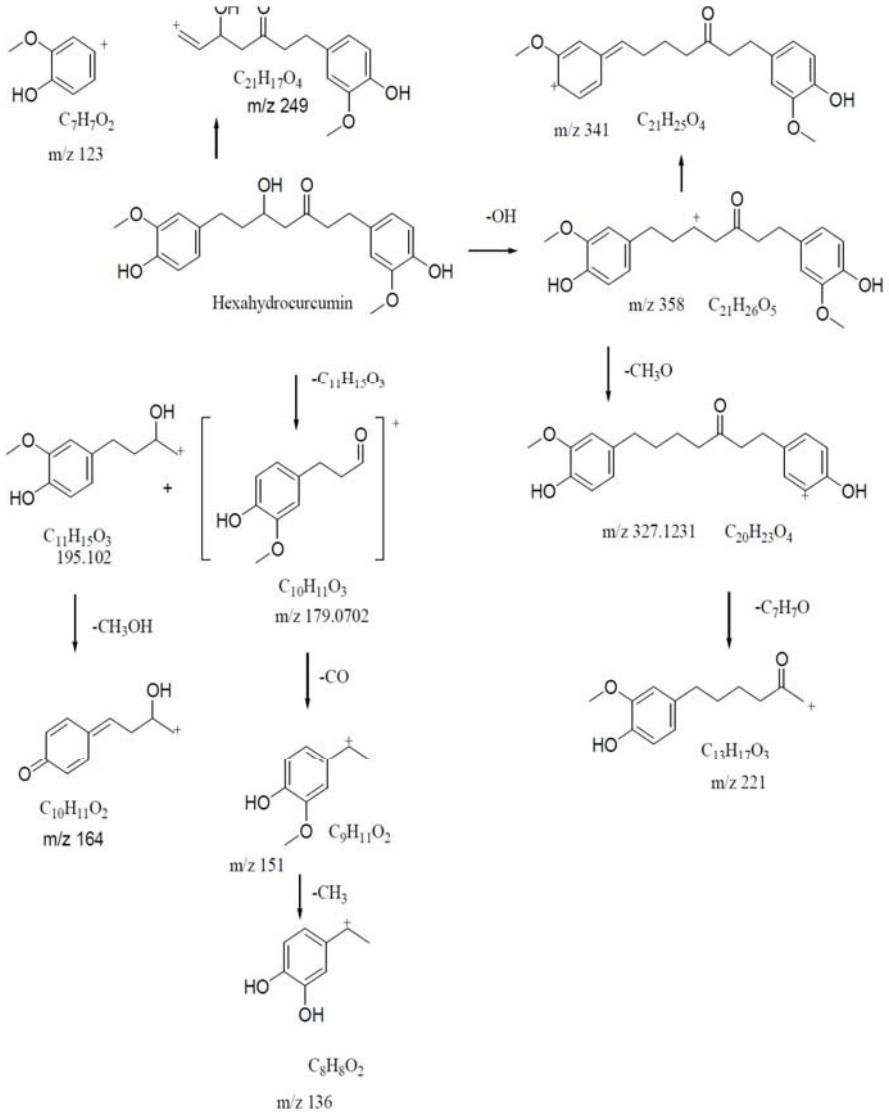


Figure 6.80. UPLC-ESI-QTOF- Mass spectrum of Hexahydrocurcumin



Scheme 6.6. Mass fragments of Hexahydrocurcumin

Compound 6 with retention time 4.266 forms a molecular ion, M-H^+ at m/z 373.1258(100%). The mass fragment at m/z 358(15%), is due to the removal of OH from the molecular ion. Further elimination of CH_3O produces the fragment 327.1231(5%). The fragment at m/z 221 was produced from 327 by breaking of C1-C2 bond. The major fragment at m/z 179.0702 (72%) and the peak at m/z 195 were formed from the precursor ion with the cleavage of C3-C4 bond. The peak at m/z 163 formed from the mass peak at m/z 195 with the loss of methanol. The mass peak at m/z 151 was formed by the loss of CO molecule from the mass peak at m/z 179.Elemental analysis and the fragmentation given below ascertain the compound as Hexahydrocurcumin, 5-hydroxy-1, 7-bis(4-hydroxy-3-methoxyphenyl) heptan-3-one, with the molecular formula $C_{21}H_{26}O_6$[67]. It is the compound present in the *Curcuma longa* L. and *Zingiber officinale* Rosc. It is the validation of the HRLCMS identification of Hexahydrocurcumin.

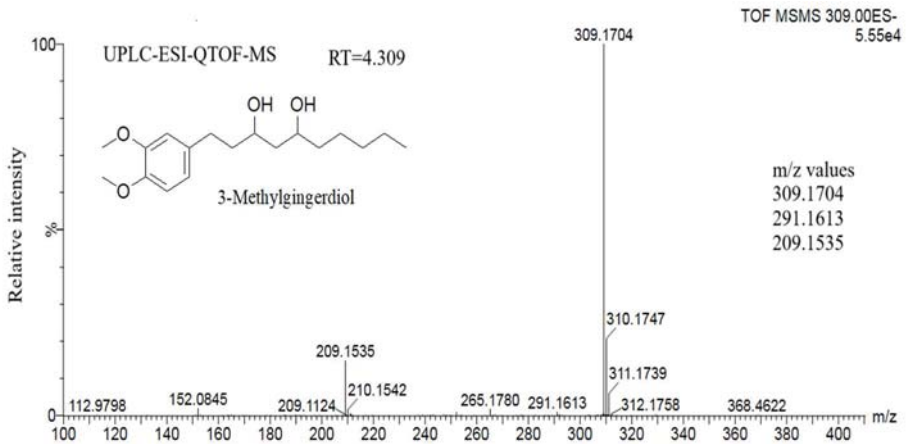
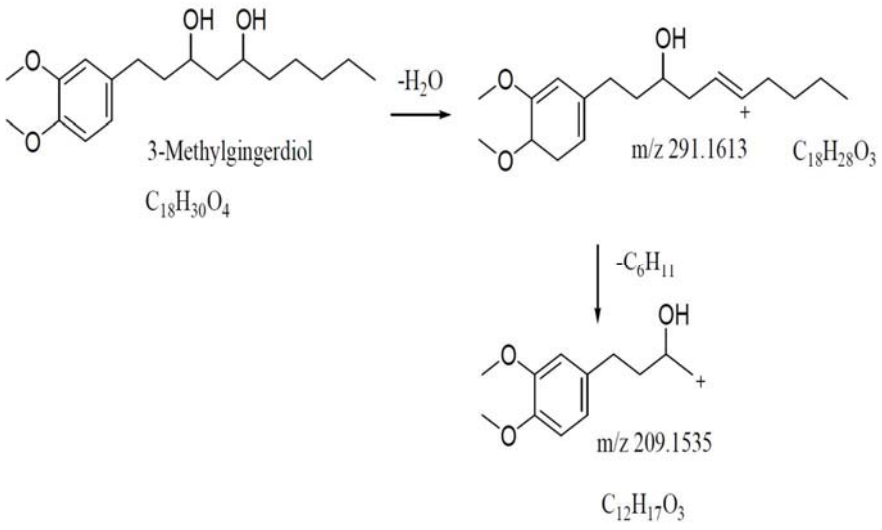


Figure 6.81. UPLC- ESI-QTOF- Mass spectrum of 3-Methyl Gingerdiol



Scheme 6.7. Mass fragments of 3-Methygingerdiol

The compound 7 with retention time 4.309 produce a molecular ion at m/z 309.1704(100%) in ESI spectrum. There are two weak peaks at m/z 291.1613(1%) and 209.1535(15%). The peak at m/z 291.1613 formed by the loss of water molecule from the molecular ion. The peak at m/z 209.1535 explained as result of the cleavage of C4-C5 bond. This compound is identified as 3-Methylgingerdiol with the molecular mass $C_{18}H_{30}O_4$.It is the constituent of *Zingiber officinale* Rosc.

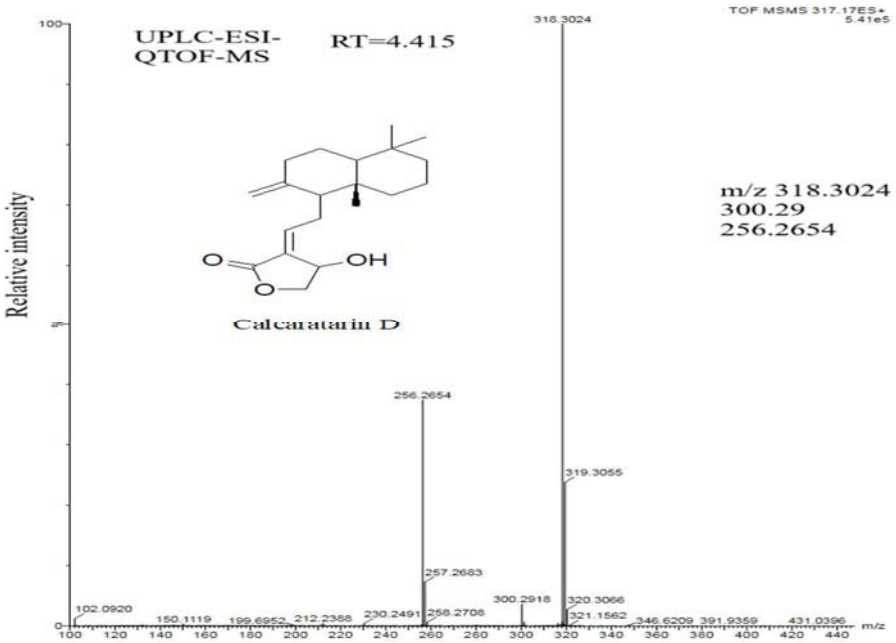
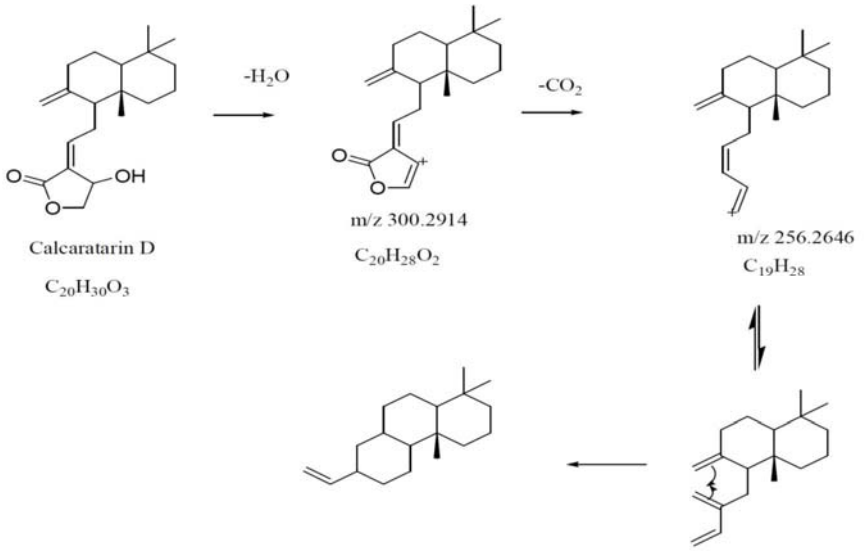


Figure 6.82. UPLC-ESI-QTOF- Mass spectrum of Calcaratarin D



Scheme 6.8. Mass fragments of Calcaratarin D

Compound 8 with the retention time 4.415 produces a molecular ion peak at m/z 318.3016(100%). The product ion peak at m/z 300.2914(2%) and 256.2646(38%). The ion peak at m/z 300.2914 formed by the elimination of water molecule on the lactone ring and subsequent loss of CO_2 which leads to the opening of the ring to form an ion at m/z 256. 2646.This may cyclize to a perhydro phenanthrene ring system substituted at 3- and 12 position. The elemental analysis and the fragmentation pattern observed confirmed the compound as Calcaratarin D, (3*Z*, 4*S*)-3-[2-[(1*S*, 4*aS*, 8*aS*)-5, 5, 8*a*-trimethyl-2-methylidene-3, 4, 4*a*, 6, 7, 8-hexahydro-1*H*-naphthalen-1-yl]ethylidene]-4-hydroxy oxolan-2-one. It is a terpenoid identified in the plant *Alpinia calcarata* Roscoe.

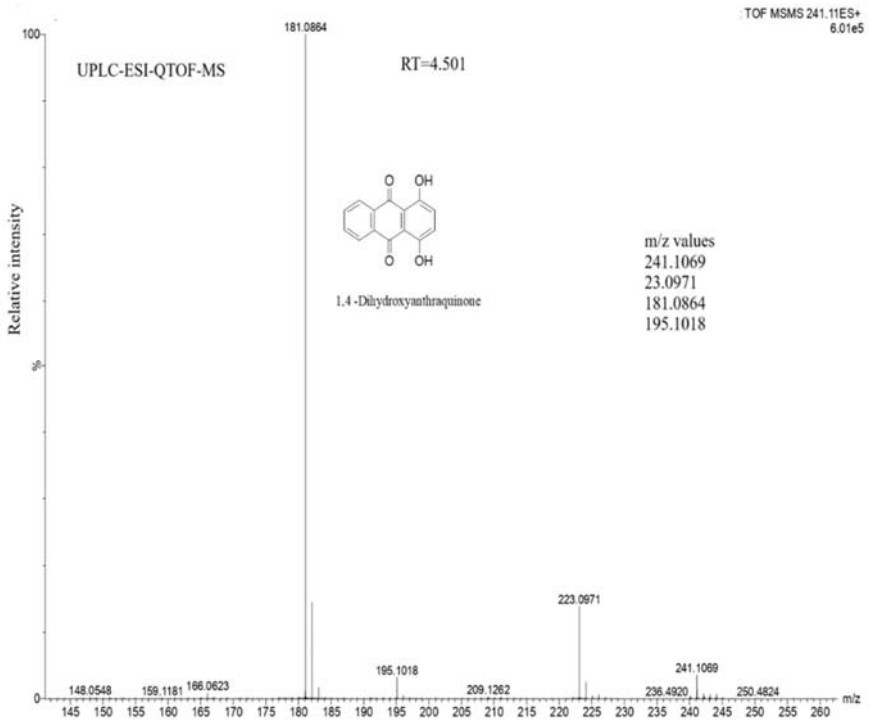
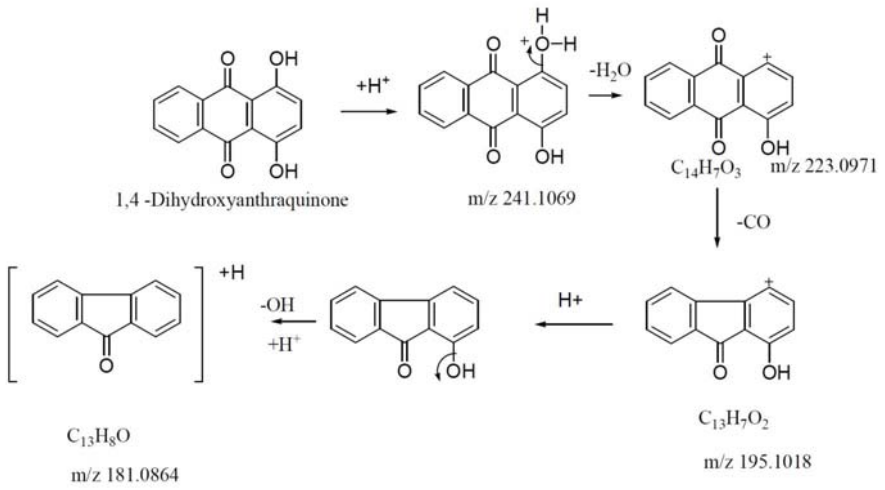


Figure 6.83. UPLC-ESI-QTOF- Mass spectrum of 1, 4-Dihydroxy anthraquinone.



Scheme 6.9. Mass fragments of 1, 4-Dihydroxy anthraquinone

Compound 9 with retention time 4.501 form a molecular ion, $M+H^+$ peak at m/z 241.1069 (2%) and base peak at m/z 181.0864 (100%). Other fragment peaks are at m/z 223.0971 (15%) and 195.1018 (2%). The peak at m/z 223.0971 formed by the loss of water molecule from the protonated molecule. This fragment will undergo a second molecule of water removal by protonation to form Fluorene -9- one(- Fluorenone) a stable molecule that forms the base peak at 181.0864.From the elemental analysis and the fragmentation pattern the compound was found to be 1, 4-Dihydroxy anthraquinone (1,4 Dihydroxyanthracene-9,10-dione) with the molecular formula $C_{14}H_8O_.$ It is the compound present in *Rubia cordifolia* L. Identification of 1, 4 –Dihydroxy anthraquinone with UPLC QTOF is the confirmation with HRLCMS.

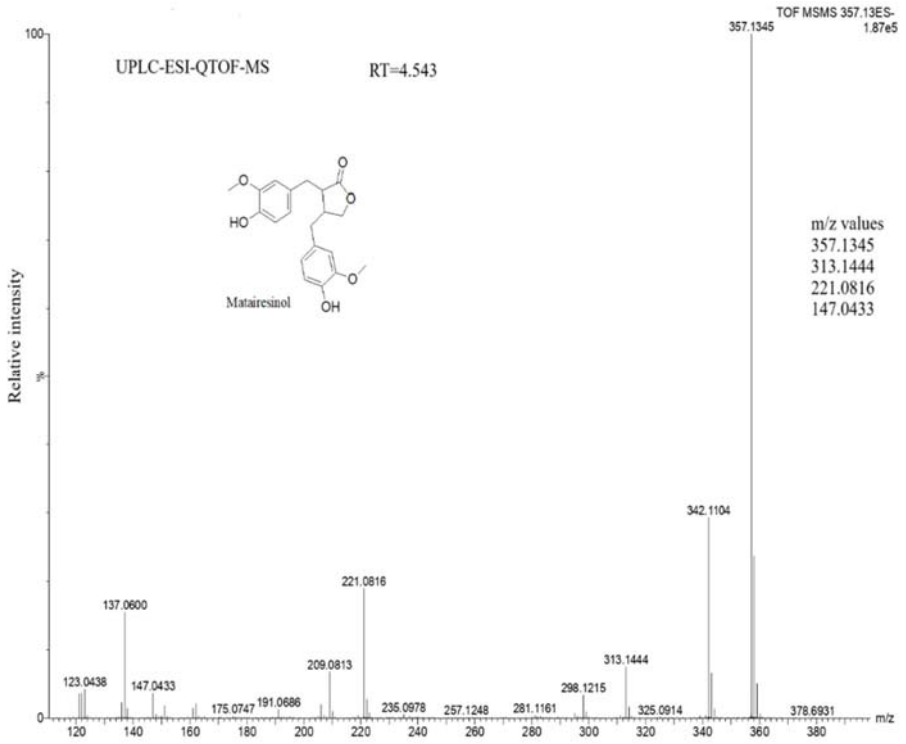
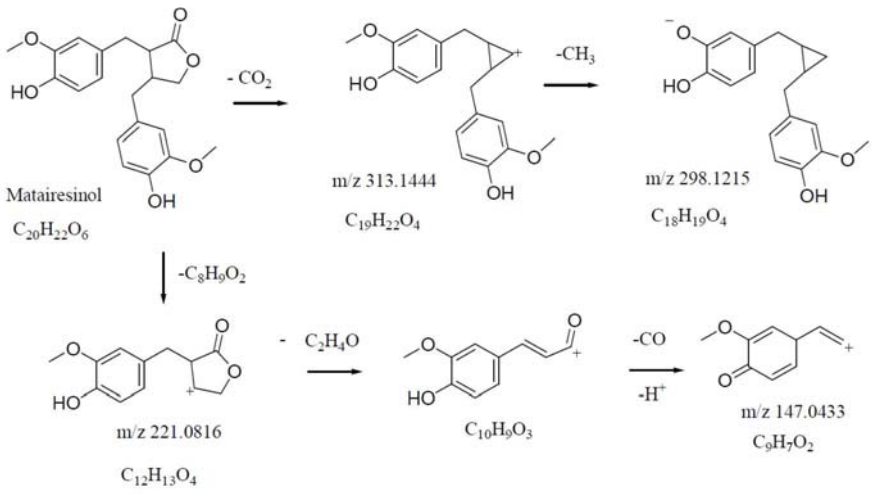


Figure 6.84. UPLC-ESI-QTOF- Mass spectrum of Matairesinol



Scheme 6.10. Mass fragments of Matairesinol

Compound 10 with retention time 4.543 forms a molecular ion peak, M- $H^{+,}$ at m/z 357.1345 (100%) and product ions at m/z 342.1104(30%), 313.1444, 298.1215 (2%), 221.0816 (20%) and 147.0433 (2%). The peak at m/z 313.1444 is due to the loss of carbon dioxide from the lactone ring. The peak at m/z 298.1215 is formed due to the loss of the methyl group from the ion with m/z 313.1446. the peak at m/z 221.0816 is due the cleavage of benzylic bond with the loss of $C_8H_9O_2$.This product ion undergoes a loss of $^{+}CH_2CH{=}O$ to form the ion at m/z 177.This compound found to be Matairesinol, (3*R*, 4*R*)-3, 4-bis[(4-hydroxy-3-methoxyphenyl) methyl] oxolan-2-one. This molecule is a constituent of *Cedrus deodara*(Roxb.ex D.Don)G.Don with molecular formula $C_{20}H_{22}O_6$.Identification of Matairesinol with HR LCMS confirmed the UPLC QTOF analysis.

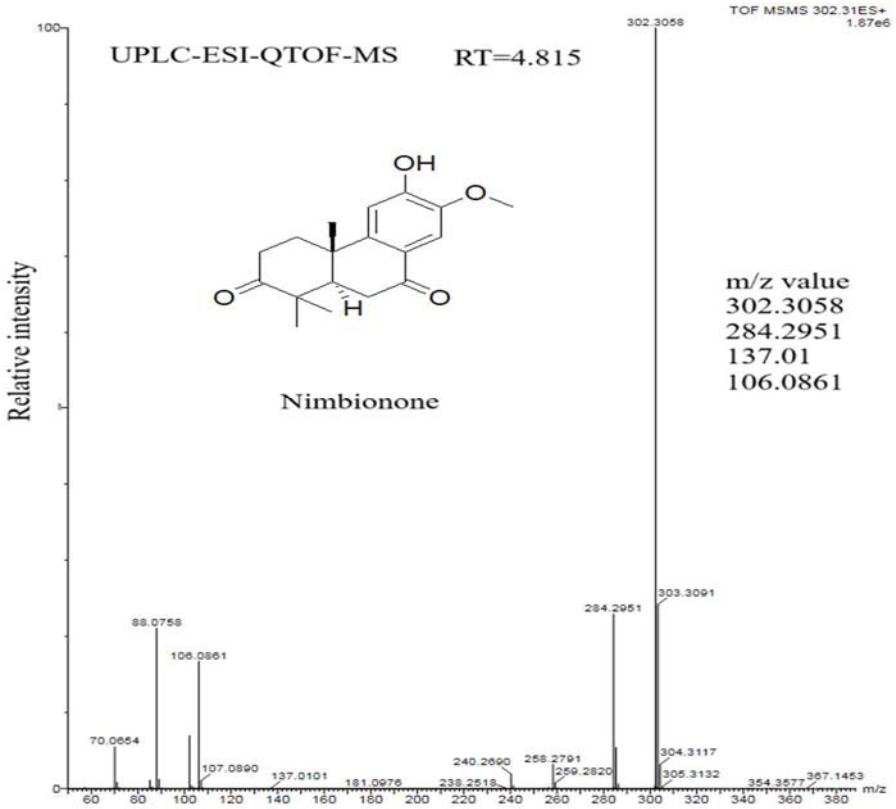
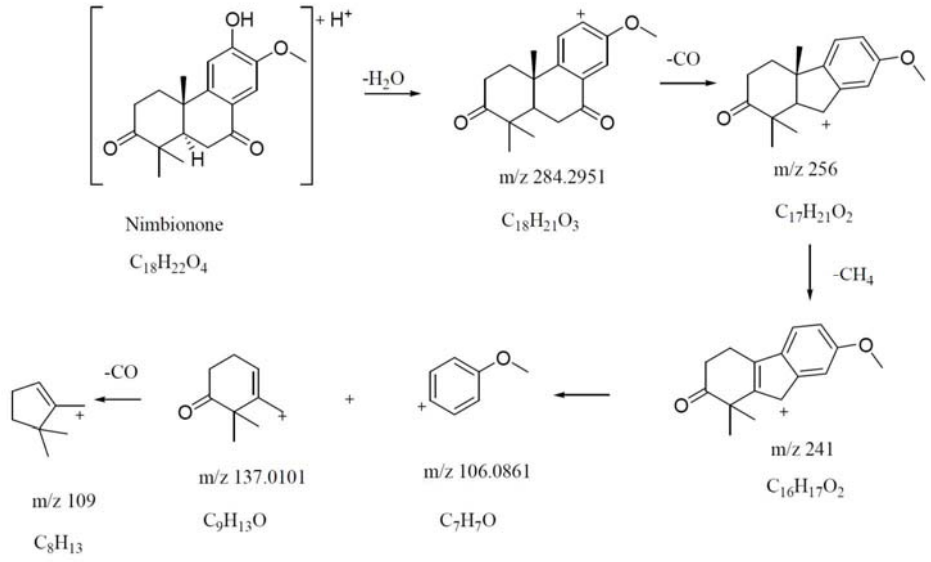


Figure 6.85. UPLC-ESI-QTOF- Mass spectrum of Nimbionone



Scheme 6.11. Mass fragments of Nimbionone

The compound 11 with the retention time 4.815 produces a molecular ion peak at m/z 302.3058 (100%). The product ions peak at m/z 284.2951(21%), 137.0107 and 106.0861(18%). The ion peak at m/z 284.2951 formed by the loss of protonated OH group as water molecule. The mass peaks 137.0107 and 106.0861are formed from the fragment ion 241 with the cleavage of cyclopentane ring. The peak at m/z 241 is formed by the remote hydrogen rearrangement with the loss of methane from the fragment 256. From the fragmentation pattern and molecular ion peak and its elemental analysis this compound identified as Nimbionone, (4*aS*, 10*aR*)-6-hydroxy-7-methoxy-1, 1, 4*a*-trimethyl-3, 4, 10, 10*a*-tetrahydrophenanthrene-2, 9-dione with the molecular formula $C_{18}H_{22}O_4$.It is the compound identified in the plant *Azadirachta indica* (L.) A.Juss.

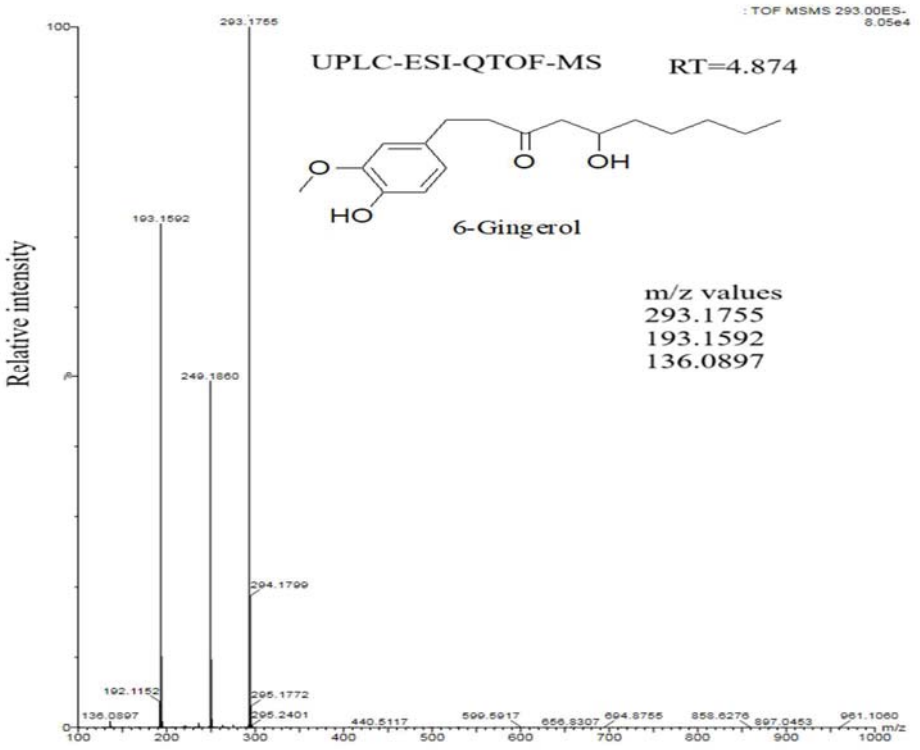
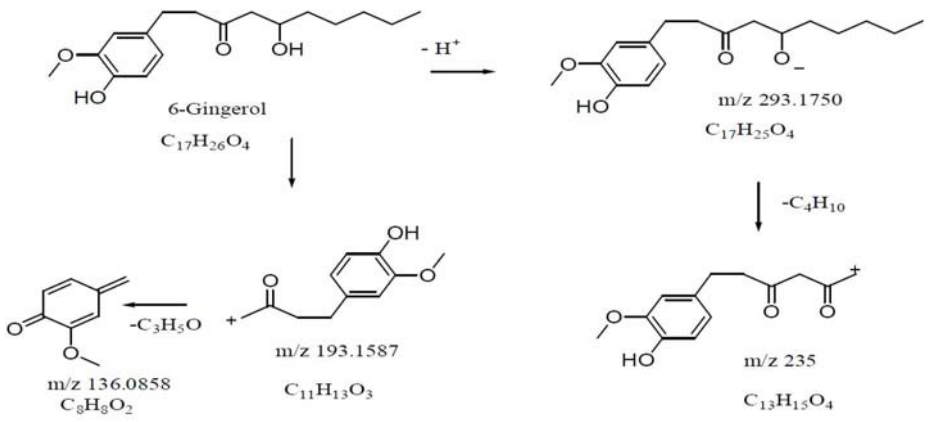


Figure 6.86. UPLC-ESI-QTOF- Mass spectrum of Gingerol



Scheme 6.12. Mass fragments of 6-Gingerol

The compound 12 with the retention time 4.874 produces a molecular ion M-H^+ peak at m/z 293.1755(100%) and major fragments at m/z 193.1592(72%) and 136.0897(2%). Molecular ions formed by the removal of H^+ ion from the OH group in the side chain. The peak at 193.1592 resulted from the loss of $C_6H_{13}O$ due to the cleavage of the C4-C5 bond. The peak at 136.0897 is formed by the loss of C_3H_5O from 193.1592. From the fragmentation patterns given above this compound identified as 6-Gingerol with the molecular formula $C_{17}H_{26}O_4$. 6-Gingerol, (5*S*)-5-hydroxy-1-(4-hydroxy-3-methoxy phenyl) decan-3-one, is a phenolic compound identified from the plant *Zingiber officinale* Rosc. It is the further confirmation of HRMS identification.

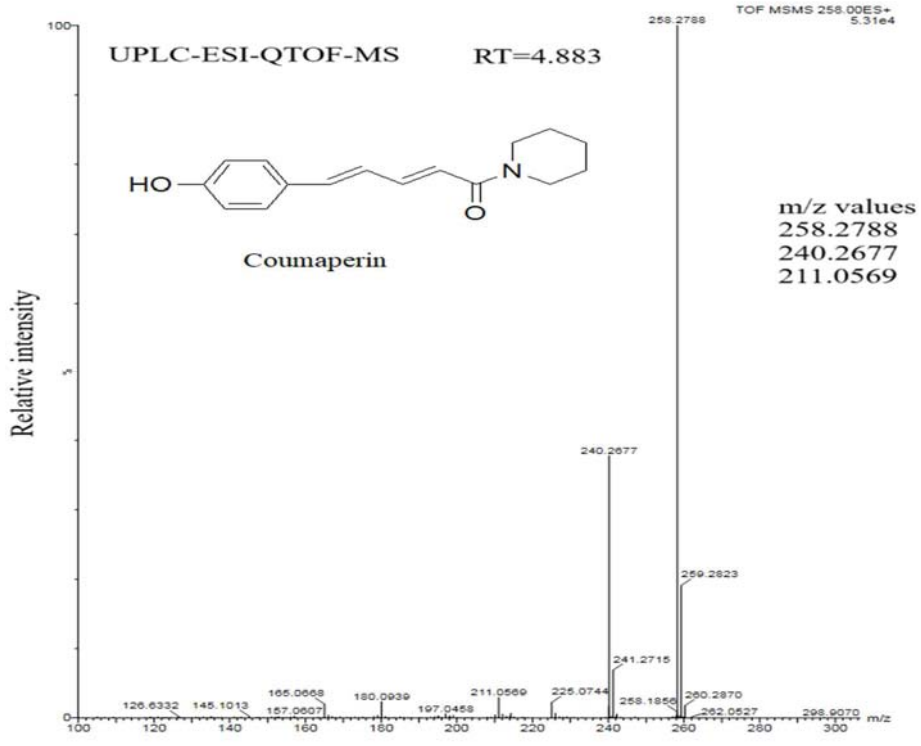
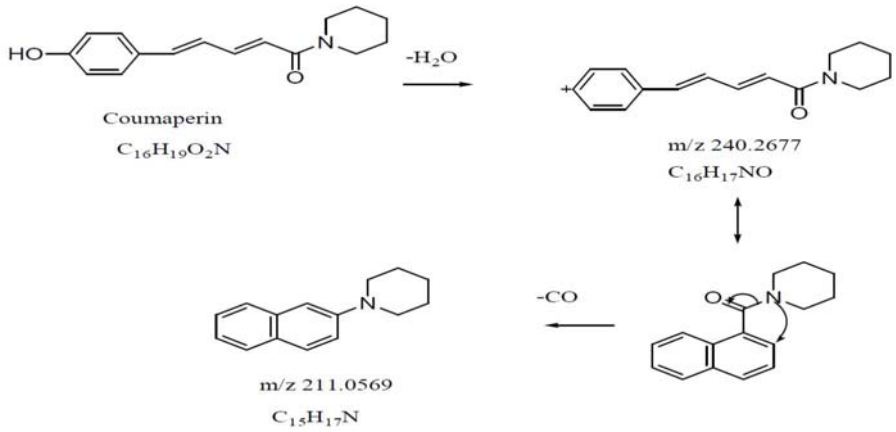


Figure 6.87. UPLC-ESI-QTOF- Mass spectrum of Coumaperine



Scheme 6.13. Mass fragments of Coumaperine

Compound 13 with retention time 4.883 produces a molecular ion peak at m/z 258.2788(100%). The product ion fragments at m/z 240.2677(38%) and 211.0569(2%). The fragments formed at m/z 240.2677 with the loss of water molecule from the protonated molecule. This molecule is undergoing cyclization and the elimination of the CO molecule results in the peak at m/ z 211. 0569.From the elemental analysis the molecular formula of compound 15 was found to be $C_{16}H_{19}NO_2$. From the fragmentation pattern it is the compound Coumaperine, (2*E*, 4*E*)-5-(4-hydroxyphenyl)-1-piperidin-1-ylpenta-2, 4-dien-1-one. It is the alkaloid found in Piper species. It is further confirmation of tentative identification of Coumaperine by HRLCMS.

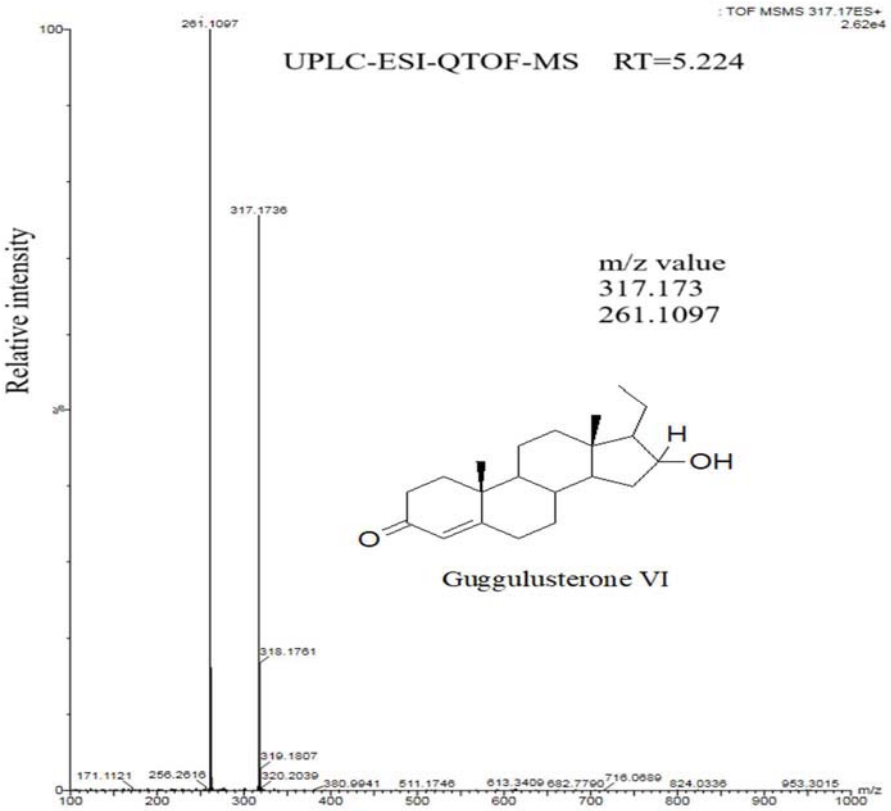
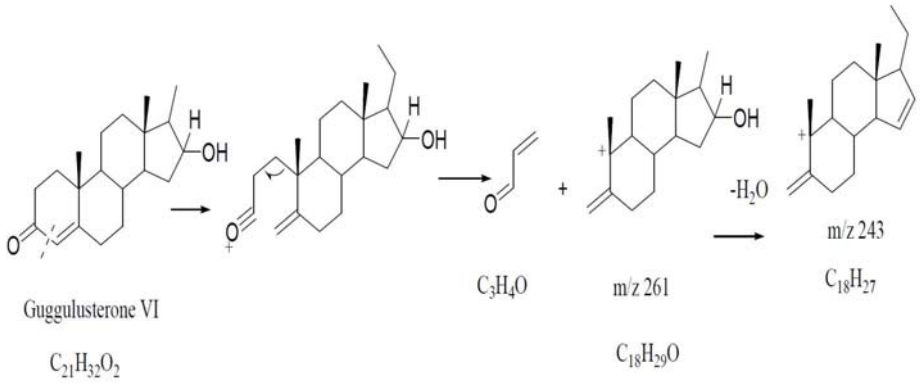


Figure 6.88. UPLC-ESI-QTOF- Mass spectrum of Guggulusterone VI



Scheme 6.14. Mass fragments of Guggulusterone VI

The compound 14 with the retention time 5.224 produces a molecular ion peak at m/z 317.1736(76%). The base peak formed at m/z 261.1097(100%). The base peak is formed by the removal of C_3H_4O from ring A of the structure as shown in the scheme. Alpha beta unsaturated cyclic ketone shows this characteristic reaction in ESI MS reactions. The compound is identified as Guggulusterone VI. It is the steroid found in the plant *Commiphora mukul* (Hook.ex Stocks) Engl.

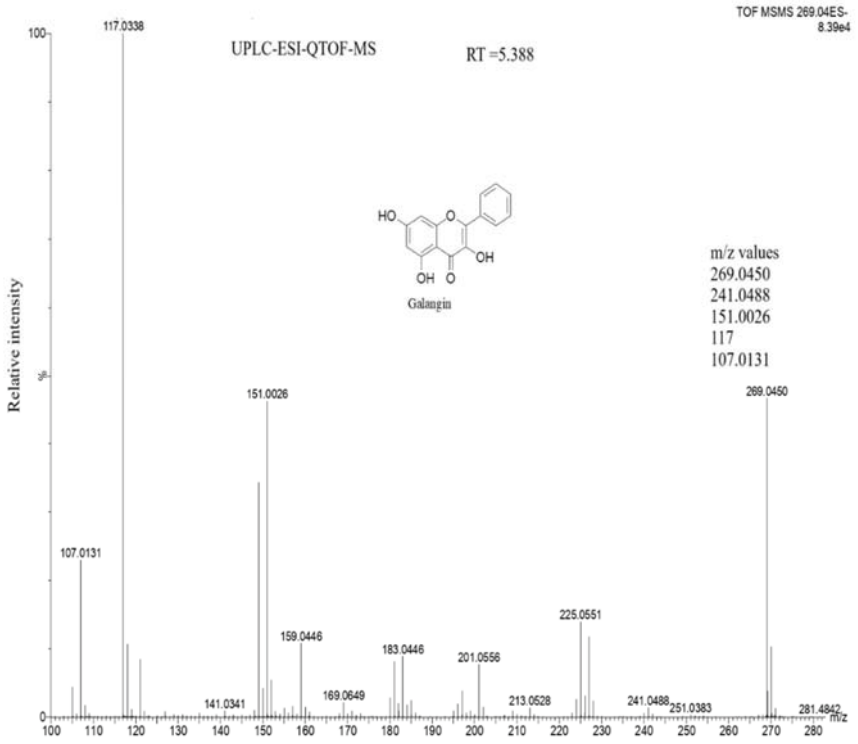
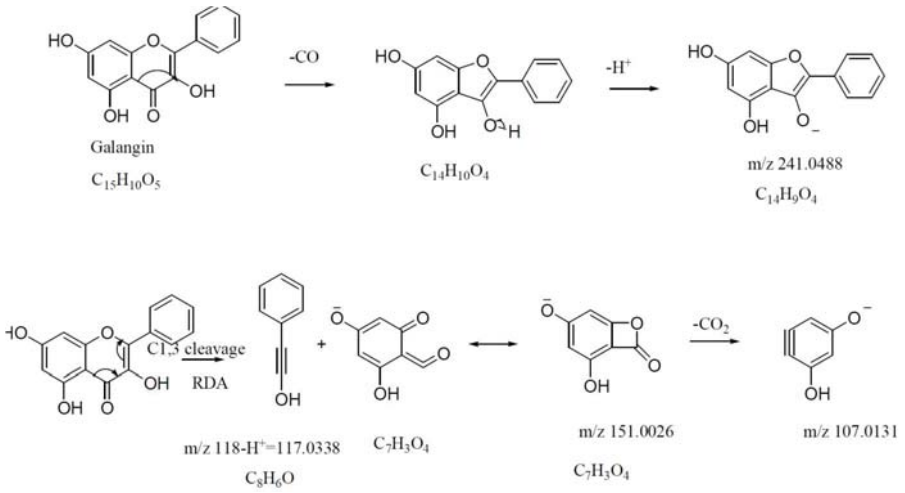


Figure 6.89. UPLC-ESI-QTOF- Mass spectrum of Galangin



Scheme 6.15. Mass fragments of Galangin

Compound 15 with the retention time 5.388 produce a molecular ion M-H^+ at m/z 269.0450(46%) and the base peak at m/z 117.0338(100%). The fragment at m/z 241.0488, 117.0338,107.0131 and 151.0026(48%). The fragment ion at m/z 241.0488 is the result of the loss of carbon monoxide from the ring C and the elimination of H+ ion. This is a characteristic reaction pattern of flavanols in ESI MS fragmentation. The peak at m/z 117.0338 is formed by RDA reaction of C- ring opening to produce 2-phenylacetylene -2-ol and the ion at m/z 151.0026 ($C_7H_3O_4$). The peak at m/z 107.0131 formed by the loss of CO_2 from the fragment m/z 151. 0026.The reaction sequence is depicted in the scheme. The compound is identified as Galangin, 7-Hydroxy flavanol, with the molecular formula $C_{15}H_{10}O_5$. It is the flavonol found in *Alpinia calcarata* Roscoe. It is the confirmation of the HRLCMS identification of Galangin(3,5,7-trihydroxy-2-phenyl chromen-4-one).

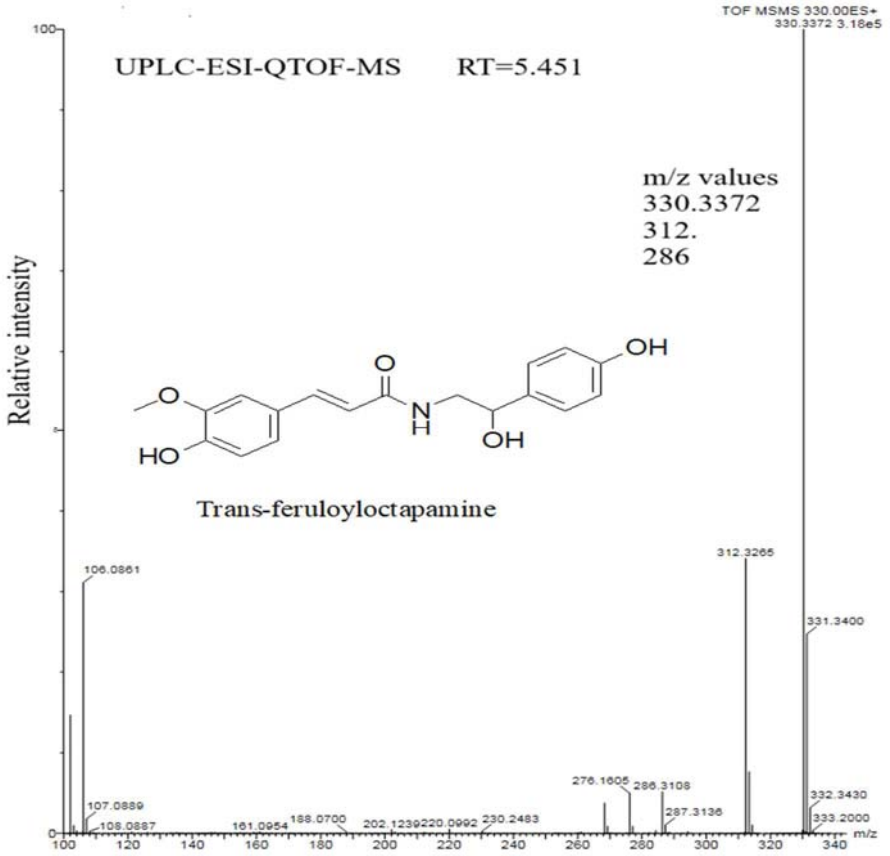
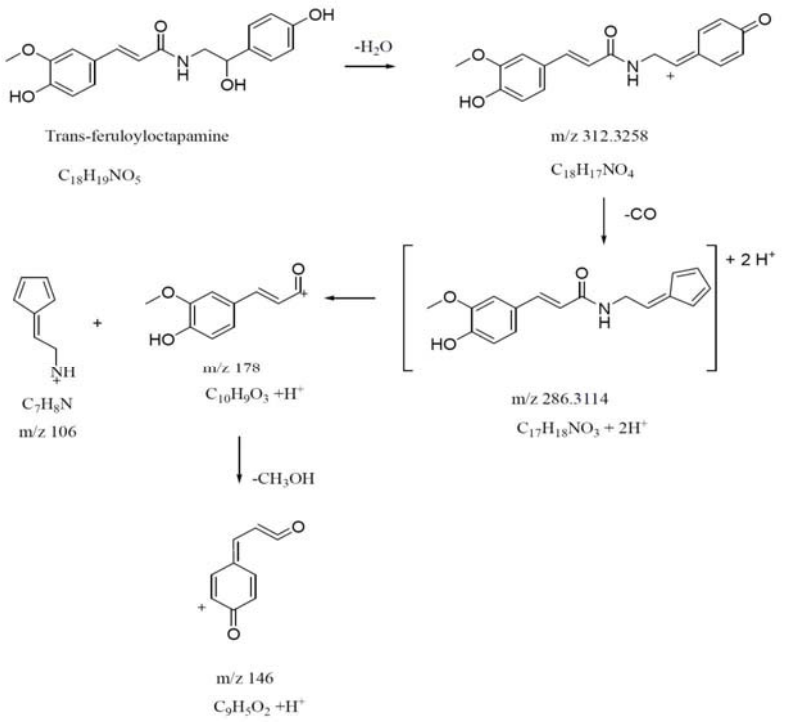


Figure 6.90. UPLC-ESI-QTOF- Mass spectrum of Trans feruloyl octopamine



Scheme 6.16. Mass fragments of Trans feruloyl octopamine

Compound 16 with retention time 5.451 produces a protonated ion peak, $M+H^+$, at m/z 330.3372(100%). The important fragments at m/z 312.3265(33%) and 286.3108(3%). The mass peak at m/z 312.3265 formed by the loss of water from the protonated molecule, which then eliminates a CO moiety with ring contraction to form m/z 286.3114. This fragment ion then undergoes the amide C-N fission to give the ion at m/z 178 ($177+H^+$). The pathway is explained in the scheme. These are the characteristic fragments of Trans feruloyl octopamine ((E)-N-[2-hydroxy-2-(4-hydroxyphenyl) ethyl]-3-(4-hydroxy-3-methoxy phenyl) prop-2-enamide) with the molecular formula $C_{18}H_{19}NO_5$.It is the alkaloid reported from the plant *Tinospora cordifolia*(Willd) Miers ex Hook.f.&Thoms. So, it is confirmation of the identification of Trans feruloylctopamine.

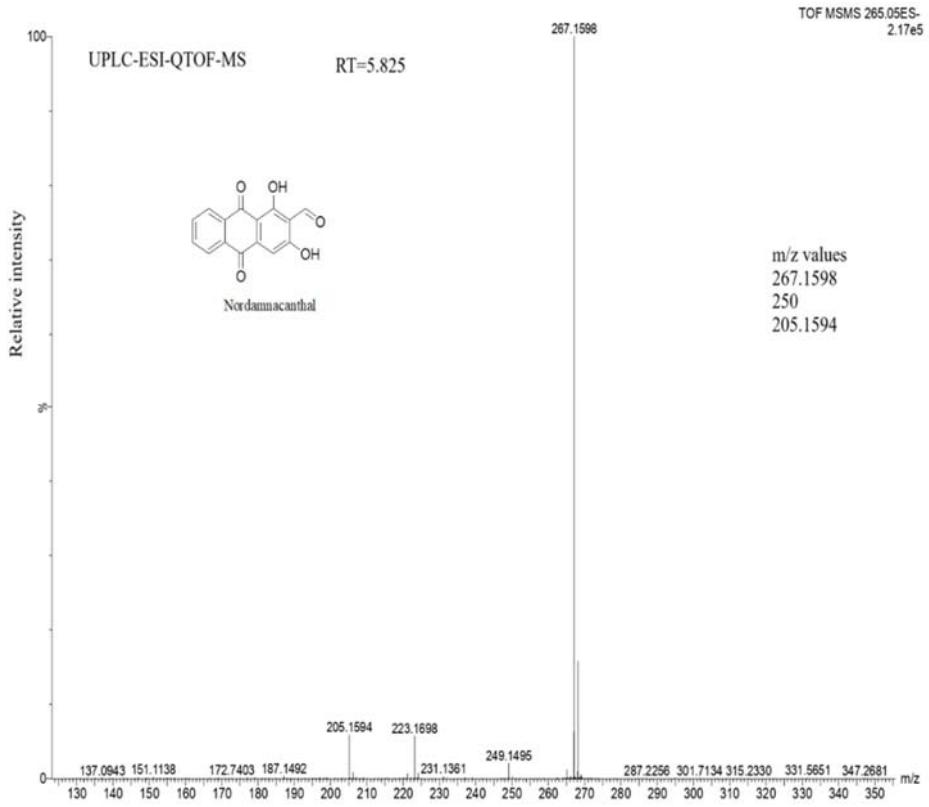
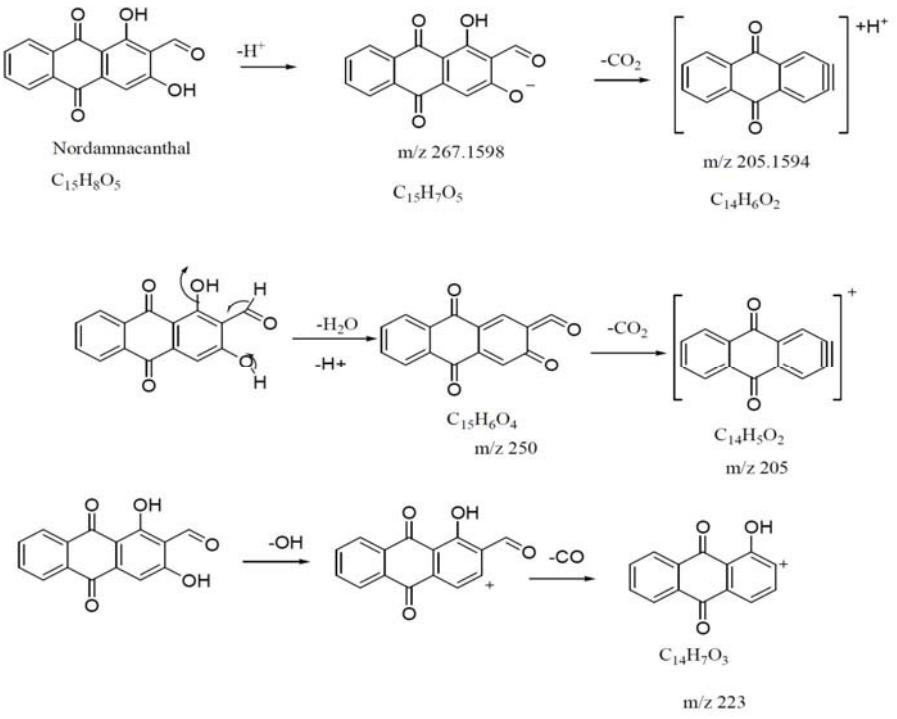


Figure 6.91. UPLC-ESI-QTOF- Mass spectrum of Nordamnacanthal



Scheme 6.17. Mass fragments of Nordamnacanthal

The Compound 17 with the retention time 5.825, form a molecular ion M-H^+ peak at m/z 267.1598(100%). Secondary fragments formed are at m/z 205.1594 (8%), and 249.1495(2%). The fragments formed at m/z 205.1594 is due to the loss of CO_2 from the ring C of the molecule. The fragment at m/z 250 is formed by the loss of water from M^+ ion. Further elimination of CO produces the peak at m/z 205 From the fragmentation pattern and its elemental analysis, it is confirmed as Nordamnacanthal with the molecular formula $C_{15}H_8O_5$, 1, 3-dihydroxy-9, 10-dioxoanthracene-2-carbaldehyde. It is a phenolic compound reported from *Rubia cordifolia* L. It validated the identification of Nordamnacanthal with HR LCMS.

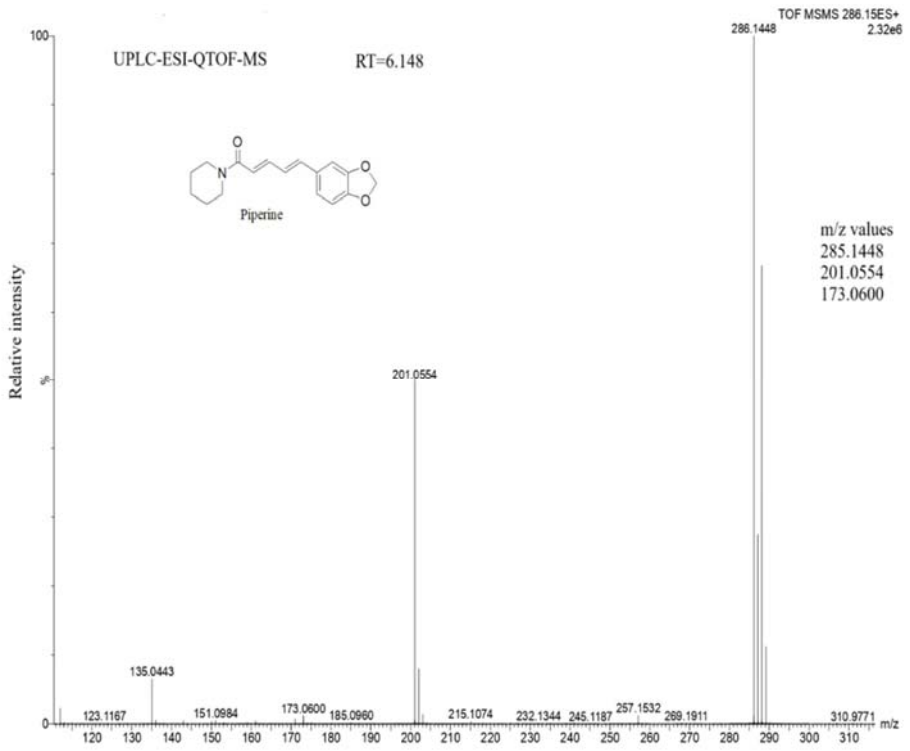
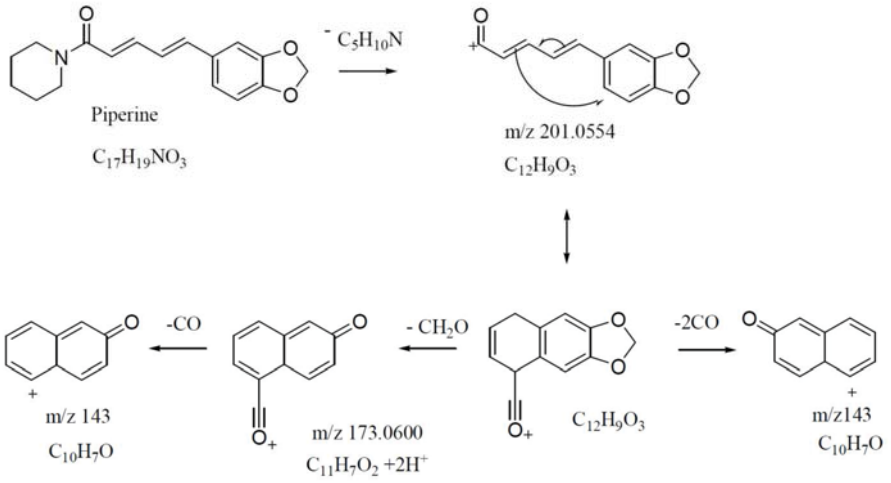


Figure 6.92. UPLC-ESI-QTOF- Mass spectrum of Piperine



Scheme 6.18. Mass fragments of Piperine

The compound 18 with retention time 6.148 produces a molecular ion peak at m/z 286.1448(100%). Other fragments are at m/z 201.0554(50%) and m/z 173.0600 (1%). The fragment with m/z 201.0554 formed by the loss of piperidine molecule by the cleavage of the amide linkage and the formation of the acylium cation. This moiety has a conjugated carbonyl system attached to a phenyl ring, and by resonance activation cyclization with the α carbon of the carbonyl and the benzene moiety occurs. This ion loses a $-CH_2O$ to give the ion at m/z 171(171+2=173). The mass peak at m/z 143 formed from the fragment at m/z 201.0554 by the removal of two molecules of CO. The mass fragmentation pattern and its elemental analysis confirmed the compound as Piperine. (2E,4E)-5-(2H-1,3-Benzodioxol-5-yl)-1-(piperidin-1-yl) penta-2,4-dien-1-one, with the molecular formula $C_{17}H_{19}NO_3$. It is the confirmation of HR LCMS identification.

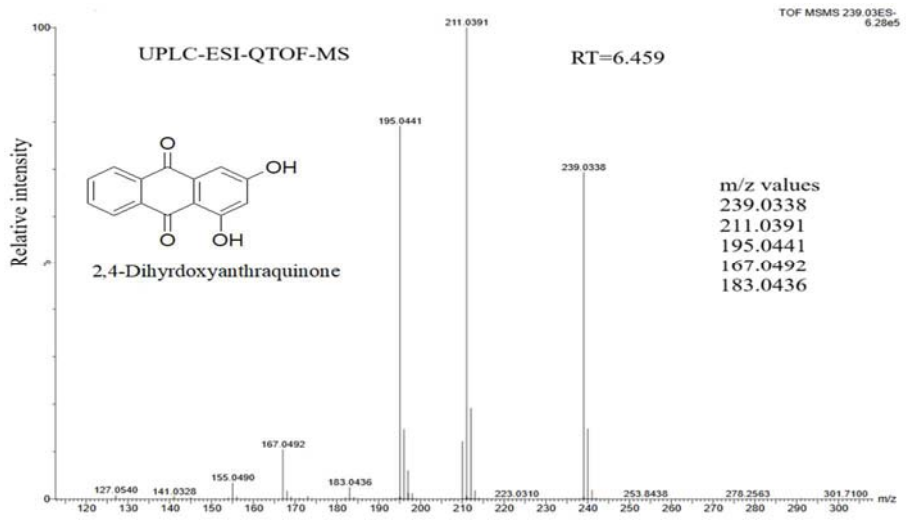
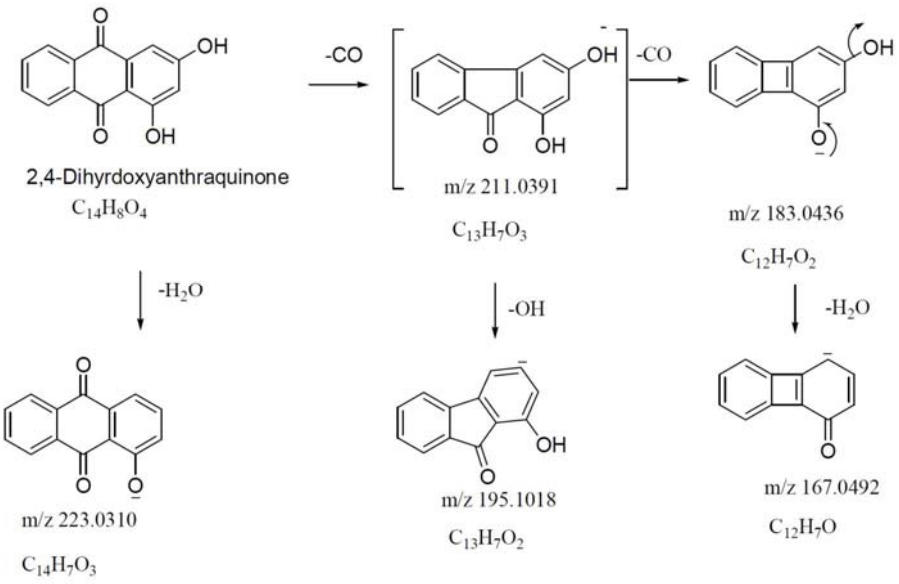


Figure 6.93. UPLC-ESI-QTOF- Mass spectrum of 2,4- Dihydroxyanthraquinone



Scheme 6.19. Mass fragments of 2, 4-Dihydroxy anthraquinone

The compound 19 with retention time 6.459 in the negative ionization mode produce a molecular ion peak at m/z 239.0338(70%). The base peak formed at m/z 211.0391(100%). Other fragments formed are at m/z 195.0441(80%), 167.0492(10%), 183.0436(2%) and 155.0490(3%). The base peak at m/z 211 formed by the elimination of CO molecule with ring contraction from the molecular ion. For most of the anthraquinones, a loss of 28 amu was observed, corresponding to a loss of CO [68].

The fragment at m/z 195.0441 is formed by the consecutive elimination of OH from the fragment at m/z 211. The peak at m/z 183 is formed by the continuous loss of two molecules of CO from the ring B of the molecular ion. The peak at m/z 167.0492 is due to the further elimination of water from the peak at m/z 183. Elemental analysis and the fragmentation pattern confirmed the identification of 2, 4 – Dihydroxyanthraquinone(2,4-Dihydroxyathracene-9,10-dione). It has been reported from *Rubia cordifolia* L.

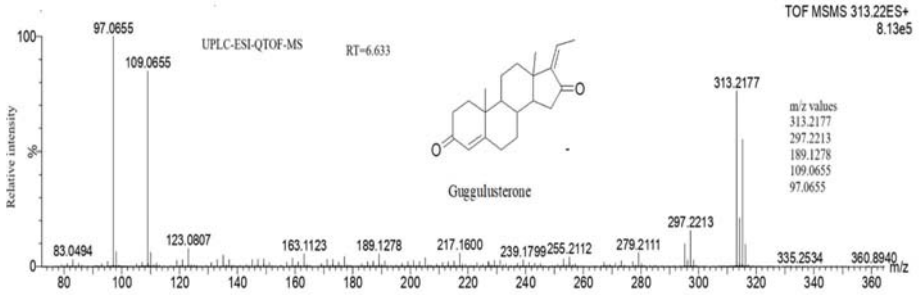
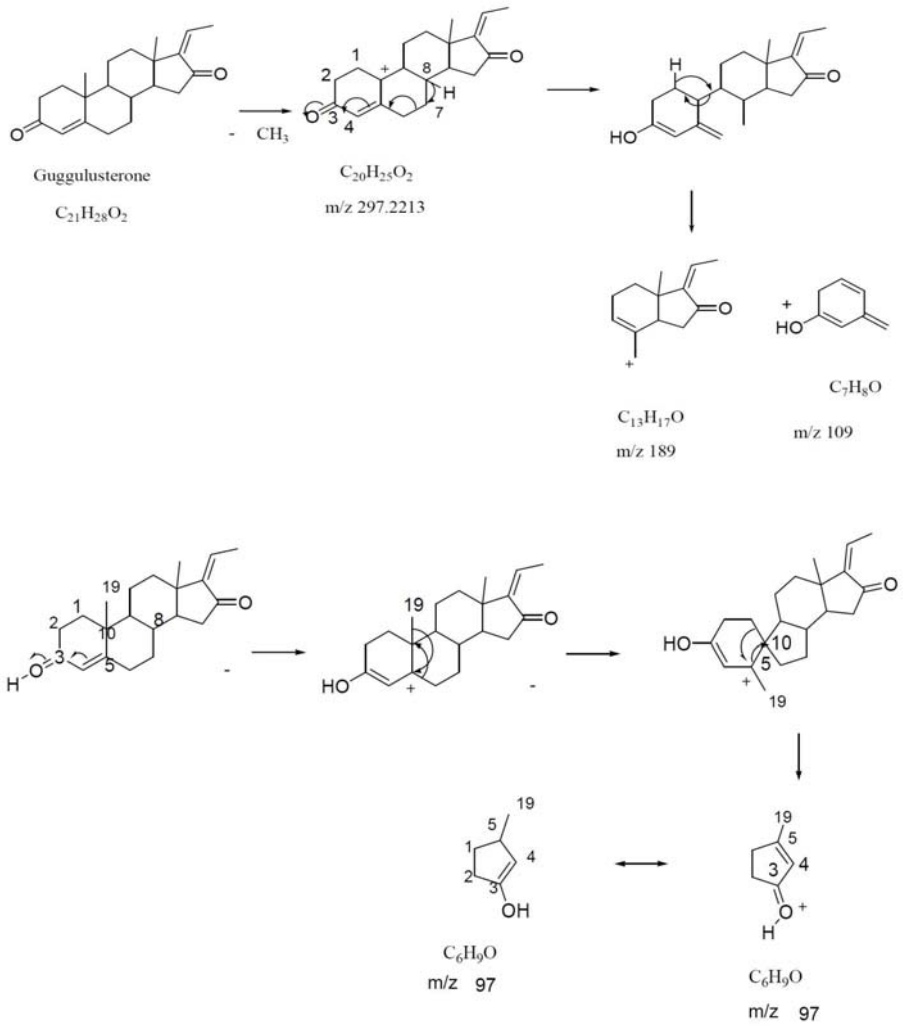


Figure 6.94. UPLC-ESI-QTOF- Mass spectrum of Z –Guggulusterone



Scheme 6.20. Mass fragments of Z- Guggulusterone

Compound 20 with retention time 6.633 produces a molecular ion peak at m/z 313.2177 (75%). Secondary fragments appear at m/z 109.0655 (89%) and 297.2213(15%). The peak at m/z 297 was formed by the loss of CH_3 from the molecular ion. Migration of a hydrogen from C8 is proposed to accompany the cleavage of the bond between C9 and C10. This results in the peaks at m/z 189 and 109.This mechanism is supported by deuterium labelling experiments by Shapiro and Djerassi [64] using α, β unsaturated 3-β steroids.

Following protonation of the C3 keto functional group location of the charge at C5 is energetically equivalent enabling the migration of C19 towards C5 in a pinacol like(Wagner Meerwine) rearrangement as reported for steroidal compounds. The simultaneous shift of the C6-C7 bond to C6-C10 allows the retention of charge in the conjugated π electron system. The subsequent rearrangement C-C bond between C1 and C10 generating a C1-C5 linkage accompanied by the disintegration of C10 results in formation of an ion at m/z 97which (has the resonance structure) forms the base peak [65]. From the information given above it is confirmed as Z- Guggulusterone((10R,1 3S,17Z)-17-ethylidene-10,13-dimethyl-1,2,6,7,8,9,11,12,14,15-decahydrocyclopenta[a]phenanthrene-3,16-dione), $C_{21}H_{28}O_2$. It is a steroid reported in the plant *Commiphora mukul*(Hook.ex Stocks) Engl. It is the further confirmation of HRMS identification.

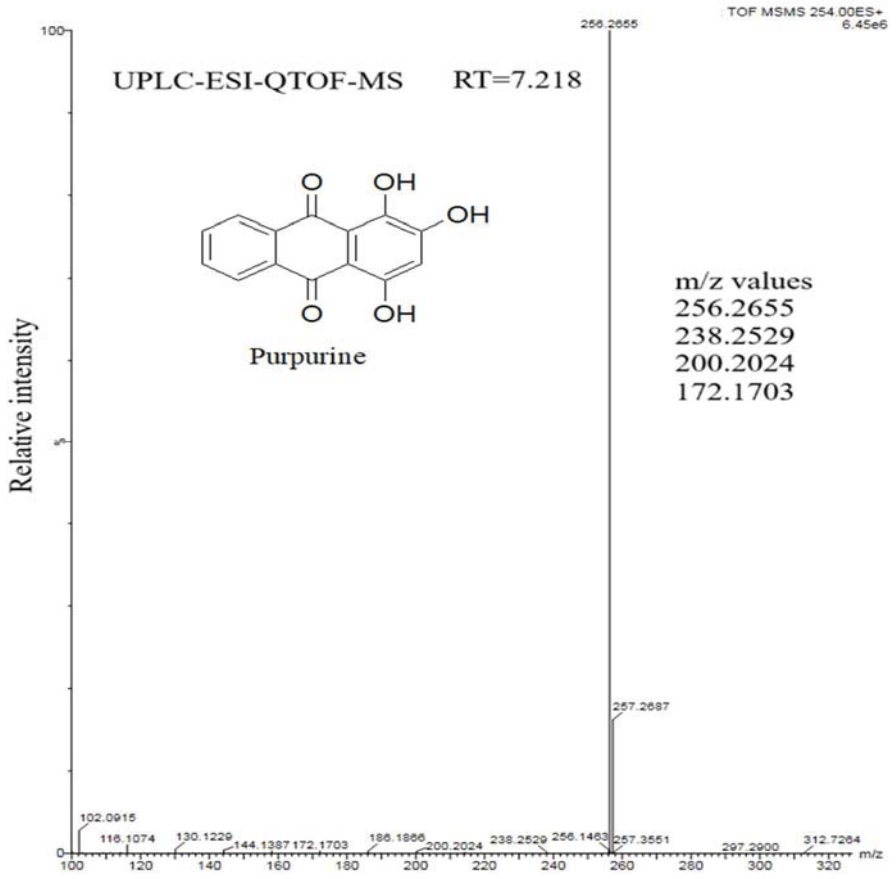
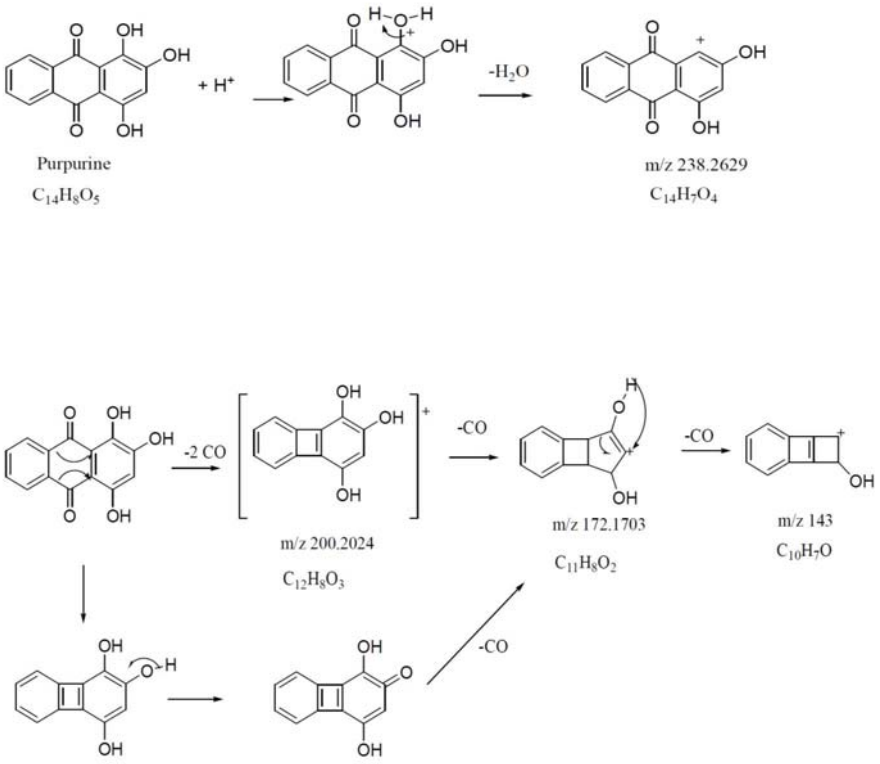


Figure 6.95. UPLC-ESI-QTOF- Mass spectrum of Purpurin



Scheme 6.21. Mass fragments of Purpurin

Compound 21 with retention time 7.218 form a molecular ion peak at m/z 256.2655(100%). The fragments are formed at m/z 238.2529, 200.2024 and 172. The mass fragments formed at m/z 238.2529 by the loss of water molecules from the molecular ion. The peak at m/z 200.2024 is due to the ion formed by the loss of two molecules of carbon monoxide from m/z 256. The peak at m/z 172 is the result of CO removal with ring contraction from 200.2024. The elimination of the CO group from m/z 200 produces the peak at m/z 172.The elemental analysis, molecular mass and fragment peaks observed confirmed it as Purpurin(1,2,4-trihydroxyanthracene-9,10-dione) with the molecular formula $C_{14}H_8O_5$. It is the tri hydroxy anthraquinone reported from *Rubia cordifolia* L.

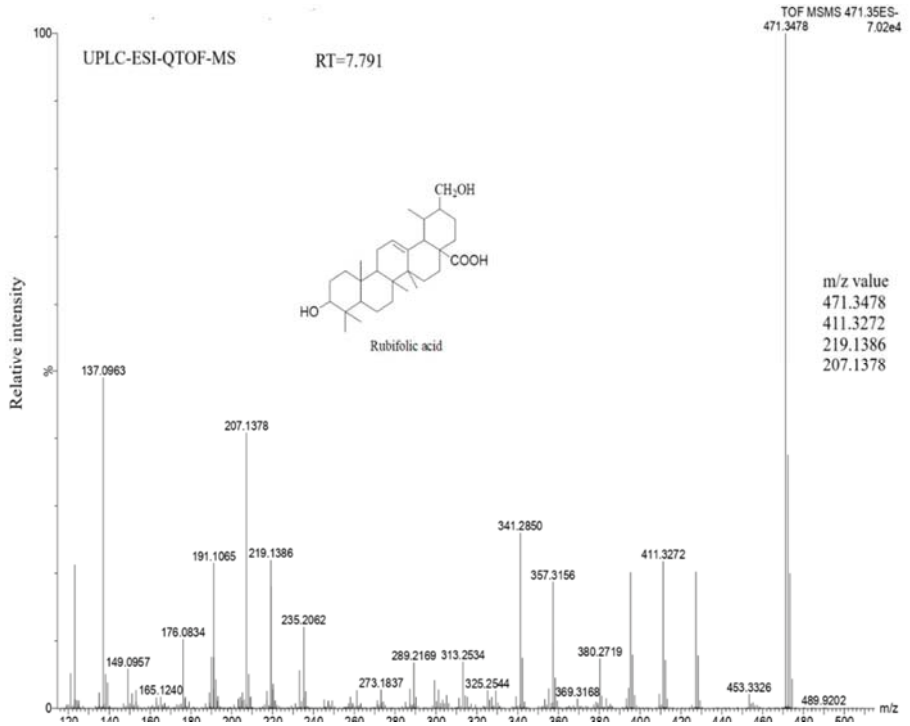
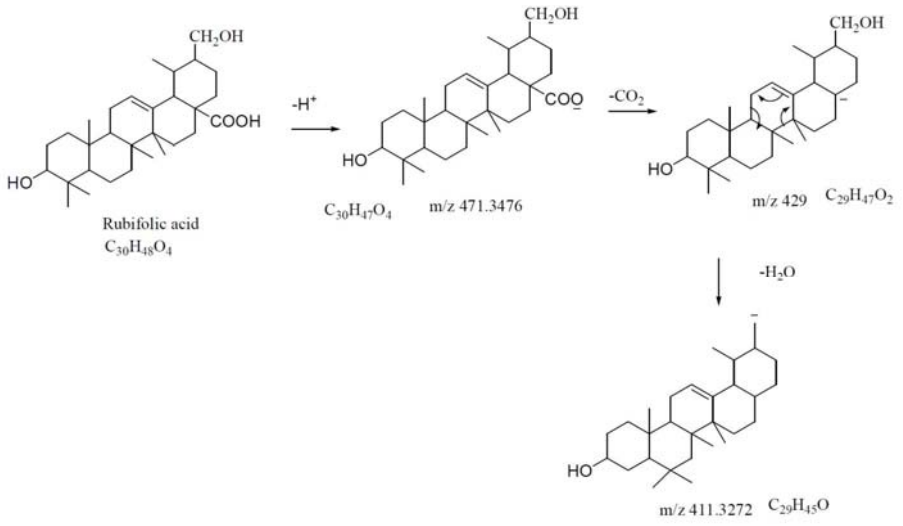
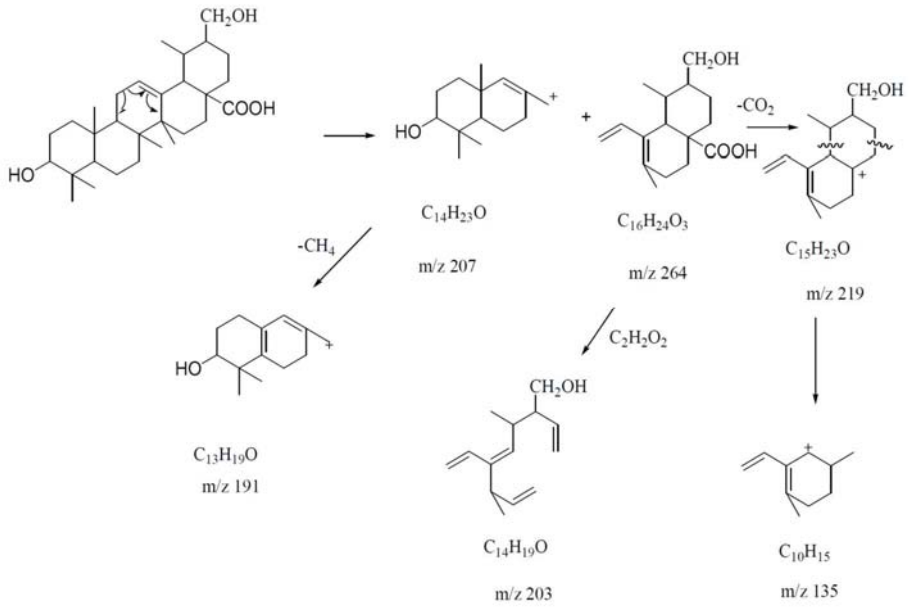


Figure 6.96. UPLC-ESI-QTOF- Mass spectrum of Rubifolic acid





Scheme 6.22. Mass fragments of Rubifolic acid

Compound 22 with retention time 7.791 produces a molecular ion ($M-H^+$) peak at m/z 471.3478 (100%). It is the deprotonated molecule formed by the removal of H^+ ion from the carboxylic group. Secondary fragments formed at m/z 429. (1%), 411.3272(21%), 207.1378(41%) and 219.1386(21%). The fragment formed at m/z 429 is formed by the removal of CO_2 from the COOH group in the molecule. The mass peak at 411.32formed by the loss of water molecule from the CH_2OH in the ring E. The mass peaks at 207.1379 and 219.1386 are formed by the ring opening of ring C by an RDA reaction. The peak at m/z 191 results from the peak at m/z 207 by the loss of methane molecule. Detailed fragmentations are given in the scheme. The elemental analysis and fragmentation pattern confirmed it as Rubifolic acid with the molecular formula $C_{30}H_{48}O_4$, 10-hydroxy-2-(hydroxymethyl)-1, 6*a*, 6*b*, 9, 9, 12*a*-hexamethyl-2, 3, 4, 5, 6, 6*a*, 7, 8, 8*a*, 10, 11, 12, 13, 14b-tetra decahydro-1H-picene-4a-carboxylic acid. It is identified in the plant *Rubia cordifolia* L. It is the additional confirmation of HRMS identification of Rubifolic acid.

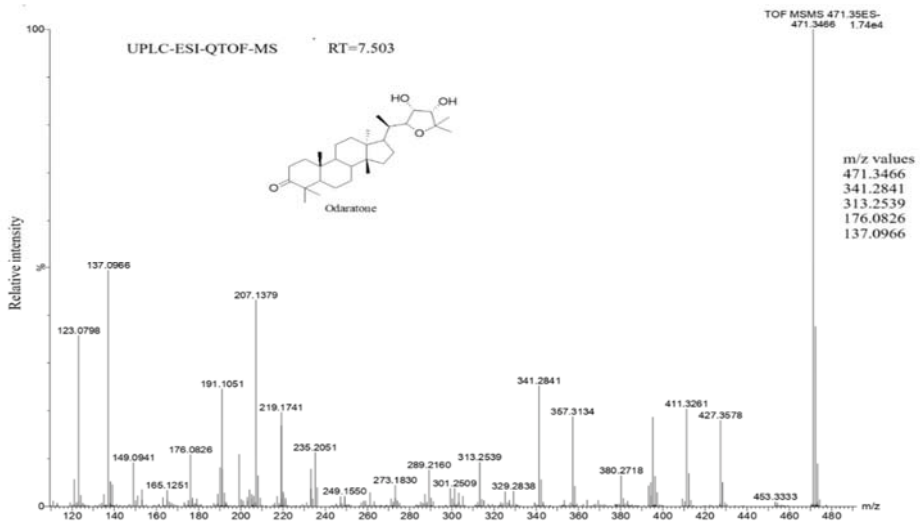
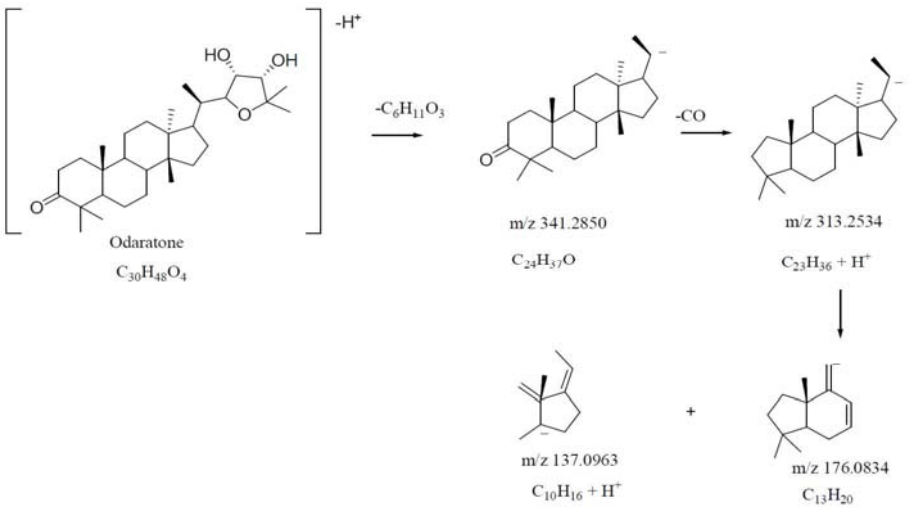


Figure 6.97. UPLC-ESI-QTOF- Mass spectrum of Odaratone



Scheme 6.23. Mass fragments of Odaratone

Compound 23 with retention time 7.503 produce a molecular ion ($M\text{-}H^+$) peak at m/z 471.3478 (100%). The product ions are formed at m/z 341.2850(28%), 137.0963(50%), 313.2534(5%) and 176.0834(10%). The fragment at m/z 341.2850 formed from the molecular ion with the elimination of the furan ring. The peak at m/z 313.2534 is due to the

removal of CO with ring contraction from the mass peak 341. The peak at m/z 176.0834 and 137.0963 are formed by the ring opening of ring C. The elemental analysis and fragmentation pattern confirmed this compound as Odaratone ((5R,9R,10 R,13S,14S, 17S)-17-[(1R)-1-[(2R,3R,4S)-3,4-dihydroxy-5,5-dimethyloxolan-2-yl] ethyl]-4,4,10,13,14-pentamethyl-1,2,5,6,9,1 1,12,15,16,17-decahyd rocyclopenta[a]phenanthren-3-one) with molecular formula $C_{30}H_{48}O_4$.It is the compound reported in the plant *Azadirachta indica* (L.)A.Juss..

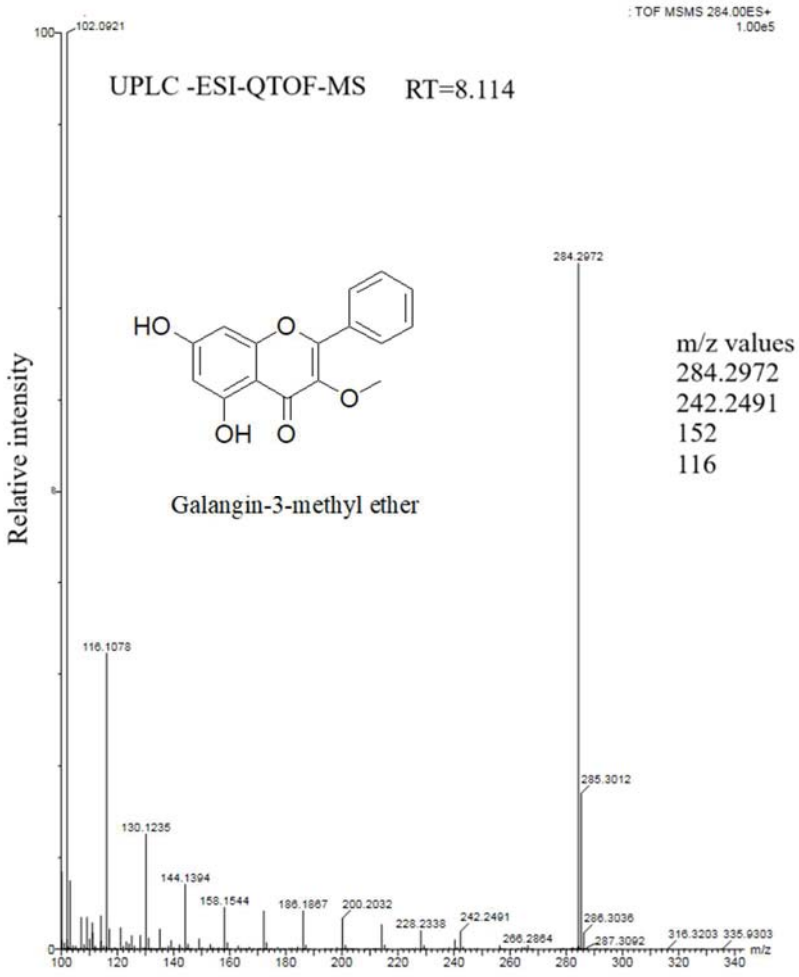
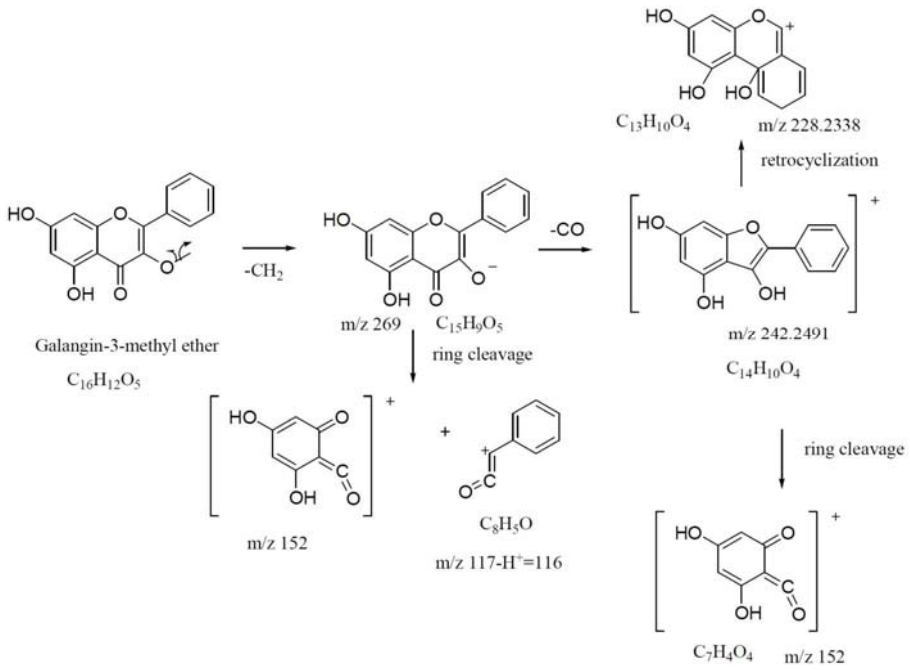


Figure 6.98. UPLC-ESI-QTOF Mass spectrum of Galangin -3-methyl ether



Scheme 6.24. Mass fragments of Galangin-3-methyl ether

The compound 24 with retention time 8.114 produces a molecular ion at m/z 284.2974(75%). The loss of CH_3 molecule from the molecular ion produces the peak at m/z 269.Subsequent elimination of CO from the ring C produces the peak at m/z 242.2491. Which on retro-cyclization produces the peak at m/z 228.Ring cleavage of the ring C of peak at m/z 269 produces the peak at m/z116 and 152. From the mass fragments and the elemental analysis this compound is identified as Galangin 3-methyl ether(5,7-dihydroxy-3-methoxy-2-phenylchromen-4-one), $C_{16}H_{12}O_5$. Galangin 3-methyl ether is a monomethoxy flavone. It is the natural flavonoid reported from *Alpinia calcarata* Roscoe.

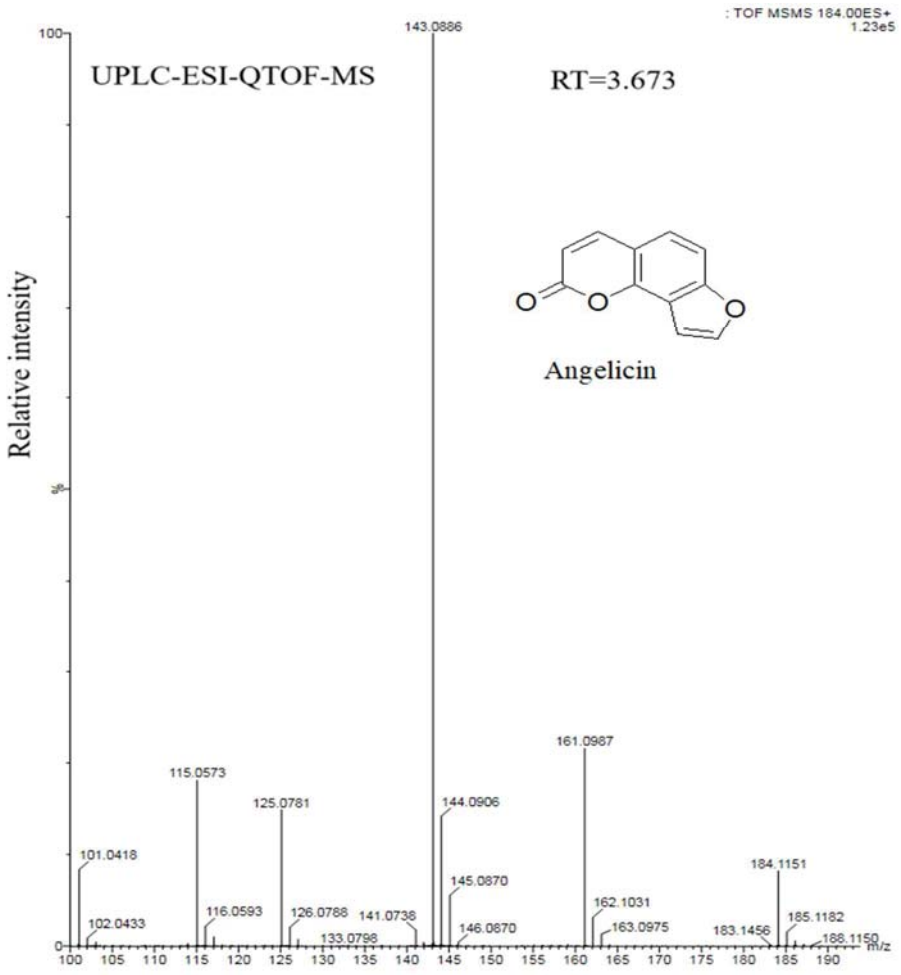


Figure 6.99. UPLC-ESI-QTOF- Mass spectrum of Angelicine

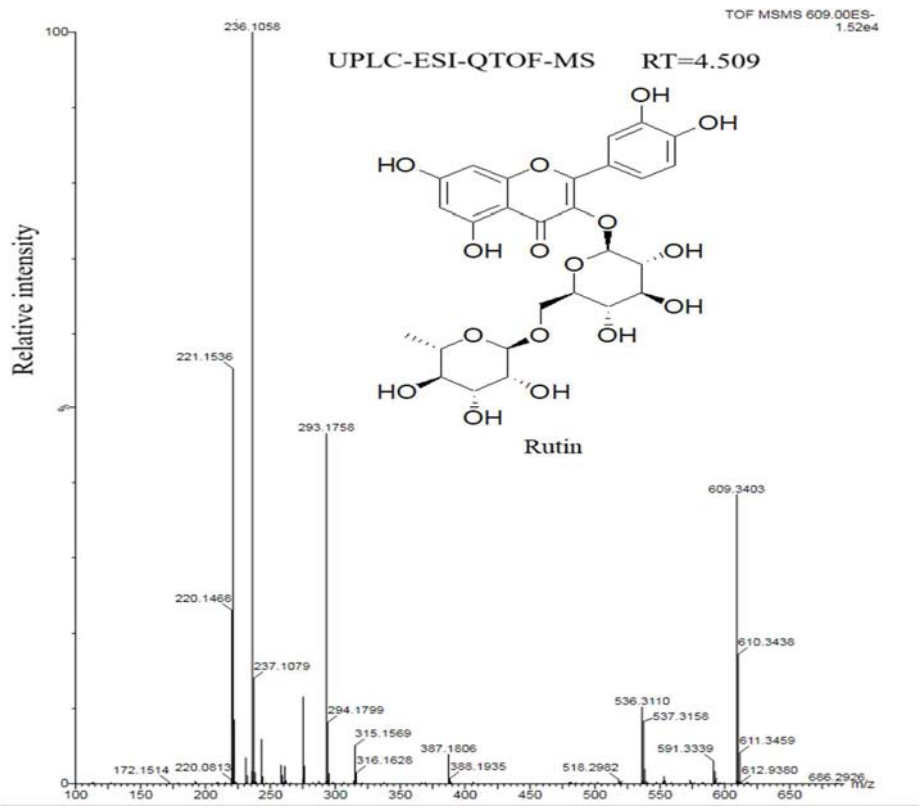


Figure 6. 100.UPLC-ESI-QTOF- Mass spectrum of Rutin

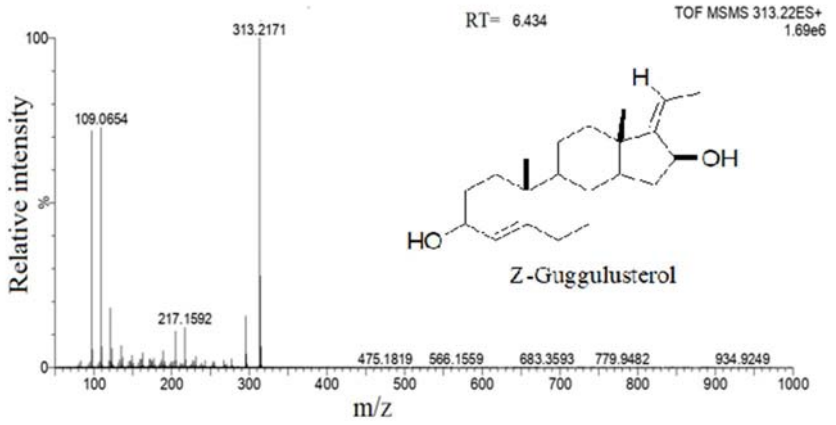


Figure 6. 101.UPLC-ESI-QTOF- Mass spectrum of Z-Guggulsterol

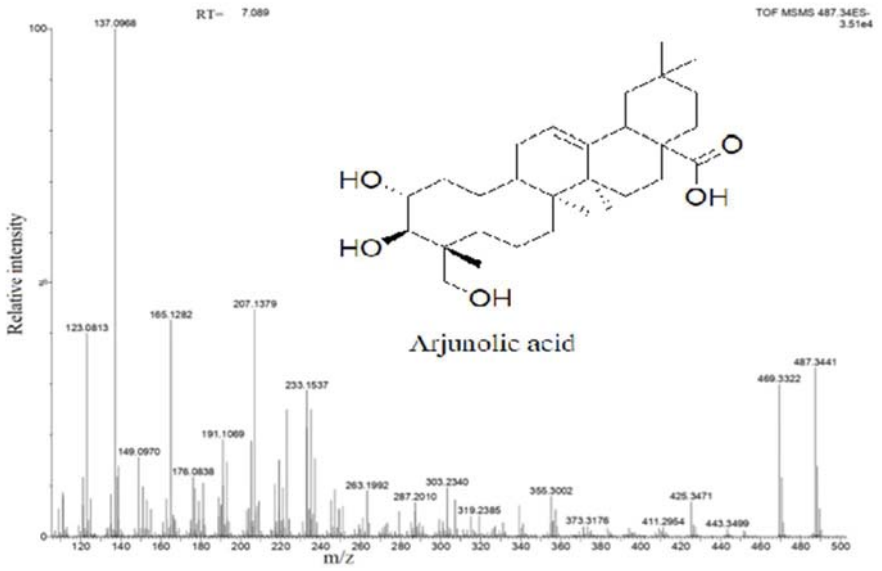


Figure 6. 102.UPLC-ESI-QTOF- Mass spectrum of Arjunolic acid

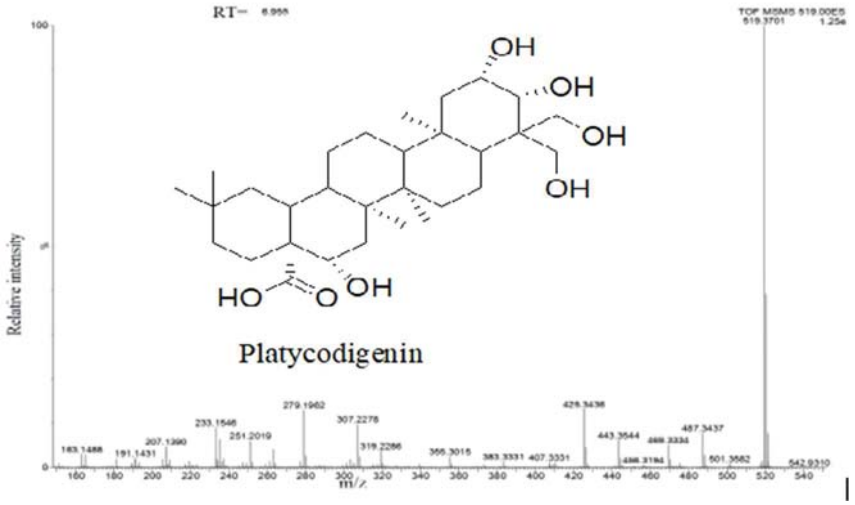


Figure 6. 103.UPLC-ESI-QTOF- Mass spectrum of Platycodigenin

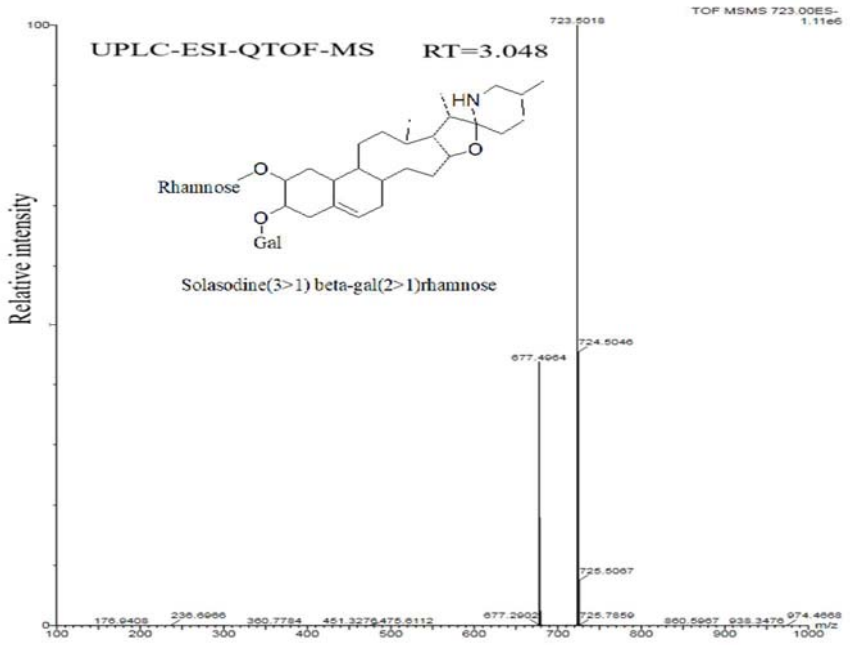


Figure 6. 104. UPLC-ESI-QTOF- Mass spectrum of Solasodine (3->1) β-gal-(2->1) rhamnose

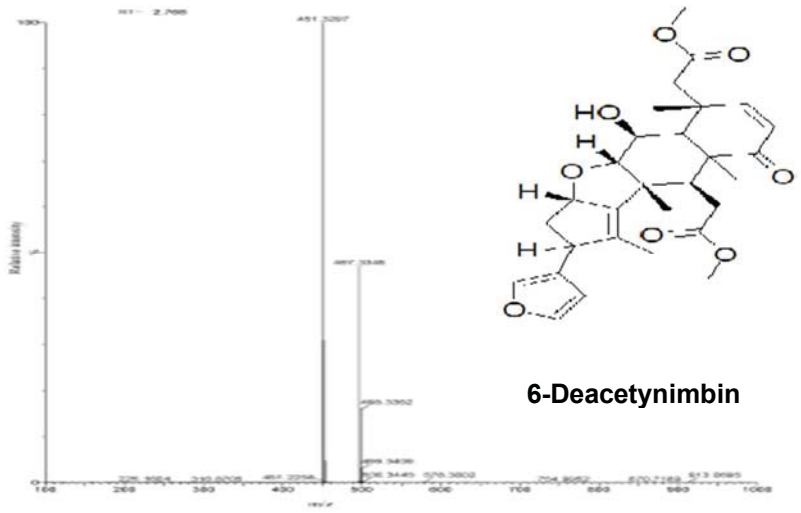


Figure 6. 105.UPLC-ESI-QTOF- Mass spectrum of 6-Deacetynimbin

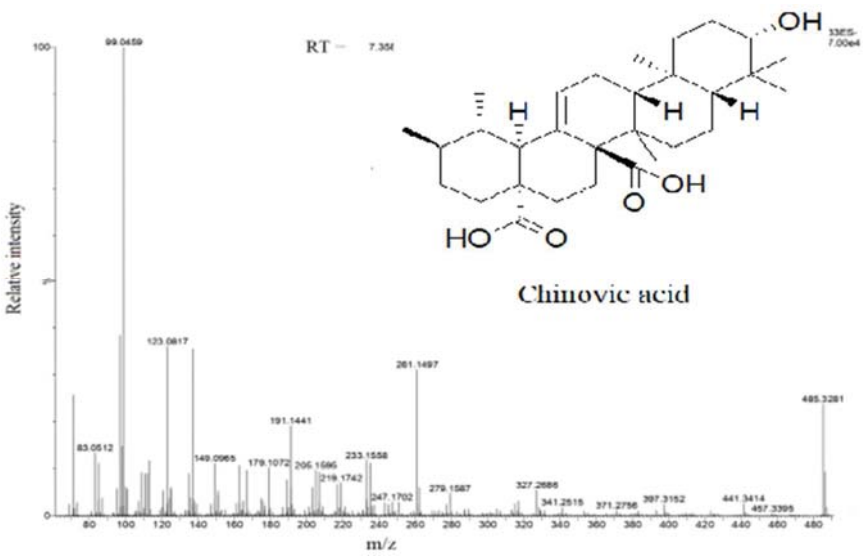


Figure 6. 106.UPLC -ESI-QTOF- Mass spectrum of Chinovic acid

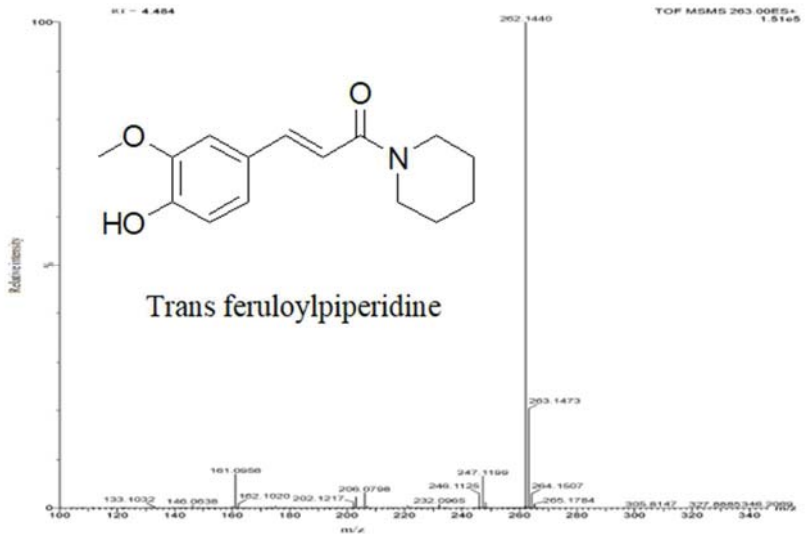


Figure 6. 107.UPLC-ESI-QTOF- Mass spectrum of Trans feruloyl piperidine

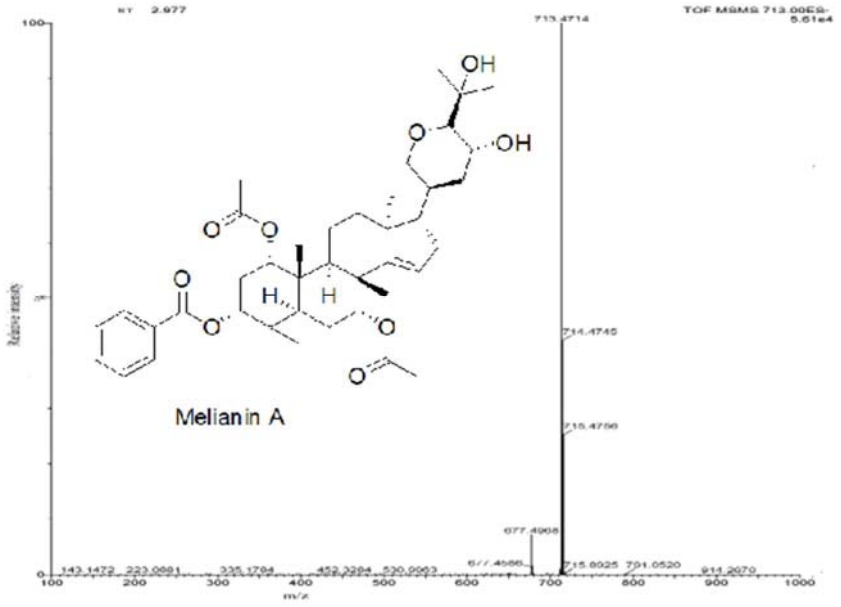


Figure 6.108. UPLC-ESI-QTOF- Mass spectrum of Melianin A

6.3. Experimental

LC-MS analysis

The chemical fingerprint of the extracts was determined using LC-MS analysis. The LC-MS was performed on a SHIMADZU-LC-MS machine (Model: 2010A) using the solvent system of formic acid – acetonitrile gradient and RP-C18 analytical column (240 mm × 2 cm) with a flow rate of 0.5 mL/min. samples were nebulized with nitrogen gas, and the ion mass (electrospray ionization [ESI]) was generated in both positive mode and negative mode.

High Resolution Liquid Chromatography –Mass Spectrometry (HRLCMS) study

HR-LCMS analysis of samples was carried out in Sophisticated Analytical Instrument Facility (SAIF), IIT Bombay, Pawai, Mumbai.HR LCMS study conducted on methanol extract of both the kashayam.

Preparation of methanol extract explained in chapter 2. Chromatographic separation was performed on Hypersil GOLD C18 column (100 x 2.1mm) and 3-micron particle size. Applying the following gradient at a pressure 1200.00 bar; solvent A (0.1%Formic acid in water) and solvent B (90% ACN +10%H_2O+ 0.1% Formic acid) ;1.00 min 95%A and 5%B at a flow rate 0.300 mL/min; 20.00 min 0.00%A and 100%B at a flow rate 0.300 mL/min;25.00min 0.00%A and 100%B at a flow rate 0.300 mL/min;26.00min 95%A and 5%B at a flow rate 0.200 mL/min; 30.00 min 95%A and 5%B at a flow rate 0.300 mL/min; Injection Volume 5.00 µL.

UPLC-ESI-QTOF-MS

300 ml of the well shaken Kashayam is centrifuged at 4000 rpm (3000g) in a REMI 8C centrifuge for 30 minutes. The Clear supernatant was carefully decanted (220ml). This was analyzed in Q-TOF -MS/MS UPLC-Q-ToF-MS analysis carried out at MG University Kottayam. The chromatographic separation and detection of analytes was carried out with ultra-performance liquid chromatography coupled to quadrupole time of flight mass spectrometry(UPLC-Q-ToF-MS).The acquity UPLC system (Waters)consists of a TUV detector(JI2TUV750A), a column chamber(JI2 CHA730G), a quaternary solvent manager(HI2 QSM632A), and a sample manager FTN (KI2 SDI069G).A reversed -phase BEH C18 column (of dimension 50mm X 2.1mm X 1.7 µm) with a flow rate of 0.3mL min -1 was used for chromatographic separation(Waters)The mobile phase was a mixture of 0.1% Formic acid in water (A) and acetonitrile (B) in a gradient elution as follows initial 95% A, 0.1min 95%A, 6.00 min 5%A, 6.5 min 5%A, 9min 95%A and 10 min 95%A.The UPLC system was connected to the quadrupole time of flight mass spectrometer (Waters Xevo G2 QTOF) with electrospray

ionization(ESI)interface working in positive and negative ionization modes .The injection volume was 10µL**.**The scanning m/z range was between 50 and 1000 .The desolvation gas flow and the temperature were 900L/h and 350 ^{0}C, respectively. The mass spectra were obtained using collision energy ranging from 5 to 30eV.The instrument control and data acquisition was done using Mass Lynx software (v 4.1).

6.4. Conclusion

From the HRLC/MS study the presence of about seventy-three components could be identified. Out of these fifty-two molecules exhibit pro anti-inflammatory and anti-inflammatory activity. This is highlighted in table-6.2 above and the reference there off. All the pharmacological studies have been carried out on the individual medicinal plants. So, a comprehensive study of the combined effect of all the constituents in any of the Ayurveda formulations has not been reported in the literature so far. Since this formulation is used therapeutically as a drug in many of the diseases caused by chronic inflammatory conditions in Ayurveda regime, the identification of the molecular entities with the target activities validates its use. Whether all the 52 constituents are essential for the therapeutic effects requires more detailed study. In the present work also no quantitative assessment of these constituents has been worked out, because the aim was to understand only the number of molecules that is present in the formulation with the target activity. The enhancement or stimulation of the immunological defense mechanism of the human body is one of the primary approaches in the Ayurveda form of treatment protocol. The immune modulatory/immunostimulant activities of many of the molecules identified in this study also proves the pharmacological basis for the therapeutic potential of these formulations. UPLC -Q-TOF-MS analysis validated the identification of compounds with the HR LCMS analysis

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Chapter 7
Conclusion

The present investigation involves a search for the phytochemical components which are responsible for the curative or ameliorative effects of two Ayurvedic formulations used to treat the various disease conditions caused by chronic inflammation. Inflammation involves a complex series of protective and reparative responses to tissue injury of the human system caused by mechanical and/or autoimmune stimuli. It can be either acute or chronic. Although inflammation has a protective role, many diseases have the etiological origin in chronic (silent) inflammatory processes. Chronic systemic inflammation is not confined to a particular tissue, but also affects many internal organs and systems. This inflammatory process is often associated with free radical damage and oxidative stress. There is a general concept that inflammation triggered by oxidative stress is the cause of many chronic diseases. Almost all of the acute and chronic diseases are driven or regulated by inflammation. The inflammatory response are complicated processes that involve the synthesis and release of large numbers of inflammatory factors. Currently the anti-inflammatory drugs approved for clinical use include non-steroidal anti-inflammatory Drugs (NSAIDs) and corticosteroids. However, the long-term administration of these compounds causes side effects and poor prognosis. Moreover, this causes serious gastrointestinal ailments and specific tissue damage. In the Ayurvedic system of therapeutics there are many formulations that were used to ameliorate oxidative stress and chronic inflammatory ailments. Medicinal plants have been the source of a wide variety of biologically active compounds. They elicit their pharmacological activity by the synergetic action of the various component molecules. Hence this investigation is undertaken to

probe and identify the molecular components of the two formulations that are prescribed to treat the derangement of *Pita Dosha* (Ayurveda classifies inflammation as the derangement of pita dosha – the fire principle). The two formulations are 1) *Prasarinyadi Kashayam*, and 2) *Gulguluthikthakam Kashayam*, the former is prepared with six medicinal herbs and, the second one uses twenty-nine plants.

The formulations under this study were acquired commercially. The methanol extracts of the two kashayams were analyzed for the identification of the different classes of phytochemicals present by classical chemical tests and by HPTLC (High Performance Thin-layer Chromatography). The result shows phenolics, alkaloids and steroids are present in Prasarinyadi kashayam and Gulguluthikthakam kashayam. The methanol extract of Gulguluthikthakam kashayam residue contain secondary metabolites like phenolics (57.9764 µg EGA/mL), flavonoids (80.75 µg equivalent to rutin /mL), alkaloids (45.66%) and steroids (8.765) in higher amount. Total amount of phenolics found to be 42.5294 µg EGA/mL in Prasarinyadi kashayam filtrate. In the Prasarinyadi kashayam residue and Gulguluthikthakam kashayam filtrate contains phenolics in the range 35-39EGA/mL. Total flavonoids in the Gulguluthikthakam kashayam filtrate found to be 51.93 µg equivalent to rutin /mL and that in the Prasarinyadi kashayam filtrate 53.22 µg equivalent to rutin /mL. Comparatively less amount of alkaloid found in Prasarinyadi kashayam filtrate (3.0833%). The alkaloidal contents of Gulguluthikthakam kashayam filtrate and Prasarinyadi kashayam residue are 31.916%and 13.875 % respectively. The total steroids in the Prasarinyadi kashayam residue, Gulguluthikthakam kashayam filtrate and Prasarinyadi kashayam filtrate are in the order 7.669%, 5.977 %and 0.1%.

Plants rich in antioxidants have been suggested as potential sources of anti-inflammatory compounds. Antioxidants regulate the oxidant–antioxidant profile of body systems by nullifying pro-oxidant molecules. Antioxidant action is associated with reduced production of ROS and free radicals and it also reduces oxidative stress, modification of proteins, and DNA damage. Since synthetic drugs are target specific, prolonged use of it causes increased risk of gastrointestinal and cardiovascular complications. It is the challenge for researchers to prepare safe antioxidants. Most of these defensive antioxidants are available from medicinal plants. The most characterized phytochemicals for antioxidant and anti-inflammatory activities are polyphenols, flavonoids and alkaloids and terpenoids to a lesser extent. Plants possessing these phytochemicals have served as sources of potent anti-oxidant and anti-inflammatory agents.

Antioxidant studies of the kashayam are carried out using approved standard protocols such as FRAP assay, hydroxyl radical scavenging assay and DPPH assay. All the extracts showed concentration dependent radical scavenging activity. Gulguluthikthakam kashayam residue exhibited high radical scavenging property than other extracts in all methods (FRAP, hydroxyl radical scavenging and DPPH assay). Quantitative analysis revealed higher concentration of alkaloids, phenolics, flavonoids and steroids in the Gulguluthikthakam kashayam residue. Other extracts also contain alkaloids, phenolics, flavonoids and steroids in a sufficient amount to exhibit radical scavenging activity. Finally, the results in this study proved that both the kashayams are a perfect source of radical scavengers and anti-inflammatory activity.

Volatile principles isolated from the kashayam using Clevenger apparatus. The percentage of volatile constituents obtained from both the

kashayam is about 0.05%. The constituent volatile principles of two kashayam have been identified with GCMS analysis. The Prasarinyadi kashayam contains 8 volatile compounds. The identified compounds are reported as antioxidant and anti-inflammatory. Twelve compounds were identified from the volatile oil of the Gulguluthikthakam kashayam. The mass spectral pattern of each of the compounds were ascertained by degradation reactions. All the identified compounds were reported to have antioxidant, anti-inflammatory and immunostimulant activities by many earlier investigators. Hence, the results of this study may be considered as a scientific validation of the therapeutic utility of Prasarinyadi kashayam and Gulguluthikthakam kashayam as an anti-inflammatory agent.

High resolution liquid chromatography/Mass spectroscopy is the state of the art analytical and diagnostic technique used to identify molecules in complex mixtures and plant extracts. LCMS, a hyphenated system which is a selective and sensitive technique for the identification and quantification of complex mixtures. Accurate mass data which allows for identification of molecular formulas of compounds could be obtained. The components are identified by matching the spectra with reference spectra of the library software.

Total Ion Chromatogram (TIC) of methanol extract of Prasarinyadi kashayam has given a thickly packed mass peaks with the relative intensity that vary from 5 to 100. This when subjected to HR LCMS analysis could identify more than 100 peaks. Out of them, 42 compounds have been identified tentatively. Out of 42 compounds identified 24 are phenolic compounds, 11 alkaloids, 5 terpenes, 1 alkane and an acid. Out of the identified compounds, 28(or 66%) compounds were reported as antioxidants, immunomodulatory/ immunostimulant and anti- inflammatory activity by several groups of researchers.

The kashayams are suspensions and the aqueous supernatant part will contain the dissolved constituents. To ascertain whether this supernatant also has a comparable component with that of the whole extract of the kashayam, analysis was carried out with the centrifuged supernatant. Then this supernatant is subjected to UPLC-ESI-QTOF MS analysis. This sophisticated technique is a very efficient analytical tool for the separation and high accuracy mass measurements. Nineteen (19) compounds were identified from the supernatant of Prasarinyadi kashayam. Phenolic compounds identified from the supernatant include 6-Gingerol, Tetrahydrocurcumin, 4-Methyl-6-gingerol, 7-Hydroxyhinokinin, Tectochrysin-5-β-glc, Kaempferol-8C-glc-3, 5-glc, glc, 4-O-methyl syringic acid, Marmesin, Quercetin and Syringaresinol. Phenolic compounds except 7-Hydroxy hinokinin and Techtochrysin 5-β-glc were sighted as anti-inflammatory. Vasicinolone and Berberine, the alkaloids, reported as anti-inflammatory which were identified from the supernatant. The terpenoids identified from the supernatant include Ecdysterone, Curzerenone, and Asaraldehyde , were reported as anti-inflammatory. These identified compounds were also analyzed by fragmentation reactions to ascertain the structural characteristics. Result shows that the supernatant is rich in phenolic compounds, possessing anti-inflammatory activity. Anti-inflammatory property of the identified compounds validates the efficacy of this Ayurvedic formulation's therapeutic use.

Densely packed mass peak was obtained in the TIC of methanol extract of Gulguluthikthakam kashayam with the relative intensity which varies from 5 to 100. More than 100 mass peaks were observed in HRLCMS analysis conducted on the same extract. 73 mass peaks were tentatively identified. This includes different class of biologically active

compounds such as alkaloids, terpenoids and phenolics. Out of 73 compounds identified, 30 compounds were terpenoids, 23 were phenolic and 20 were alkaloids. The 66% of the identified compounds were reported as immunostimulant, antioxidants and anti-inflammatory activities by many groups of researchers. Remaining compounds may increase the activity of the lead compound, stabilize the compound or help their absorption. From the supernatant, thirty-four compounds were identified when subjected to UPLC-ESI-QTOF-MS analysis. Identified compounds contain 8 alkaloids, 12 terpenes and 14 phenolic compounds. Twenty-four identified compounds were further validated with the mass fragmentation reaction pattern. Marmesin , Rutin, Hexahydrocurcumin, Purpurin, Galangin, Matairesinol, 3-Methylgingerdiol, Galangin 3-methyl ether, 6-Gingerol, 1, 4-Dihydroxy anthraquinone, 2, 4 – Dihydroxyanthraquinone, Nordamnacanthal, Rubiasin C and Angelicin were identified from the supernatant. It is already reported that all the phenolic compounds identified by UPLC-QTOF are anti-inflammatory. Terpenes identified from the supernatant include Melianin A, Chinovic acid, 6-Deacetylnimbin, Platycodigenin, Arjunolic acid, Z-Guggulsterol, Rubifolic acid, Odaratone, Guggulusterone VI, Guggulusterone, Calcaratarin D and Nimbionone. Rubifolic acid reported as anti-oxidant. Except Nimbionone and Odoratone all other compounds were reported as anti-inflammatory compounds. Piperine, Trans feruloylctapamine, Vasicinone, Coumaperine, Tetrahydropiperine, Vasicinolone, Solasodine (3->1) β-gal (2->1) rhamnose and Trans-feruloyl piperidine were the eight alkaloids identified from the supernatant. Alkaloids identified were reported as anti-inflammatory except Solasodine (3->1) β-gal (2->1) rhamnose. Thus, it has been confirmed that the supernatants separated from the Gulguluthikthakam kashayam are rich sources of anti-inflammatory and antioxidant compounds.

Both kashayam were rich in phenolic compounds, terpenoids and alkaloids. Phenolic compounds were the compound, which were widely studied for its anti-oxidant and anti-inflammatory properties. Polyphenols target multiple inflammatory components and lead to anti-inflammatory mechanisms. Phenol's control or prevent the inflammation related disease (cancer, diabetes, Alzheimer's, aging) by different mechanisms. They involve radical scavenging, metal chelating, NOX inhibition, tempering the mitochondrial respiratory chain, inhibition of certain enzymes involved in ROS production, like xanthine oxidase and upregulation of endogenous antioxidant enzymes. Terpenoids and alkaloids are other classes of phytochemicals to lighten inflammation related diseases through diverse mechanisms.

The findings of the study recommend that the kashayam which investigated for the present study are highly effective free radical scavengers and possess anti –inflammatory properties.

In the present work no quantitative assessment of these constituents has been worked out, because the aim was to understand only the number of different phyto-chemical constituent molecules that is present in the formulation with the target activity. The enhancement or stimulation of the immunological defense mechanism of the human body is one of the primary approaches in the Ayurvedic form of treatment protocol. The immune modulatory / immunostimulant activities of many of the molecules identified in this study also proves the pharmacological basis for the therapeutic potential of these formulations.

From the observations and the results obtained it may be concluded that the Prasarinyadi kashayam and Gulguluthikthakam kashayam are rich source of secondary metabolite such as alkaloids,

terpenes and phenolics having anti-inflammatory activity, and both are formulations that proved their efficacy in treatment. Anti-inflammatory activity is a complex cascade of reactions that finally produce that effect by acting by multitude of reaction pathways. The present work is the identification of the molecules that produce the target activity using the modern technique of HRLCMS and ESI-QTOF- MS and MSMS. Hence a discussion of the various mechanisms and multitude pathways in which the final effect is produced is not presented here. However, no quantitative assessment of the active constituents has been carried out. Also, no search is made to ascertain whether all the identified molecules are essential for the therapeutic effect of the formulations. A moot question is whether all these constituents are required for the therapeutic efficacy of the formulations?

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Publications

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