Validation and Evaluation of Some Indian Medicinal Plants Using Chromatography and Near Infrared Spectroscopy

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Declaration

I hereby declare that my research work embodied in this Ph.D (Pharmacy) thesis entitled "**Validation and Evaluation of Some Indian Medicinal Plants Using Chromatography and Near Infrared Spectroscopy**" have been carried out by me in the Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, India, Under the Supervision of **Prof. Pulok K. Mukherjee**, Director, Institute of Bioresources and Sustainable Development, Dept. Of Biotechnology, Ministry of Science & Technology, Govt. of India, Takyelpat, Imphal-795001, Manipur, India & Professor (on lien), Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, India & **Prof. Rajib Bandyopadhyay**, Department of Instrumentation & Electronics Engg. Jadavpur University, Kolkata-700098. I also confirmed that this work is original and has not been submitted partly or in full for any other degree or diploma to this or other University or Institute.

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Statement of Originality

I, Shibu Narayan Jana registered on 18.06.2019 to do hereby declare that this thesis entitled "**Validation and Evaluation of Some Indian Medicinal Plants Using Chromatography and Near Infrared Spectroscopy**" contains literature survey and original research work done by the undersigned candidates as part of Doctoral studies. All information in this thesis have been obtained and presented in accordance with existing academic rules ethical conduct. I declare that as required by thesis rules and conduct. I have fully cited and referred all materials and results that are not original to this work. I also declare that I have checked this thesis as per the "Policy on Anti-plagarism, Jadavpur University, 2019", and the level of similarity as checked by iThenticate software is 9%.

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List of Publication/s:

1. Quantification of piperine in different varieties of *Piper nigrum* by a validated high-performance thin-layer chromatography–densitometry method. **Shibu Narayan Jana**, Dilip Sing, Subhadip Banerjee, Pallab Kanti Haldar, Barun Dasgupta, Amit Kar, Nanaocha Sharma, Rajib Bandyopadhayay, Pulok K Mukherjee, JPC–Journal of Planar Chromatography–Modern TLC, 34, 2021, 521-530.
2. Quantification and Standardization of andrographolide in *Andrographis paniculata samples* by validated RP-HPLC and HPTLC methods. **Shibu Narayan Jana**, Subhadip Banerjee, Sayan Biswas, Dilip Sing, Amit Kar, Rajib Bandyopadhayay, Pallab K Haldar, Nanaocha Sharma and Pulok K. Mukherjee, Journal of Chromatographic Sciences, Oxford University Press. (**Accepted, "in press"**).
3. Marker analysis and Antidiabtic potential of *Piper nigrum* L. seeds. **Shibu Narayan Jana**, Dilip Sing, Subhadip Banerjee, Pallab Kanti Haldar, Barun Dasgupta, Amit Kar, Nanaocha Sharma, Rajib Bandyopadhayay, Pulok K Mukherjee, Orient Pharm Exp Med (Communicated).
4. Quality evaluation and quantification of gallic acid in different varieties of *Emblica officinalis* by a validated high performance thin-layer chromatography-densitomety method. **Shibu Narayan Jana**, Amit Kar, Barun Dasgupta, Subhadip Banerjee, Pallab Kanti Haldar, Rajib Bandyopadhyay, and Pulok K. Mukherjee, Journal of Planar Chromatography. ("Under review").
5. Estimation of gallic acid in *Emblica officinalis* samples using Near infra-red spectroscopy and Reverse-phase high performance liquid chromatography. **Shibu Narayan Jana**, Dilip Sing, Subhadip Banerjee, Rajib Bandyopadhyay, and Pulok K. Mukherjee, Phytochemical analysis. (Communicated).

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List of Presentations in National and International Conference

1. Participated and presented an Oral presentation at 5th Convention Society for Ethnopharmacology, India (SFE 2018, Sept 7-8) at Jadavpur University, Kolkata "Quality evaluation and standardization of Piperine in *Piper nigrum* samples using RP-HPLC and NIRs" **Shibu Narayan Jana**, Dilip Sing, Barun Dasgupta, Subhadip Banerjee, Rajib Bandyopadhyay, and Pulok K. Mukherjee.
2. Participated and presented a poster at 7th International Congress of Society for Ethnopharmacology, India (SFEC 2020, Feb 15-17) at Jamia Hamdard, New Delhi "HPLC and HPTLC method for quantification and standardization of andrographolide in *Andrographis paniculata* extract'' **Shibu Narayan Jana**, Dilip Sing, Rajib Bandyopadhyay, Subhadip Banerjee, and Pulok K. Mukherjee.
3. Participated and presented a poster at 8th convention Society for Ethnopharmacology, India (SFE 2021, Dec 10) "Marker profiling and antidiabetic potential of *Emblica officinalis*" **Shibu Narayan Jana**, Amit Kar, Barun Dasgupta, Dilip Sing, Rajib Bandyopadhyay, Subhadip Banerjee, Shibu Narayan Jana, and Pulok K. Mukherjee.
4. Participated and presented an Oral presentation at 9th convention Society for Ethnopharmacology, India (SFE 2022, Sept 23-24) at Jadavpur University, Kolkata "Estimation of gallic acid in *Emblica officinalis* samples using Near infra-red spectroscopy" **Shibu Narayan Jana**, Amit Kar, Dilip Sing, Barun Dasgupta, Rajib Bandyopadhayay, Pallab K Haldar, and Pulok K. Mukherjee.

Content

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Shibu Narayan Jana

Abbreviations Used

µL	Microliter
µM	Micromolar
Mg	Miligram
µm	Micrometer
mL	Mililiter
Ng	Nanogram
HPLC	High-performance liquid chromatography
HPTLC	High performance thin layer chromatography
RP-HPLC	Reverse phase high performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
Σ	Standard deviation
S	Slope
RSD	Relative standard deviation
%	Percentage
LQC	Low quality control
MQC	Medium quality control
HQC	High quality control

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This thesis is dedicated to my family and labmates…..

Chapter 1

Introduction

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1.1. Importance of quality evaluation and validation of medicinal plants

Medicinal plants prevent and manage different diseases, also provide nutritional benefit. World Health Organization stated that, 80% of the world population depend on medicinal plants (Mukherjee et al., 2012). There is a great demand for herbs and medicinal plants in developed and developing countries and therefore a great potential in the global market (Sofowora et al., 2013; Ekor, 2014). Medicinal plants are used in various forms i.e. leaves, flowers, fruits, woods, barks, roots, gums, resins, essential oil etc. and formulations such as extracts, tinctures etc. for prevention and management of diseases (DeFilipps & Krupnick, 2018). In India, AYUSH systems of medicines are used for preventing and managing of diseases (Mukherjee & Wahile, 2006; WHO, 2005; Veeresham, 2012). Over the past few decades, public interest and acceptance of natural remedies has increased in both developing and developed countries (Mukherjee et al., 2010). No single herb is recommended for a particular health condition, nor is there a single ailment associated with a particular herb (Wachtel & Benzie, 2011). Plants contain various chemicals responsible for their medicinal properties, and these chemicals are known as active ingredients (Mukherjee et al., 2013). These moieties depends on factors such as plant species, harvesting time, nature of soil (Chen et al., 2016). In most cases, the active ingredients behind the biological activities of herbs are unknown and their potency is lower compared to synthetic drugs. These drugs are used very effectively against chronic diseases with a low incidence of side effects (Wink, 2015). Biomarkers are important in determining the efficacy and potency of medicinal plants. Phytomarkers are used in a variety of field of studies, such as validation of original species, exploration for novel products and materials, extraction and purification process

optimization, structural elucidation, and quality evaluation (Mukherjee et al., 2015). Phytomarkers through scientific investigation can contribute to the identification and discovery of novel medications (Mukherjee et al., 2011; Mickymaray, 2019).

Quality control importance to product safety, efficacy and acceptance and is an essential process in the pharmaceutical industry (Mukherjee & Houghton, 2009; Yu et al., 2014). Essentially, quality control is based on three key pharmacopoeial definitions: identity, purity and content of active botanicals (Mukherjee & Verpoorte, 2003, Mukherjee et al., 2016). Harvesting, drying, storage, transportation, and processing methods (extraction type, extraction solvent polarity, component instability, etc.) also affect herb quality (Zhang et al., 2018). Regulation of norms of the medicinal products are lenient when compared to the synthetic medicines. Because of this, quality of the herbal products are decreasing by adulteration, spurious drugs, substitution of drugs (Sahoo et al., 2010). It can lead to adverse effects on the health of the consumers. So controlling the quality of the herbal products and its preparations is very much required for the betterment of the mankind (Mukherjee et al., 2011). Standardization measures during the manufacturing process and quality control result in reproducible quality of a given product, pre-defining the quantity, quality and therapeutic efficacy of each dose molecule (Mukherjee, 2001). Standardized plant extracts containing specific compounds and quality controlled during the growing, harvesting and production process (Folashade, 2012). Phyto-marker compound selection basically depends upon a different factors such as stability, ease of analysis, time and cost of analysis, and therapeutic effect. Controversial identity and adulteration of plant material are very challenging for

standardization of botanicals. Other additional issues are also related to standardization of herbal products. Herbal products are usually mixtures of many botanicals, in most cases the active ingredients are unknown, selective analytical methods and reference compounds may not be commercially available. Herbal products vary chemically and naturally, source and quality of ingredients vary (Thomford et al., 2018; Mukherjee et al., 2014).

Marker profiling or fingerprinting of a medicinal product is a pattern of the extract of some common chemical constituents of biologically active characteristics (Mukherjee et al., 2013). Good quality control requires consideration of multiple botanicals in herbal medicines, rather than considering one or two individual marker compounds to assess product quality. However, all medicinal plant products and their extracts contain hundreds of unknown ingredients, many in very small amounts (Mukherjee, 2002; Li et al., 2010). It is very important to obtain chromatographic fingerprints that represent biologically active molecule of medicinal product. Many organizations like as WHO, USFDA have developed and approved several tools to identify and quantify the marker compound in herbs and herbal medicines (Noviana et al., 2022). RP-HPLC and HPTLC fingerprinting includes the recording of chromatograms, retention times of individual peaks, and absorption spectra in different mobile phases. Thus, quality evaluation, validation, chemoprofiling and standardization are essential approaches for determining and quantifying of the active phytomolecule(s) and linking the chemical properties to the pharmacological activities (Mukherjee et al., 2015; Ipek, 2020). A few important medicinal plants, their major active compounds, and pharmacological activity are shown in Table 1.1.

Table 1.1. Important medicinal plants, major active compounds and their pharmacological activity

Medicinal plant	Active compounds	Pharmacological activity	Reference
Andrographis paniculata	Andrographolide, Neoandrographolide, Isoandrographolide, Apigenin	Treatment in fever, inflammation, upper respiratory tract infection and hepatic disorder	Low et al., 2015; Suebsasana et al., 2009
Allium sativum	Ajoenes, allicin, and alliin	Hypoglycaemic, hypolipidaemic, antihypertensive, hepatoprotective and immunomodulatory	El-Saber et al., 2020; Foroutan et al., 2017
Aloe vera	Emodin, P-coumaric, gallic, vanilic, ferulic, cinamic and ellagic acids.	Wound healing, anti-inflammatory, hypoglycaemic and gastroprotective	Hamman, 2008
Azadirachta indica	Nimbanene, nimbiol and nimbin,	Antibacterial, antimalarial and used in skin infection	Al et al., 2017
Berberis vulgaris	Berlambine, hydroxycanthine, tejedine	Used to treat skin infections	Mahm et al., 2014

Medicinal plant	**Active compounds**	**Pharmacological activity**	**Reference**
Bacopa monnieri	Bacoside, apigenin, brahmine, herpestine, and hersapomin	Memory improver, antiinfluenzal and anti-inflammatory	Kumar et al., 2016
Boswellia serrate	Boswellic acid, lupeolic acid, and acetyl lupeolic acid	Used to treat inflammatory bowel disease, rheumatoid arthritis, and asthma	Siddiqui & Boswellia, 2011
Capsicum annum	Capsaicin, caffeic acid, luteolin, and vitexin	Hypocholesterolemic, antiviral, and antibacterial	Batiha et al., 2020
Centella asiatica	Asiaticoside, asiatic acid, Madecassic acid and centellicin	Antimicrobial and antiinfluezal	Arribas et al., 2022
Calendula officinalis	α-cadinol, cadinenes, and copane	Used in treatment of wounds and soothing skin	Arora et al., 2013
Curcuma longa	Curcumin, cyclocurmin, coumaric and sinapic acid	Antitumour, antioxidant, antiarthritic, and anti-inflammatory	Ruby et al., 1995; Bagad et al., 2013
Citrus limon	Sabinene, 3-carene, limonene and β-ocimene	Used for in coughs and sore throat	Klimek et al., 2020

Medicinal plant	**Active compounds**	**Pharmacological activity**	**Reference**
Digitalis lanata	Digitoxin, digitoxigenin and lanatoside C	Digoxin is used as an antiarrhythmic and inotrope	Gurel et al., 2017
Emblica officinalis	Gallic acid, methylgalate, ellagic acid, and linoleic acid	Antioxidant, intiinflammatory, adaptogenic and hepatoprotective	Gaire et al., 2014
Ephedra sinica	Ephedrine, pseudoephedrine, N-methylephedrine, methoxykynurenic acid	Bronchodilators and decongestants	Munafò et al., 2021
Erythroxylum coca	Cocaine	Anesthetic	Redman et al., 2011
Ferula assa-foetida	Carvacrol, α-bisabolol, b-ocimene	Hypocholesterolemic and antiasthmatic	Iranshahy et al., 2010
Fumaria officinalis	Chelidonine, protopine, and stylopine	Used in skin infections and conjunctivitis	Raafat et al., 2020
Gingko biloba	Ginkgolide A, ginkgolide B and ginkgoside B	Antioxidant, anticancer, antialzheimer and antidepressant	Rojas et al., 2011; Shi et al., 2010

Medicinal plant	**Active compounds**	**Pharmacological activity**	**Reference**
Glycyrrhiza glabra	Glycyrrhizin, glabridin, Glycyrrhizin, glabridin licochalcone A	Antiinflammatory, antiulcer, antioxidant, hepatoprotective, antibacterial, and antiviral	Yang et al., 2016; Pastorino et al., 2018
Geranium robertianum	Ellagitannin, hexahydroxydiphenylglucose	Used in wound healing, tooth decay and nasalbleeds	Catarino et al., 2017
Hypericum perforatum	Hypericin, chlorogenic acid, caffeic acid	Antidepressant	Okmen & Balpınar, 2016
Lavandula angustifolia	Camphene, sabinene and limonene	Antiseptic and antipsychotic	López et al., 2017
Lawsonia inermis	Eugenol, hexadecanoic acid, phytol and α-terpineol	Antibacterial, analgesic and anti-inflammatory	Nesa et al., 2014
Momordica charantia	Momordicine I, momordicine II,	Hypoglycaemic, and adaptogenic	Jia et al., 2017
Morinda citrifolia	Americanin A, asperuloside, borreriagenin, citrifolinin B	Used in arthritis, joint pain and skin infections	West et al., 2018

Medicinal plant	**Active compounds**	**Pharmacological activity**	**Reference**
Nelumbo nucifera	Betulinic acid, nelumnucifoside A and nelumnucifoside B	Antiinfluenzal, anti-inflammation, anticancer	West et al., 2018; Bishayee et al., 2022
Ocimum sanctum	Carvacrol, oleanolic acid, ursolic acid, rosmarinic acid, eugenol, carvacrol, linalool, β-caryophyllene	Used in oxidative stress, microbial infections, and hypertension	Zhao et al., 2017; Cohen, 2014
Piper nigrum	Piperine, α-pinene, β-pinene, Sabinene, Myrcene and pipericide	Antiageing, adaptogenic, and immunomodulator	Meghwani et al., 2018; Bajad et al., 2001; Pathak & Khandelwal, 2009
Piper methysticum	Kavain, methysticin, yangonin	Used in tradional medicine to treat fever, respiratory problems, convulsion and urogenital problems and asthama	Selvendiran & Sakthisekaran, 2004; Salehi et al., 2019; Bian et al., 2020

Medicinal plant	**Active compounds**	**Pharmacological activity**	**Reference**
Silybum marianum	Silibinin, silychristin, isosilybin a, taxifolin, and silidianin	Hepatoprotective	Vargas et al., 2014
Terminalia belerica	Gallic acid	Anti-atherosclerotic, hepatoprotective, cardioprotective,	Parveen et al., 2018
Terminalia chebula	Punicalagin, terflavin A, terchebulin, terchebin, and neo-chebulic acid	Liver protective, cardiotonic, anticancer and antimicrobial	Bag et al., 2013; Choi et al., 2015
Withania somnifera	Withanolide A, withanolide B, withanone, withasomnine, withanoside iv	Antitumour, antituberculosis and antiulcer	Saleem et al., 2020
Youngia japonica	Chlorogenic acid, chicoric acid, caftaric acid	Antitussive, used in treatment of boils and snakebites	Badalamenti et al., 2022
Zingiber officinale	Gingerol, β-bisabolene, α-curcumene, zingiberene, α-farnesene, and β-sesquiphellandrene	Antiviral, antimicrobial, antinausea, antiasthmatic	Rahmani et al., 2014; Khan et al., 2014

1.2. Chromatographic approaches for quality evaluation and validation of medicinal plants

Chromatographic techniques are used in process development, identification, standardization and detection of adulterants in medicinal products, and in quality control of plants and health products (Pratiwi et al., 2021). Chemical fingerprinting helps to authenticate, identify and quantify plant molecules and can link chemical composition to aspects of bioactivity for standardization and validation of products (Mukherjee et al., 2015). Marker compounds are used for maintaining quality of natural products derived from plant sources (Cristians et al., 2018). A Metabolomics approach in drug research and development by identifying and profiling of secondary metabolites from plant sources will be a great tool (Breitling et al., 2013). There are over 200000 plant secondary metabolites known from plant sources (Gomez et al., 2013). Metabolomics study divided into categories: (a) target compound analysis (b) the metabolomic profiling (c) metabolite chemo-analysis (d) metabolomic fingerprinting (Aderemi et al., 2021). Chemo profiling of the metabolites by various techniques such as RP-HPLC, HPTLC, LC-MS, GC-MS, NIRs, NMR and Raman spectroscopy can estimate the metabolites and nature of compounds (Piasecka et al., 2019; Castelli et al., 2022).

High performance thin layer chromatography (HPTLC), is a highly sophisticated analytical technology used for the analysis, fingerprinting and marker analysis of herbal extracts. HPTLC densitometry standardization and marker profiling of different medicinal plants have been reported from our laboratory (Biswas &

Mukherjee, 2019; Pandit et al., 2011; Gantait et al., 2012; Kumar et al., 2007). Different advantages of HPTLC are:

a) It is a high throughput and non-destructive technique,

b) Compounds present in minute quantities (µg or ng) may be visualized,

c) Separate systems and visual inspection reduces grouping errors,

d) The comparisons are achieved through visual inspection.

HPTLC is an improvised and highly sensitive version of conventional TLC technique. Modern HPTLC is performed on Aluminium-backed HPTLC plates. The thickness of the Silica Gel layer in this type of plate is 200-250 µm (Chandel et al., 2012). In food industry, HPTLC can used for identification of complex mixtures and detection of adulteration. HPTLC is quick, risk-free, effective, and inexpensive, and it can analyze a large amount of samples per day without generating a lot of waste (Baetz et al., 2021). Pharmaceutical industries use HPTLC to ensure safety, efficacy quality, content uniformity, identity of phyto-active molecules, detection of adulterants, analysis of medicinal plants and herbs, analysis of pesticide mixtures (Mukherjee et al., 2019). HPTLC is a highly flexible analytical approach that provides excellent separation potency through the use of precise sample application, software-controlled chromatographic estimations, chromatogram creation scanning, and photo documentation (Attimarad et al., 2011). In recent years, HPTLC fingerprint analysis used for naturally occurring products is the perfect approach for quality evaluation and validation therapeutic products (Frommenwiler et al., 2019).

High Performance Liquid Chromatography (HPLC) is an advanced technique of column liquid chromatography. This is a common analytical technique used in the pharmaceutical industry to separate, identify and quantify individual components of mixtures (Coskun, 2016). Reverse-phase High performance liquid chromatography (RP-HPLC), is an advanced separation and analytical technique used in quality assurance, quality control, and natural product research laboratories (Chen et al., 2021). The most widely column used is C18, which is better known as the octadecylsilane column. The column is packed with silica particles, which are modified hydrophobically by adding an eighteen carbon chain to it through a siloxane bonding. The mobile phase contains a mixture of two solvents. One of the solvents is usually an aqueous phase, consisting of water (HPLC grade), whose pH has been adjusted with an orthophosphoric acid or glacial acetic acid to a desired pH and another organic solvent, such as Methanol or Acetonitrile (HPLC grade) (Li et al., 2010). Currently, this fingerprinting analysis is the main choice for quality control and validation of herbs and its preparations (Bārzdiņa et al., 2022). Several authors reported the use of RP-HPLC for characterization and quantification and quality control of secondary metabolites in plant extracts, mainly phenol, phenolics, steroids, flavonoids, glycosides and compounds (Bruck et al., 2020). HPLC fingerprint of herbal products is essential to authenticate plant material, maintain quality, safety and quatify of phytochemically active molecules (Mukherjee et al., 2019). For separation of the phytoconstituents from polyherbal preparations, HPLC one of the well accepted method. For the quality evaluation and standardization of the thermo-liable substance, one of the target method is HPLC (Zhang et al., 2018). RP-HPLC can be effective for quality evaluation of multi-phytoconstituents medicinal compounds.

The technique RP-HPLC serves high reproducibility and automation in the quantification and identification of multiple phyto-constituents of herbs (Abbas et al., 2021; Sharma et al., 2020).

1.3. Near Infra-red spectroscopic technique for quality evaluation and validation of medicinal plants

The near-infrared spectroscopy (NIR) region was the first known invisible portion of the electromagnetic spectrum, discovered by Herschel in 1800 (Manley, 2014; Krzysztof & Christian 2019). Near Infrared (NIR) spectroscopy is a popular analytical approach which has been described in the European Pharmacopoeia since 1997 (Zuo et al., 2020; Leong et al., 2020). Near infra-red spectroscopy is used to predict the content of total polyphenols, flavonoids, alkaloids, and catechins (Wu et al., 2020; Bai et al., 2022). This method shows great importance for authentication, quality evaluation and validation purposes (Beć et al., 2020). It also shows high potential for classifying medicinal compounds and estimating adulteration (Couto et al., 2021). This spectroscopy is applied for estimation of single or multiple components in herbs or herbal products (Gavan et al., 2018). Analytical spectra in the frequency range 4000–12,500 cm^{-1} (800–2500 nm) contain a lot of relevant information about organic molecules (Sankom et al., 2021). International Standards Committees have accepted NIR as the official analytical method for quantifying many compounds (Kasemsumran et al., 2022). Near infra-red spectroscopy (NIRs) has been successfully used in pharmaceutical industry, tea industry for assessment of quality of tea leaves, food industry for food safety assessment, environmental chemistry, microfluidics, biomolecules, cancer diagnosis and explosive detection (Sing et al., 2021). The use of NIR spectra

to detect and infer specific chemical constituents has been proven in various fields (Ma et al., 2019). NIR spectroscopy uses liquid and solid samples without any pretreatment (Fu et al., 2008). In recent times, NIR spectroscopy is widely applying in pharmaceutical purposes for raw material testing, and quality control of products (Pu et al., 2021; Liu et al., 2015). As per the American Society for Testing and Materials, the NIR portion of the electromagnetic spectrum is the wavelength range from 780–2526 nm, which corresponds to the wave number range of 12820–3959 cm^{-1} (Gao et al., 2021; Zhang et al., 2022). Fast and nondestructive NIRs estimations without any sample pre-treatments may increase the analytical throughput significantly (Luypaert et al., 2007). Fiber optic probes allow in-line and on-line process monitoring. A Number of research and review articles are reported on NIR spectroscopy in medicinal research, and quality control of medicinal products (Blanco et al., 2008; Candolfi et al., 1999). Determination of mean particle size or particle size distribution by NIR spectroscopy using lactose monohydrate has been reported (Jørgensen et al., 2004), microcrystalline cellulose (Neil et al., 1998; Fix et al., 2004) , NaCl, sorbitol (Cantor et al., 2011), aspirin, caffeine and paracetamol (Grabska et al., 2021), and piracetam (Karamancheva et al., 2000), as model excipients and active ingredients, respectively.

1.4. Botanicals for the management of diabetes mellitus

Diabetes is an endocrine disorder resulting from insulin deficiency or the availability of insulin produced by the body (Tabish et al., 2007). It is caused by destruction of pancreatic beta-cell that do not secrete enough insulin for proper function, or the insulin receptors that do not respond to insulin (American Diabetes Association, 2009; Wilcox, 2021). Susurtha, the father of Indian medicine, was diagnosed in 1000 BC. Chr.Diabetes (De et al., 2018). The discovery of insulin by Banting and Best in 1922 was an important milestone in the treatment of diabetes (De et al., 2018). Different animal models for diabetes include partial pancreatectomy, alloxan or streptozotocin (STZ) models, high-fat diet models, fructose feeding models, and intrauterine growth retardation models.

The initial approach to type-2 diabetes mellitus treatment involves a combination of efficient lifestyle changes and the use of pharmaceutical drugs. The first pharmacological intervention for type-2 diabetes was with insulin isolated from the pancreas of animals in the 1920s. In recent years, many oral and injectable drugs have been developed as therapeutic agents for patients with type-2 diabetes. These drugs are intended to lower blood sugar levels, and reduce weight, and reduce the risk of cardiovascular damage. Various oral diabetes medications can be divided into the following categories: insulin sensitizers (metformin, rosiglitazone), insulin secretagogues (Tolbutamide, Chlorpropamide, Glipizide and Repaglinide), alpha-glucosidase inhibitors (Acarbose, Miglitol), main mechanism action is Decrease glucose absorption from intestine, peptide analogs (Exenatide, Sitagliptin, Saxagliptin, linagliptin, Pramlintide), acts by Increase

incretin levels which inhibit glucagon release and increases insulin secretion, and Insulin treatment, main mode action is Decrease glucose production (Lorenzati et al., 2010).

Active subsatances of animal active origin or substances extracted from herbs or herbal products can be useful in both preventing or supporting the therapy of hyperglycaemic conditions. Naturopathic products or herbal supplements for treating type-2 diabetes focus on regulating of insulin secretion, improving insulin resistance, and insulin sensitivity. They also influence hyperlipidaemic conditions, over-weight and diabetic complications, and improve health conditions. At the present scenerio, the efficacy and safety of natural products is lower than that of pharmaceutical drugs. Non-flavonoids polyphenols, phytosterols, betaine, carotenoids, fruits, vegetables and other bioactive products may improve insulin secretion (Mutha et al., 2021). Hypoglycaemic natural products and their mechanism action are described in Table 1.2.

Table 1.2. Some antidiabetic medicinal plants, their bioactive compound and mode of action

Medicinal plant	Bioactive compound	Mode of action	Reference
Curcuma longa	Curcumin	NF-κB activation and reduction of inflammatory cytokine levels	(Hewlings & Kalman, 2017)
Polygonum cuspidatum	Resveratrol	Protects β-cells from oxidative stress	(Park et al., 2018)
Solanum paniculatum	Tannins	Hypoglycaemic, stimulate glucose transport	(Vijayakumar et al., 2005)
Aegle marmelos	Marmelosine	Increase activity of pancreatic β-cells	(Narendhira & Subramanian, 2010)
Allium sativum	Allicin	Improve plasma lipid metabolism	(Najman et al., 2022)
Aloe borbadensis	Beta-sitosterol	Improvement in impaired glucose tolerance	(Alinejad et al., 2015)
Andrographis paniculata	Andrographolide	Increases the glucose utilization	(Akhtar et al., 2016)
Black rice	Anthocyanins	prevent free radical production	(Lobo et al., 2010)
Emblica officinalis	Gallic acid	Decreases lipid peroxidation, antioxidant	(Kapoor et al., 2019)

Medicinal plant	**Bioactive compound**	**Mode of action**	**Reference**
Green tea	Epigallocatechin gallate	Antioxidant and anti-inflammatory abilities, enhances glucose-stimulated insulin secretion, hypolipidaemic and hypocholesterolemia	(Ohishi et al., 2016)
Glycyrrhiza glabra	Quercetin	Enhanced glucose uptake by insulin-dependent MAPK mechanism, increased phosphorylation of PI3K/Akt signaling pathway, interaction with PPARγ receptor	(Zhang et al., 2019; Liu et al., 2018)
Arabidopsis thaliana	Naringin	Reduces blood glucose, cholesterol levels and antioxidant	(Aghajanyan et al., 2017)
Eucalyptus macrorhyncha	Rutin	Reduces blood glucose level, modulates and increase insulin secretion, hypolipidaemic and antioxidant	(Kreznar et al., 2017)

Medicinal plant	Bioactive compound	Mode of action	Reference
Ginkgo biloba	Kaempferol	Increases insulin sensitivity by inhibiting proinflammatory cytokines, reduces inflammatory response and inflammatory liver lesions	(Li et al., 2021; Kotakadi et al., 2008)
Carica papaya	Papain	Reduce fasting blood glucose, triglyceride and cholesterol	(Juárez et al., 2012)
Cassia auriculata	Limonene	Regulates hepatic hexohinase	(Nille et al., 2021)
Piper nigrum	Piperine	Prevent free radical production	(Saetang et al., 2022)
Catharanthus roseus	Vincristine	Lowering of glycaemia	(Nammi et al., 2003)
Coriandrum sativum	p-cymene	Increases the function of β-cells and decrease serum glucose	(Eidi et al., 2009)
Cinnamomum tamala	β-caryophyllene	Insulin release from pancreatic β–cells, increase insulin activity	(Rorsman & Ashcroft, 2018)

Medicinal plant	Bioactive compound	Mode of action	Reference
Eugenia jambolana	Ellagic acid	Inhibited insulinase activity from liver and kidney	(Achrekar et al., 1991)
Mangifera indica	β-carotene	Reduction in the intestinal absorption of glucose	(Shah et al., 2010)
Momordica charantia	Charantin	Activates PPARs α	(Chao et al., 2003)
Punica grantum	Punicalagin	Inhibit α - glycosidase activity and decrease glucose absorption	(Laaraj et al., 2022)
Ocimum sanctum	Eugenol	Regulates insulin release	(Pattanayak et al., 2010)
Terminalia arjuna	Arjunic acid	Decrease the blood glucose level and decrease the activities of G6P	(Ragavan et al., 2006)
Zingiber officinalis	Gingerol	Increases insulin level	(Li et al., 2012)

The combination therapy of pharmaceutical drugs and herbs or herbal preparations provides a more effective treatment for type 2 diabetes mellitus. Moreover, synergistic effect is helpful in reducing the dose of antidiabetic drugs. These combination therapy associated with minimal numbers of side effects. Possible combinations of pharmaceutical agents and herbs or herbal products are

mentioned below. Combination therapies with their synergistic effects are described in Table 1.3.

Table 1.3. Combination therapies with their synergistic effects

Combination therapy	**Synergistic effects**	**Reference**
Glibenclamide + *Allium sativum* extract	Decreases plasma glucose levels	(Poonam et al., 2013)
Gliclazide + *Allium sativum* extract	Hypoglycaemic	(Alam et al., 2022)
Glimepiride + *Curcuma longa* extract	Hypolipidaemic and antioxidant	(Ali, 2002)
Repaglinide + *Curcuma longa* extract	Inhibits lipid peroxidation, enhances serum insulin levels	(Lee et al., 2017)
Acarbose + green tea extract	Inhibits alpha amylase and alpha glucosidase activities	(Yilmazer et al., 2012)
Exendin-4 + epigallocatechin gallate	Hypoglycaemic, enhances pancreatic beta cell function and increase insulin secretion and hypolipidaemic	(Chang et al., 2013)
Metformin + resveratrol	Hypoglycaemic, hypolipidaemic and hypocholesterolemic	(Dludla et al., 2020)

Combination therapy	**Synergistic effects**	**Reference**
Metformin + *Allium sativum* extract	Hypoglycaemic	(Moradabadi et al., 2013)
Pioglitazone + *Curcuma longa* extract	Inhibits lipid peroxidation, increase insulin sensitivity	(Farzaei et al., 2018)

Chapter 2

Scope, objectives and plan of work of the study

2.1. Scope and rationale of the present work

2.2. Objective of the work

2.3. Plan of work

2.1. Scope and rationale of the present work

Scientific validation and quality control of medicinal plant and its product is now become essential approach to assure efficacy, safety and rationalization of their use in preventive medicine. If a product is too complex, it must be standardized for better understanding of biological activity parameters. Estimation of marker compounds and secondary metabolites helps in establishing the botanical identity, uniformity, and chemical sanctity of the medicinal products.

Herbal medicines are majorly used in India for maintenance of health, prevention, diagnosis, treatment, and improvement of physical and and mental illnesses (WHO, 2005). Quality evaluation, validation, standardization and marker analysis of herbs are important approaches for estimation of amounts of concentration of phytomarkers present in the extract. The therapeutic importance of medicinal plants and nutraceuticals has been established due to their wide spectrum of pharmacological potentials, higher safety margin as well as lesser costs than synthetic drugs. Primarily, specific bioactive compounds present in the medicinal plants are responsible for their specific bioactivities. Thus, standardization of the herbal materials through several analytical methods such as HPLC, HPTLC and NIRs are essential for profiling of the marker compounds.

Medicinal plants provide a wealth of phytomolecules with various pharmacological activities, which are found to be highly effective in the management of metabolic disorders. The ingredients are extracted from herbs and therapeutically purified for specific pharmacological activity. Therefore, quality control and verification of raw herbal products and their bioactive compounds are of major importance in traditional medicines to assess their

quality, efficacy, and safety. Pharmacological property of a medicinal preparation based on its phytoconstituents. The development of analytical methods, which can carry out for profiling of phytoconstituent, including estimation of phytomarker molecules and other phytoconstituents, is very challenging to the scientists. With this background, the aim of the study was quality evaluation and validation through marker profiling using HPLC, HPTLC, and NIRs of the different extracts of three medicinal plants - *A. paniculata*, *P. nigrum* and *E. officinalis.* Further study like antidiabetic activity of the *P. nigrum* and *E. officinalis* extracts were performed through in-vivo study on streptozotocin induced diabetic rats. The diagram of the research workflow is presented in Figure 2.1.

2.1. Objectives of the work

- Phytochemical evaluation of extracts of *A. paniculata*, *P. nigrum*, and *E. officinalis.*
- Standardization, validation and quality evaluation of *A. paniculata*, *P. nigrum* and *E. officinalis.*
- Standardization and quality evaluation of selected medicinal plants with suitable marker compound through HPLC, HPTLC and NIRs, in both qualitative and quantitative manner.
- Evaluation of the probable anti-diabetic activity of the selected medicinal plants through in-vivo study .

2.2. Plan of work

This study highlights on the following aspects which has been illustrated in figure 2.1.

- Collection and authentication of the three medicinal plants (*A. paniculata*, *P. nigrum*, and *E. officinalis*).
- Shade drying, pulverization of the plant materials.
- Extraction of plant materials.
- Phytochemical evaluation of the plant extracts.
- Marker profiling of the plant extracts using High performance liquid chromatography (RP-HPLC), High performance thin layer chromatography (HPTLC), and Near infra-red spectroscopy (NIRs).
- Assessment of hypoglycaemic property of the selected medicinal plants through in-vivo study.

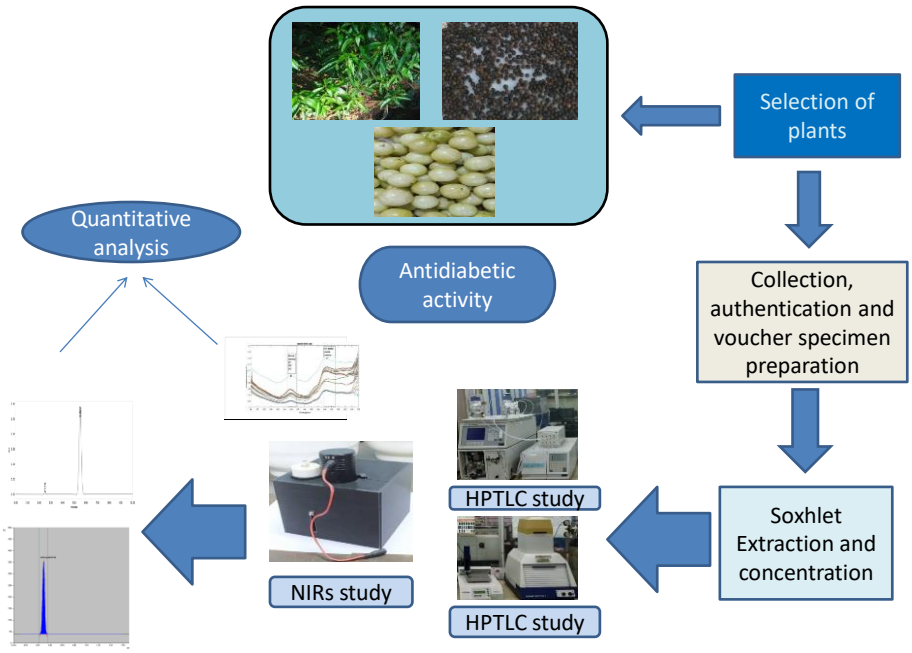


Figure 2.1. Workplan of the research work

Chapter 3

Plant profile, collection, authentication and extraction

3.1. *Andrographis paniculata*

3.2. *Piper nigrum*

3.3. *Emblica officinalis*

3.4. Extraction and phytochemical screening of plants

3.5. Phytochemical evaluation

3.1. *Andrographis paniculata*

3.1.1. Plant description

Andrographis paniculata is a very popular medicinal plant. The total number of species of this genus varied is reported to be different in different papers, and it varies from 19 to 44 species. A. paniculata is an annual, branched, and herbaceous plant which grows in moist habitat, and roadsides. It also can be cultivated in garden (Hossain et al., 2014). This plant mainly grows in Asia such as India, Java, Sri Lanka, Pakistan, and Indonesia. It is grown in India, China, Jamaica, Barbados, and the Bahamas, and southwestern Nigeria (Hossain et al., 2021).

Figure 3.1. *Andrographis paniculata* leaves

3.1.2. Botanical taxonomy

Scientific classification		Vernacular names	
Kingdom	: Plantae	**English**	: Creat , King of bitters
Clade	:Tracheophytes	**Hindi**	: Kalmegh

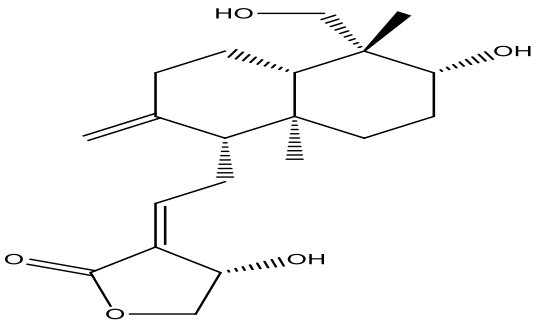
Clade	: Angiosperms	**Gujrati**	: Kiriyata
Clade	: Asterids	**Marathi**	: Olen kirayat
Order	: Lamiales	**Sanskrit**	: Bhuinimb
Family	: Acanthaceae	**Malayalam**	: Nilaveppu
Genus	: Andrographis	**Telugu**	: NelaVemu
Species	: *A. paniculata*	**Tamil**	: Nilavempui

3.1.3. Morphology of plant

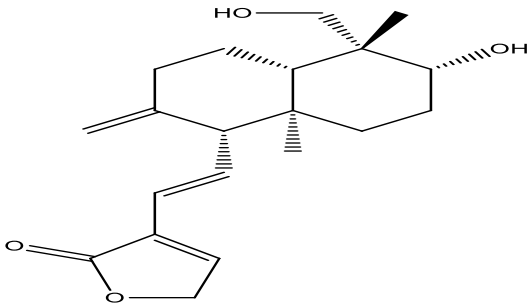
The height of *Andrographis paniculata* is typically 30-110 cm in moist shady places (Okhuarobo et al., 2014). Flowers are white with rose-purple spots on the petals, flowering and fruiting time is from December to April. Yellowish brown seeds are capsules oblong, acute 1.9 cm × 0.3 cm in size (Tong et al., 2020).

3.1.4. Phytochemical profile

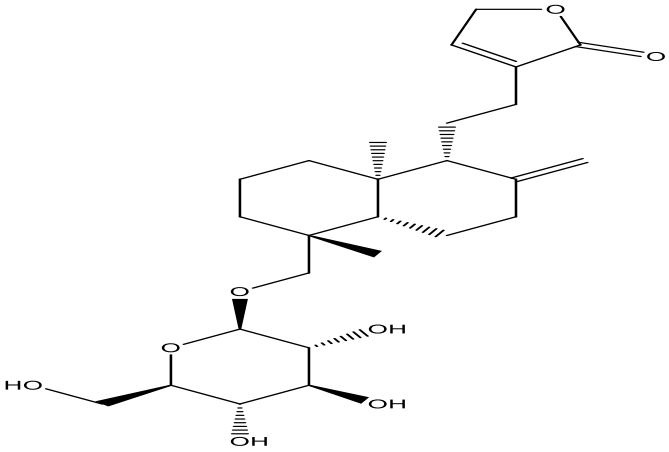
The primary phytoconstituent of *A. paniculata* is andrographolide (diterpene lactone), mp 230 - 239°C, 14-deoxy-11-oxoandrographolide, mp 100°C, 14-deoxyandrographolide, mp 175°C (Intharuksa et al., 2022). Leaves of *A. paniculata* contains several bitter glycosides such as andrographolide, panaculoside, flavonoids, andrographonin, panicalin, neoandrographolide, apigenin 7-4-dimethyl either. Deoxyandrographolide, 19β-D-glucoside and neo-andrographolide present in leaves (Li et al., 2019; Chao & Lin, 2010). Chemical structure some bioactive compounds of *A. paniculata* are shown in Figure 3.2.



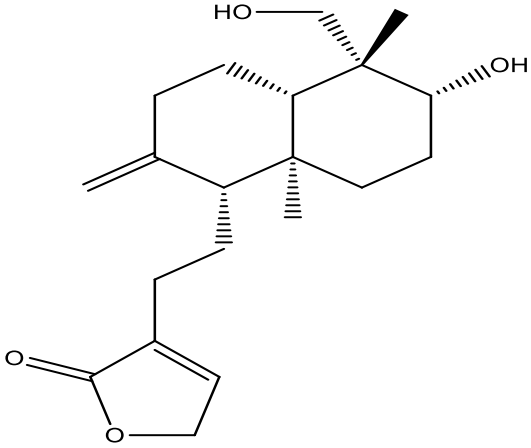
Andrographolide (1)



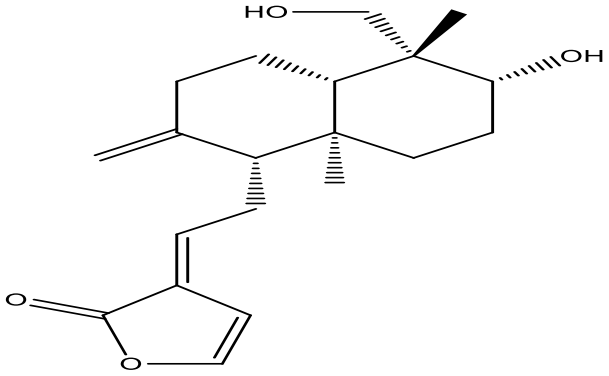
14-deoxy-11,12-didehydroandrographolide (2)



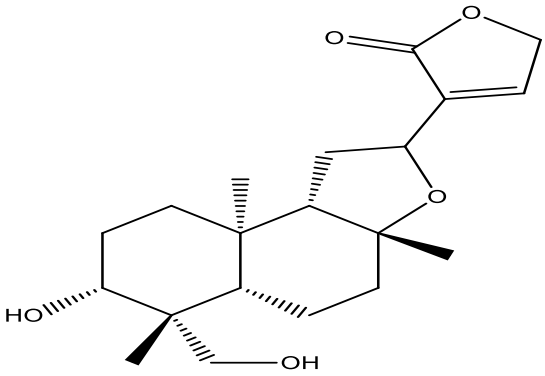
Neoandrographolide (3)



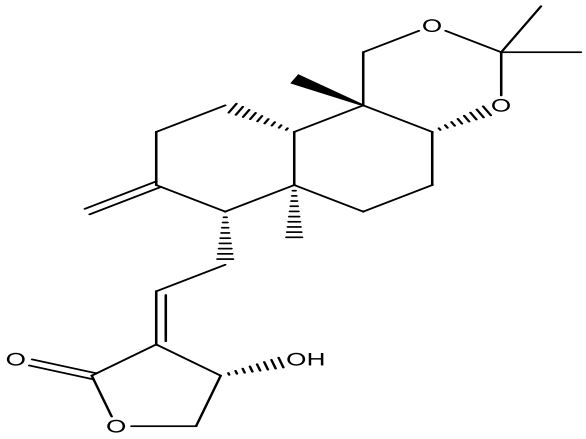
14-deoxyandrographolide (4)



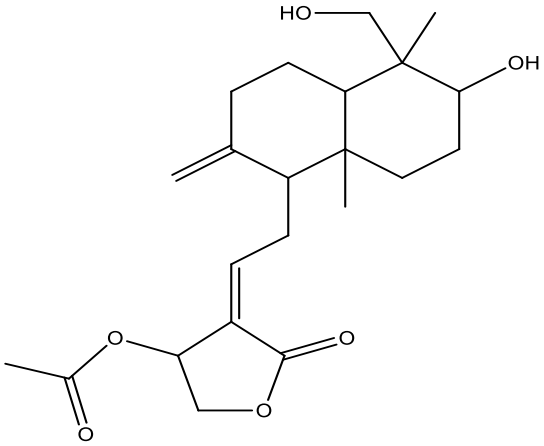
14-deoxy-14,15-dehydroandrographolide (5)



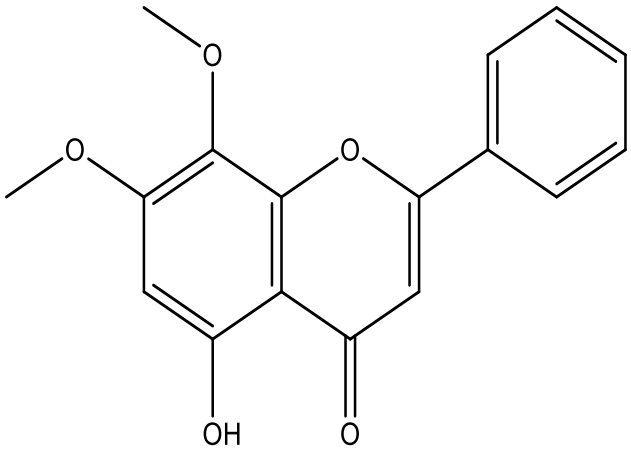
Isoandrographolide (6)



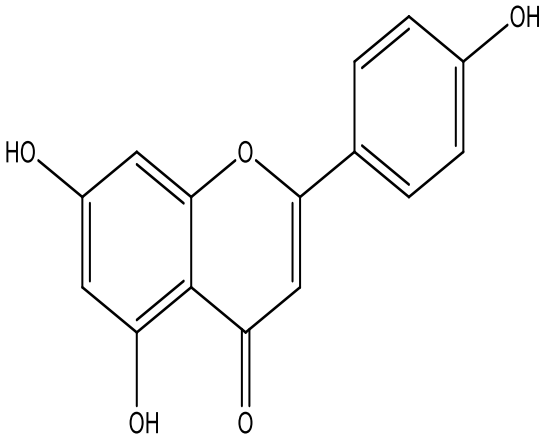
3,19-isopropylideneandrographolide (7)



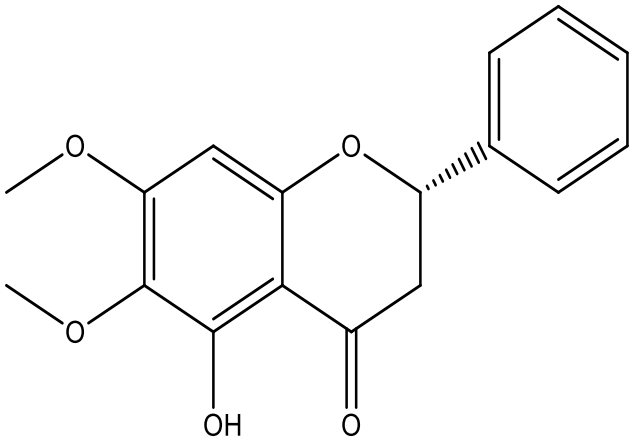
14-acetylandrographolide (8)



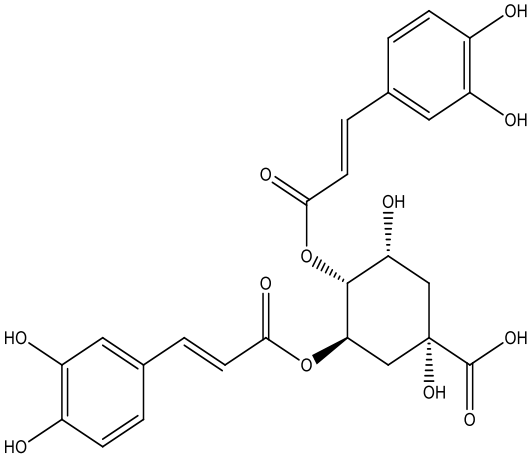
7-*O*-methylwogonin (9)



Apigenin (10)



Onysilin (11)



3,4-dicaffeoylquinic (12)

Figure 3.2. Chemical structure of few bioactive compounds from *A. Paniculata* (1-12)

3.1.5. Pharmacological activity

Several in vitro, in vivo studies confirmed various biological and pharmacological properties of *A. paniculata*. The pharmacological activities are described in Table 3.1.

Table 3.1. Pharmacological activities of *A. paniculata*

Parts used	Pharmacological activity	Reference
Leaves and stems	Hepatoprotective, antibacterial, antioxidant, anticancer, anti-inflammatory,	(Trivedi & Rawal, 2001)
Roots	Antiasthmatic and antipyretic	(Ilmi et al., 2021)
Flowers	Acute infections, pneumonia, dysentery, gastroenteritis	(Jayakumar et al., 2013)
Seeds	Immunomodulator and cardioprotective	(Rajanna et al., 2021)
Leaves	hypoglycaemic, and adaptogenic	(Wediasari et al., 2020)
Leaves	Hypolipidaemic	(Yang et al., 2012)

3.2. *Piper nigrum*

3.2.1. Plant description

Piper nigrum plant is a perennial plant that climbs and spreads. Black pepper stems are shaped like long, cylindrical vines and knuckles. This plant is dimorphic

with two main types of vines: vine tendrils and fruit-bearing tendrils. Black pepper berries are generally round or slightly oval. There are three varieties of fruit, green regular fruit, red-orange fruit when ripe (Jeong et al., 2020). Black pepper grows well in areas between 0 and 500m above sea level, but grows best at 100m above sea level. Desirable rainfall is 2000-3000 mm per year with two dry months to promote flowering (Joshi et al., 2017).

Figure 3.3. *Piper nigrum* fruits

3.2.2. Botanical taxonomy

Scientific classification		**Vernacular names**	
Kingdom	**:** Plantae	**English**	**:** Black pepper
Clade	:Tracheophytes	**Assamese**	**:**Gulmorich
Clade	:Angiosperms	**Bengali**	**:** Kali marich
Clade	: Magnoliids	**Hindi**	**:** Kali mirch
Order	: Piperales	**Gujarati**	: Kala mari

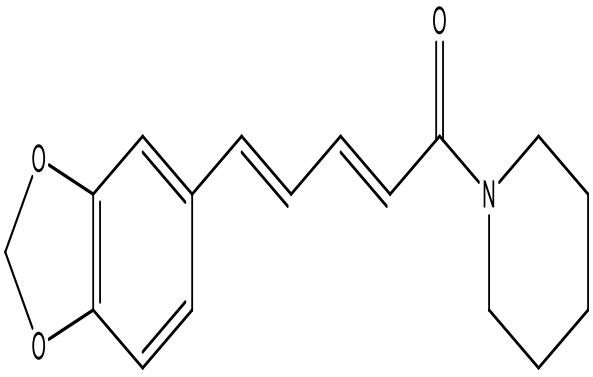
Family	: Piperaceae	**Sanskrit**	**:** Kali mirch
Genus	: *P. nigrum*	**Tamil**	:Kurumilagu

3.2.3. Morphology of fruits

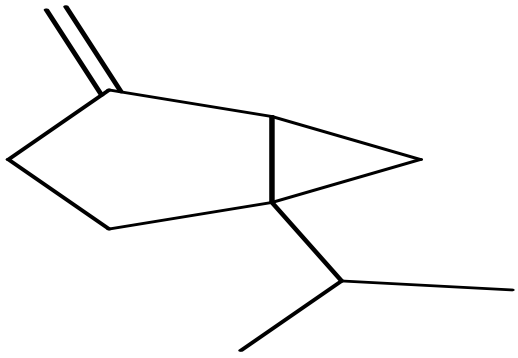
The plant grown in rainy season, high temperatures, and partial shade. Commonly, three different forms of *P. nigrum* seeds are black, green and white peppers. Black pepper is cultivated in tropical regions such as Viet Nam, Indonesia, Brazil, India, and China (Barata et al., 2021).

3.2.4. Phytochemical profile

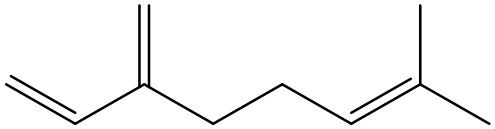
P. nigrum crude extract contains piperine as the major odorous alkaloid component. *P. nigrum* essential oil is composed of α-pinene, β-pinene, sabinene, myrcene, p-cymene, piperetine, piperanine, pipericide and others (Nakatani et al., 1986; Cho et al., 2017). Structure of some bioactive compounds of *P. nigrum* are shown in figure 3.4.



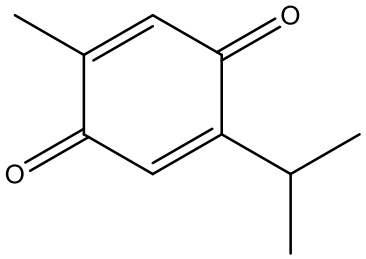
Piperine (13)



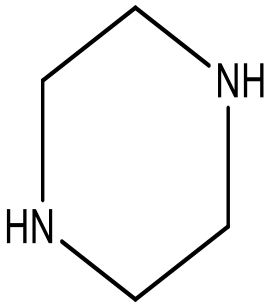
Sabinene (14)



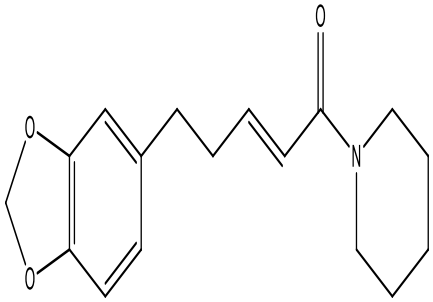
Myrcene (15)



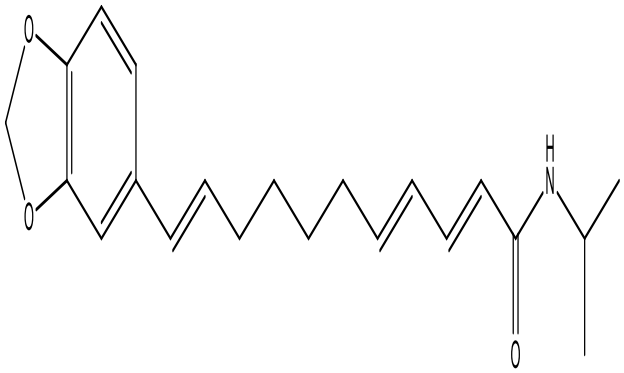
p-cymene (16)



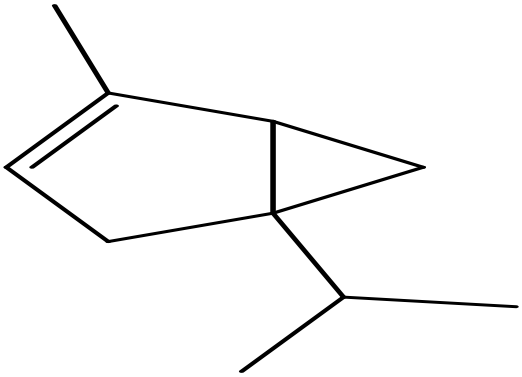
Piperazine (17)



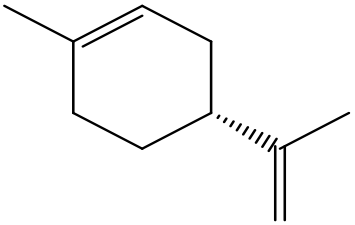
Piperanine (18)



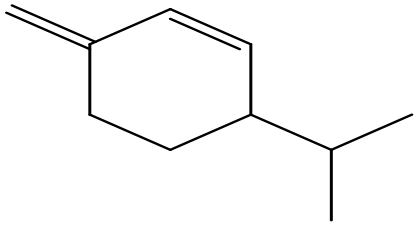
Pipericide (19)



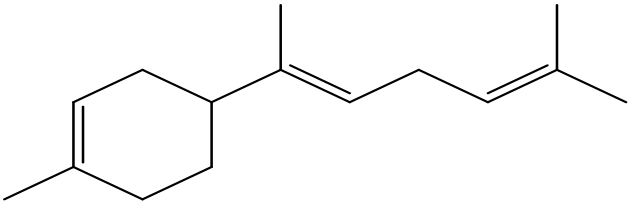
Alpha-thujene (20)

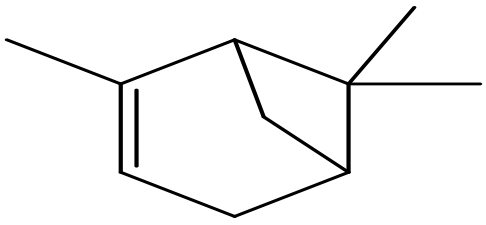


D-Limonene (21)

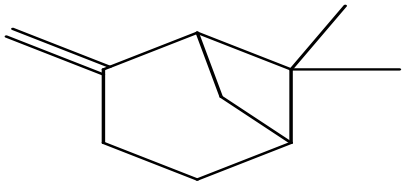


Beta-phellandrene (22)





Alpha-bisabolene (23)



Alpha-pinene (24)

Beta-pinene (25)

Figure 3.4. Chemical structure of few bioactive compounds from *P. nigrum* (13-25)

3.2.5. Pharmacological activity

pharmacological activities of *P. nigrum* are summarized in Table 3.2.

Table 3.2. Pharmacological activities of *P. nigrum*

Parts used	Pharmacological activity	Reference
Fruits and seeds	Antimicrobial, immunomodulator and hypoglycaemic	(Zahin et al., 2021)
Seeds	Antioxidant, analgesic and anti-asthmatic	(Takooree et al., 2019)
Roots	Antiplatelets, antioxidant, antihypertensive	(Bagheri et al., 2014)
Stem	Antibacterial	(Zou et al., 2015)
Seeds	Anti-inflammatory, hepatoprotective, anticancer, antidiarrhoeal, carminative, antiepileptic and adaptogenic	(Kumar et al., 2015)

3.3. *Emblica officinalis*

3.3.1. Plant description

Medium sized tree, 8-18 m tall, with light gray bark, grows in subtropical and tropical regions. Fruits of *E. officinalis* are 15-20 mm long and 18-25 mm wide (Middha et al., 2015). Fresh fruits are light green and ripe fruits turn light brown in colour (Variya et al., 2016).

Figure 3.5. *Emblica officinalis* fruits

3.3.2. Botanica taxonomy

Scientific classification		Vernacular names	
Kingdom	: Plantae	**English**	: Indian Gooseberry
Division	: Angiospermae	**Hindi**	: Amla
Class	: Dicotyledonae	**Sanskrit**	: Amalki
Order	: Geraniales	**Malayalam**	: Nelli

Family	: Euphorbiaceae	**Telugu**	: Usheeri kaya
Genus	: Emblica	**Tamil**	: Nellikai
Species	: officinalis Geartn		

3.3.3. Morphology of fruits

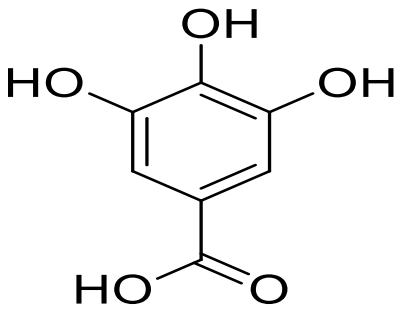
The fruit is spherical, light greenish yellow, quite smooth. The fruiting season of *Emblica officinalis* is exceptionally long.The taste of Indian gooseberry is sour, bitter and astringent (Nazish & Ansari, 2017).

3.3.4. Phytochemical profile

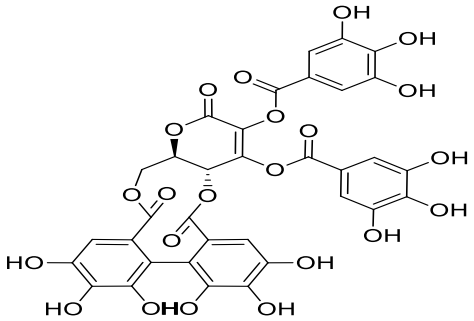
This medicinal plant contains many pharmacologically active ingredients, such as gallic acid, ellagic acid, Emblicanin A, Emblicanin B, Phyllaemblicin B found in *Emblica officinalis* (Majeed et al., 2009). The phytoconstituents present in *E. officinalis* are presented in Table 3.3. Chemical structure some bioactive compounds of *E. officinalis* are shown in figure 3.6.

Table 3.3. Phytoconstituents of *E. officinalis*

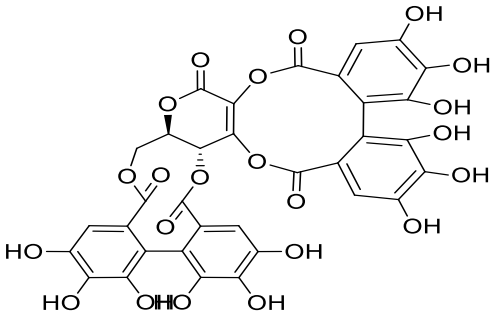
Plant part	Phytoconstituents	Reference
Fruit	Polyphenol content including gallic acid, 1-o-galloyl-β-D-glucose, gallic acid esters, methyl gallate, corilagin, furosine, geraniin, embricanin A and B, punigluconin, pedunculagin and ellagic acid.	(Patel et al., 2013)
	Glutamic acid, proline, aspartic acid, alanine, lysine, moisture, protein, fat, mineral matter, fiber, carbohydrate, calcium, potassium, iron, nicotinic acid.	(Gantait et al., 2021)
Seeds	Sterol, Linolenic acid, Linoleic acid, Oleic Acid, Palmitic acid, Myristic acid.	(Mysore & Arghya, 2022)
Leaves	Luteolin-40-O neohesperidoside,1,2,3,4,6-penta-Ogalloylglucose,Trihydroxysitosterol.	(Variya et al., 2016)
Barks	Leukodelphinidin, tannin and proanthocy-anidin.	(Nashine et al., 2019)
Roots	Ellagic acid and lupeol, Phyllaemblicin-A, B, C, Phyllaemblic acid.	(Mao et al., 2016)



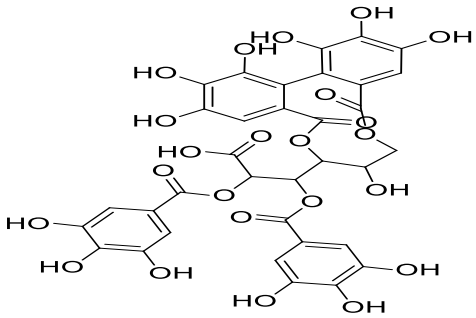
Gallic acid(26)



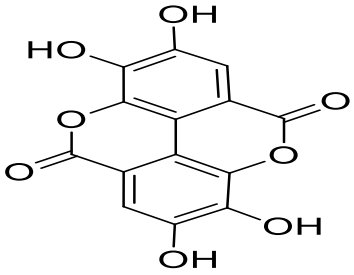
Emblicanin A(27)



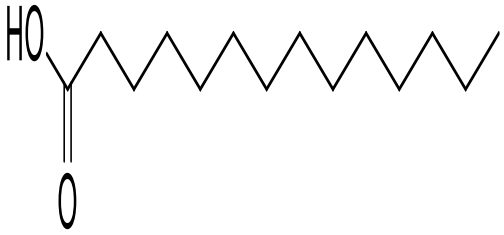
Emblicanin B(28)



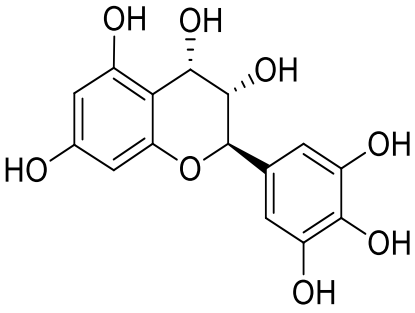
Punigluconin(29)



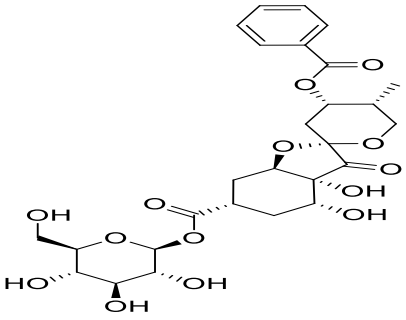
Ellagic acid(30)



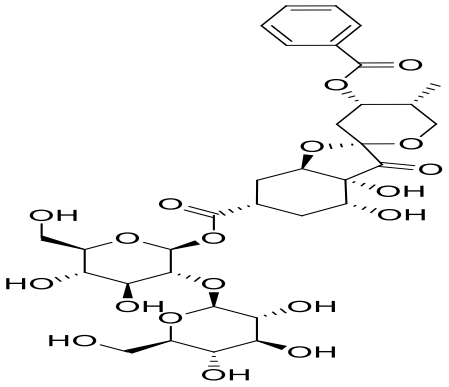
Myristic acid(31)



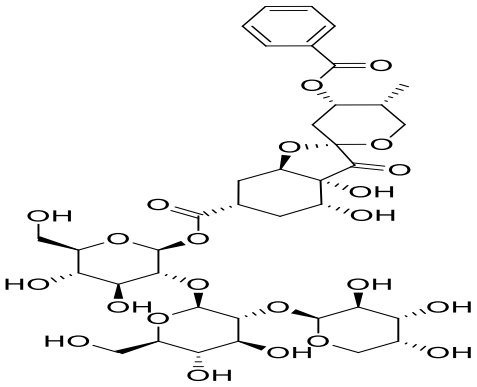
Leukodelphinidin(32)



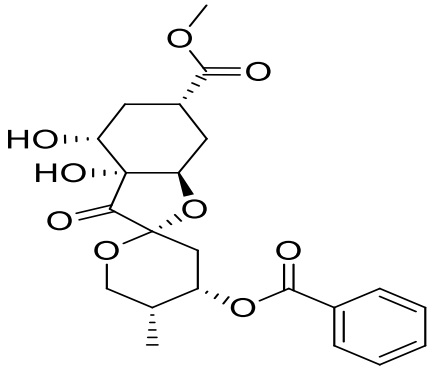
Phyllaemblicin-A(33)



Phyllaemblicin-B(34)



Phyllaemblicin-C(35)



Phyllaemblic acid(36)

Figure 3.6. Chemical structure of few bioactive compounds from *E. officinalis* (26-36)

3.3.5. Pharmacological activity

E. officinalis are used in Indian traditional medicinal system for treatment of different conditions such as tridosha, hyperglycaemia, cough, asthma, bronchitis (Ravishankar & Shukla, 2007). Different pharmacological activities of *E. officinalis* are shown in Table 3.4.

Table 3.4. Pharmacological activities of *E. officinalis*

Plant part	Pharmacological activity	Reference
Fruit	Hypoglycemic, hypolipidemic, antioxidative.	(Akhtar et al., 2011)
	Analgesic, antipyretic	(Perianayagam et al., 2004)
Leaves	Antioxidative, anti-carcinogenic, antidiabetic and anti-inflammatory.	(Malik et al., 2016; Golechha et al., 2014)
Seeds	Antioxidant, hypoglycemic	(Nain et al., 2012)
Bark	Antidiabetic, haemolytic, anti-inflammatory, anticancer	(Singh et al., 2020)

3.4. Extraction and phytochemical screening of plants

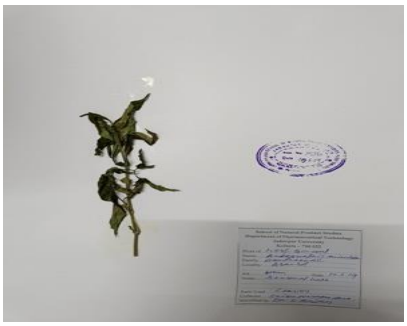
3.4.1. Collection and authentication

The leaves of *Andrographis paniculata* were collected from different districts of West Bengal (AP1a-AP1j). *Piper nigrum* fruits were collected from various outlets of Burra Bazar Spice Market (PN1-PN10), Kolkata District of West Bengal. Fruits of *Emblica officinalis* (EO01-EO20) were collected from different stores in Hamdard Nagar, New Delhi and Kolkata of West Bengal, India based on morphology, price and quality. Authentication of the plant samples by matching them with suitable herbarium samples were performed by the field botanist. The details of the collection of the plant materials are presented in Table 3.5.

Table 3.5. List of the plants and voucher specimen details

Name of the plant	Collection area	Voucher specimen number	Collection season
Andrographis paniculata	West Bengal, India	SNPS/JU/2019/1516-25	Monsoon
Piper nigrum	Burra Bazar, Kolkata	SNPS/JU/2019/1506-15	Summer
Emblica officinalis	Hamdard Nagar, Delhi and Kolkata, West Bengal	SNPS/JU/2020/1526-45	Winter

The voucher specimens of the plants are presented in Figures 3.4, 3.5, and 3.6.



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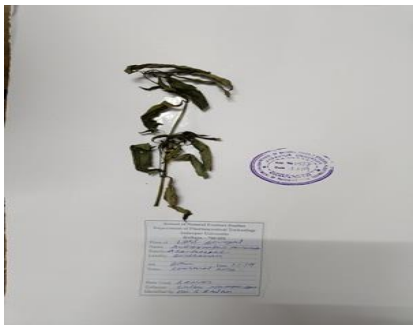

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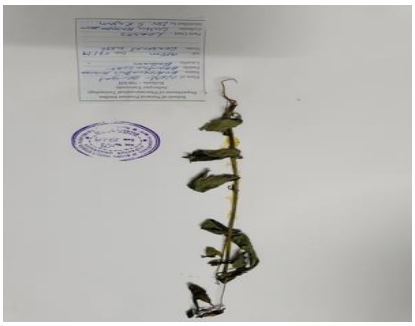

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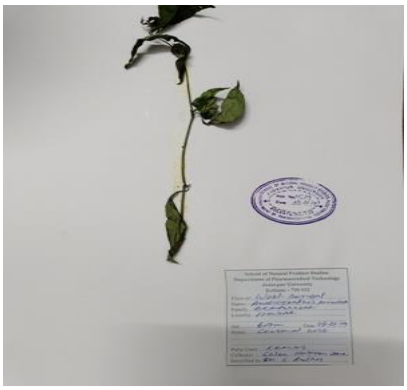
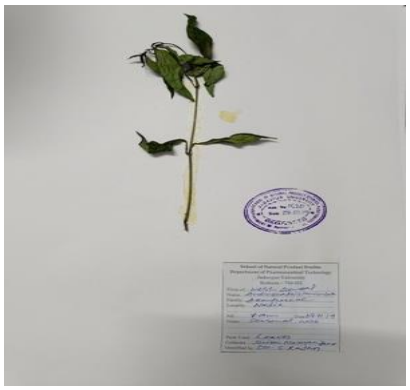
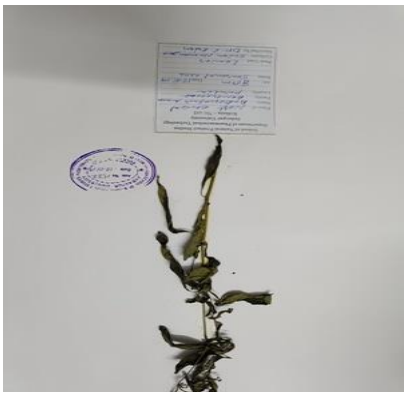


` **(D)**



(E)

(F)



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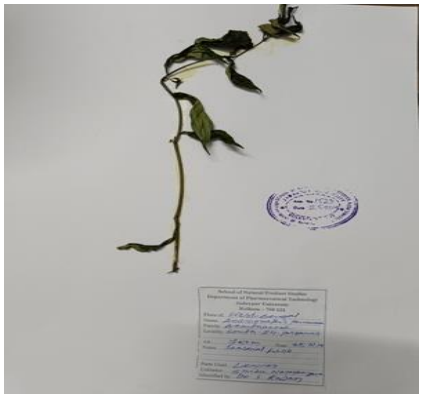
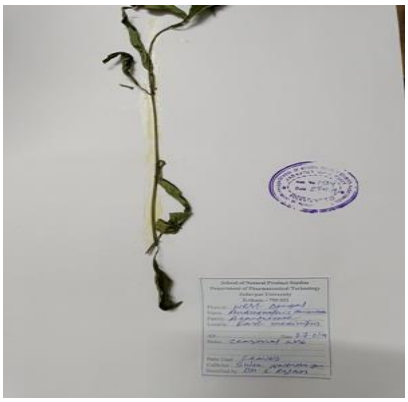


(H)



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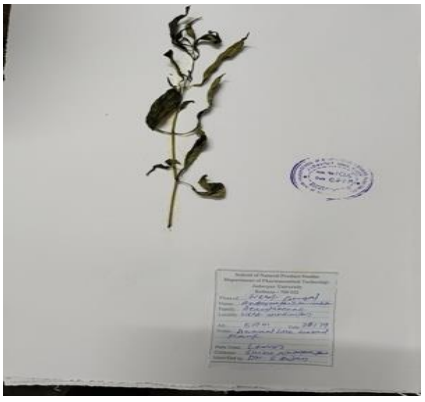
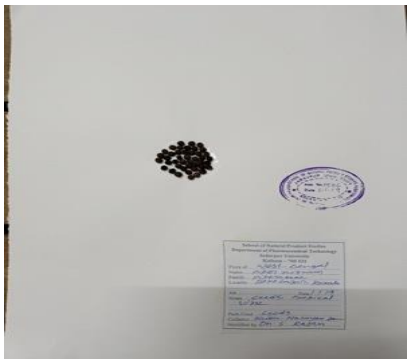


(J)

Figure 3.4. Voucher specimens of *A. paniculata* samples, A-J, (SNPS/JU/2019/1516-SNPS/JU/2019/1525).



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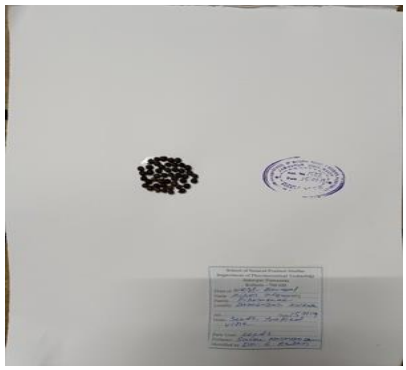
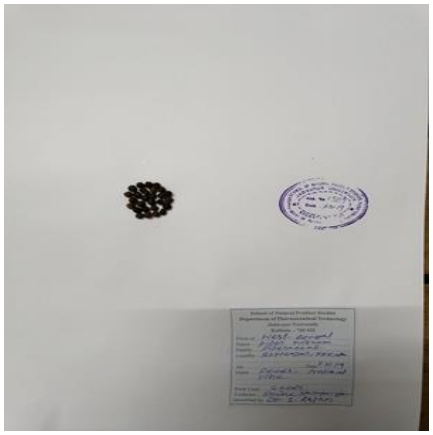


(B)

(C)



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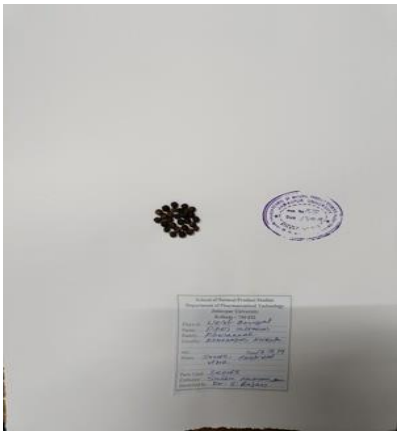

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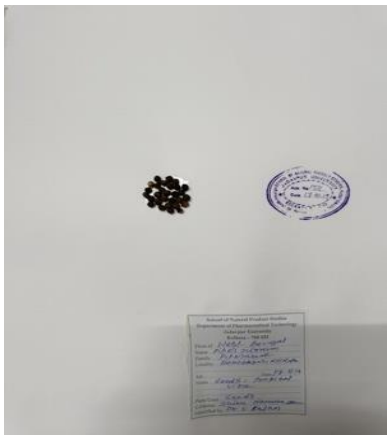

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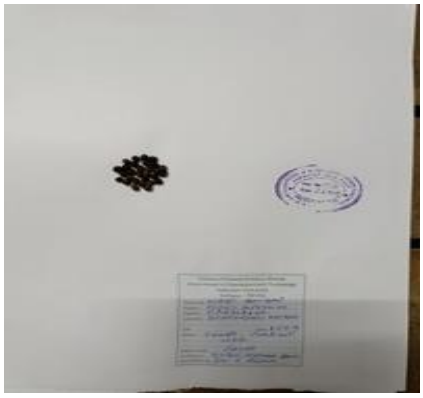
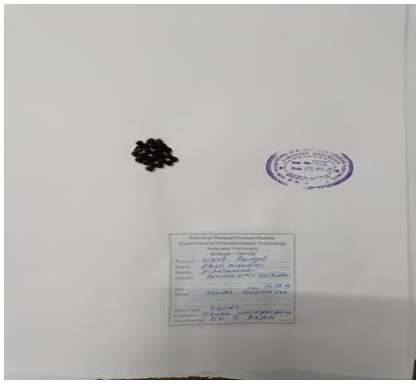


(G)

(H)

(I)



(J)

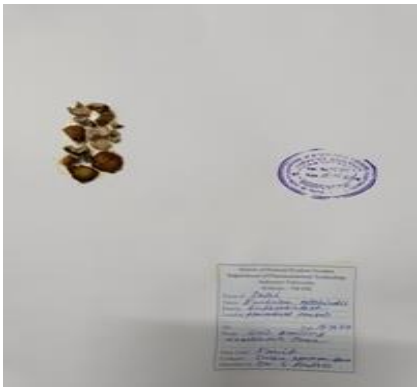
Figure 3.5. Voucher specimens of *P. nigrum* samples, A-J, (SNPS/JU/2019/1506-SNPS/JU/2019/1515).

(A)

(B)

(C)

(D)



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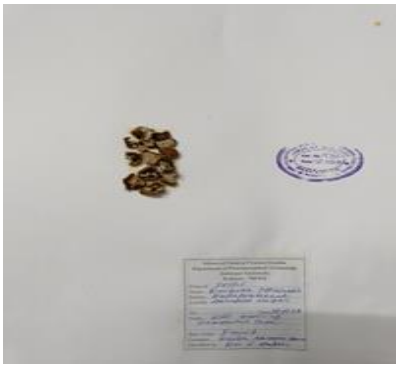

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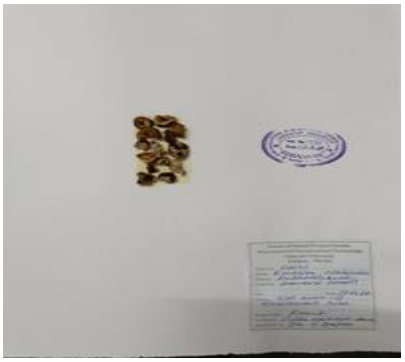

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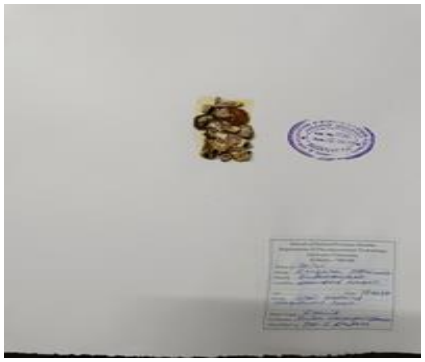

(I)

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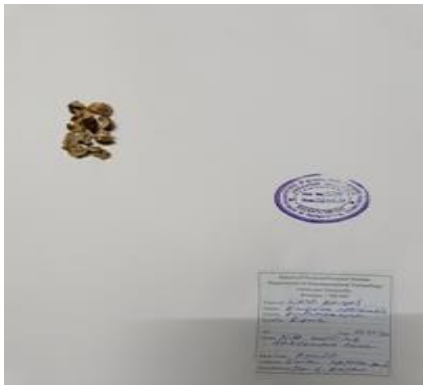

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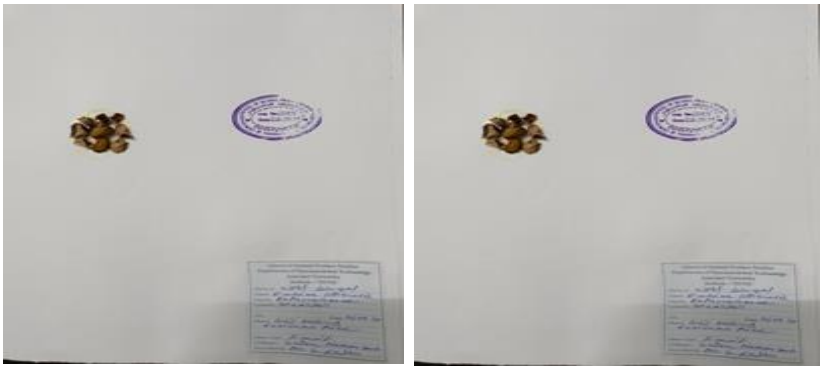


(T) (U)

Figure 3.6. Voucher specimens of *E. officinalis* samples, A-U, (SNPS/JU/2020/1526-SNPS/JU/2020/1545).

3.4.2. Extraction

All the plant materials were subjected to soxhlation using 70% methanol using soxhlet extractor. All the plant extracts were loaded in the thimble, which is placed inside the soxhlet extractor. The process were allowed to run for a total of 6-8 hours. Samples of *Andrographis paniculata* leaves were pulverized to make a moderately coarse powder by using a mechanical grinder and then used for the extraction. Shed dried fruits of *Piper nigrum* and *Emblica officinalis* were also pulverized to make a moderately coarse powder and then used for the extraction. The filtrate containing the extract was collected and concentrated at 45˚C in a rotary vacuum evaraporator (Tokyo, Japan), and the concentrated extract was freeze-dried. The % (w/w) yield for each sample was calculated and are shown in Table 3.6.

Table 3.6. Yield of each plant extract

Name of the plant	Initial quantity taken	% yield (w/w)
Andrographis paniculata		
SNPS/JU/2019/1516	100 g	25.6
SNPS/JU/2019/1517	100 g	26.8
SNPS/JU/2019/1518	100 g	30.3
SNPS/JU/2019/1519	100 g	27.1
SNPS/JU/2019/1520	100 g	29.6
SNPS/JU/2019/1521	100 g	25.2
SNPS/JU/2019/1522	100 g	28.4
SNPS/JU/2019/1523	100g	29.5
SNPS/JU/2019/1524	100 g	23.2
SNPS/JU/2019/1525	100 g	25.6
Piper nigrum		
SNPS/JU/2019/1506	100 g	23.4
SNPS/JU/2019/1507	100 g	26.1
SNPS/JU/2019/1508	100 g	29.7
SNPS/JU/2019/1509	100 g	25.9
SNPS/JU/2019/1510	100 g	26.7
SNPS/JU/2019/1511	100 g	22.3
SNPS/JU/2019/1512	100 g	25.8
SNPS/JU/2019/1513	100 g	21.5
SNPS/JU/2O19/1514	100 g	24.3
SNPS/JU/2019/1515	100 g	24.8

Name of the plant	**Initial quantity taken**	**% yield (w/w)**
Emblica officinalis		
SNPS/JU/2020/1526	100 g	30.5
SNPS/JU/2020/1527	100 g	26.1
SNPS/JU/2020/1528	100 g	29.5
SNPS/JU/2020/1529	100 g	32.2
SNPS/JU/2020/1530	100 g	25.3
SNPS/JU/2020/1531	100 g	31.6
SNPS/JU/2020/1532	100 g	29.3
SNPS/JU/2020/1533	100 g	27.1
SNPS/JU/2020/1534	100 g	25.1
SNPS/JU/2020/1535	100 g	22.8
SNPS/JU/2020/1536	100 g	22.6
SNPS/JU/2020/1537	100 g	26.7
SNPS/JU/2020/1538	100 g	28.3
SNPS/JU/2020/1539	100 g	`26.8
SNPS/JU/2020/1540	100 g	27.6
SNPS/JU/2020/1541	100 g	21.9
SNPS/JU/2020/1542	100 g	29.4
SNPS/JU/2020/1543	100 g	30.5
SNPS/JU/2020/1544	100 g	22.6
SNPS/JU/2020/1545	100 g	30.9

3.5. Phytochemical evaluation

The plant extracts underwent qualitative testing to identify of various plant constituents including carbohydrates, amino acids, vitamin C, saponins, flavonoids and more. The results of the phytochemical screening confirmed the presence of different secondary metabolites in the extracts and described in Table 3.7.

3.5.1. Qualitative phytochemical analysis

A. Detection of alkaloids

The crude aqueous ethanol extracts were individually dissolved in dilute HCl and filtered. The filtrate was used to test for the presence of alkaloids. Different test such as Mayer's, Dragendoroff's, Hager's, Wagner's test were performed for detection of alkaloids.

B. Detection of glycosides

Crude aqueous ethanol extracts were hydrolysed with dilute HCl and then assayed for glycosides. Various test were performed for detection of glycosides such as Borntrager's, Modified Borntrager's, Legal, Baljet test.

C. Detection of phytosterols and triterpenoids (Mukherjee, 2002)

For detection of triterpenoids different chemical test like Lieberman Buchard, Salkowski, Noller's test were performed.

D. Detection of flavonoids

Different chemical test like Zinc hydrochloride and reduction, Gelatin, Lead acetate were performed.

E. Detection of phenolic compounds and tannin (Mukherjee, 2002)

For detection of phenolic compounds and tannin different test such as Ferric chloride, Potassium dichromate, Alkaline Reagent test were performed.

F. Detection of saponins (Harborne, 1998)

For detection of saponins different chemical test like Foam, Potassium dichromate test were performed.

Table 3.7. Results of qualitative phytochemical tests

Metabolites	***A. Paniculata***	***P. nigrum***	***E. officinalis***
Alkaloid	**+**	**+**	**+**
Phenolics	**+**	**-**	**+**
Flavonoids	**+**	**-**	**+**
Saponins	**+**		**+**
Anthroquinone glycosides	**-**	**-**	**-**
Steroids	**-**	**-**	**-**
Triterpenoids	**+**	**+**	**+**
Tannins	**+**	**+**	**+**
Monoterpenoids	**+**	**+**	**+**

(+): Presence; (-): Absence

The plant extract was subjected to qualitative phytochemical screening which confirmed the presence of phenolics, flavonoids, glycosides and tannins etc. Further the standardization of plant extract was done by high performance thin layer chromatography, high performance liquid chromatography and near infra-red spectroscopy. Therapeutic validation was carried out with the extract against diabetes mellitus.

Chapter 4

HPTLC and RP-HPLC standardization of *A. paniculata*, *P. nigrum* and *E. officinalis* with marker compound

4.1. HPTLC and HPLC in medicinal plant analysis

HPTLC is a sophisticated, automated form of thin-layer chromatography (TLC) technique (Alam et al., 2015). It is an excellent and convenient approach for standardization and validation of herbs and herbal compounds. Applications of HPTLC include phytochemical screening, quantification, fingerprinting and estimation of adulterants (Islam et al., 2021). It is a powerful analytical method equally suitable for qualitative and quantitative analytical purposes. It uses HPTLC plates containing small particles with a narrow size distribution. This makes it possible to obtain layers with smooth surfaces (Hamidi et al., 2017). HPTLC uses small plates (10 x 10 or 10 x 20 cm), greatly reducing the development distance (typically 6 cm) and analysis time (7-20 min). HPTLC plates are used to develop in twin-trough chambers, or horizontal development chambers, fitted with filter

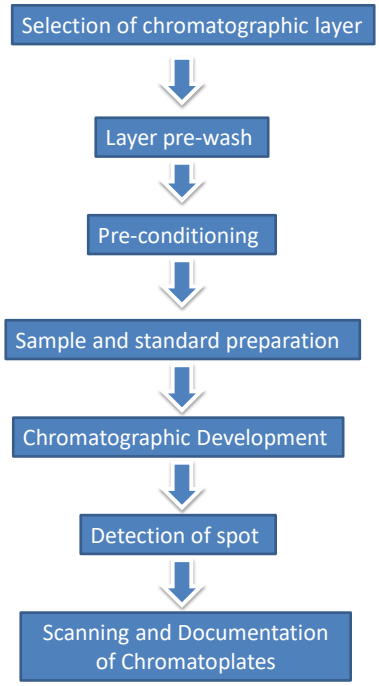


Figure 4.1. Workflow of HPTLC analysis

Paper to offer the better reproducibility. Detection is carried out in UV range (200-400 nm), specifically at 254 and 366 nm. The quantitative estimation of the biomarker is done by measuring the zones of sample based on the densitometric

scanning (Raman et al., 2014). The workflow of typical HPTLC analysis has been described in Figure 4.1.

High performance liquid chromatography (HPLC) is a versatile separation technique for qualitative and quantitative evaluation of targeted phytomolecule in plant extracts. HPLC technique is generally used in authentication, validation, standardization and quality evaluation based on the marker compounds which are ensure its quality, safety, and efficacy of medicinal products. HPLC is hyphenated with various detection techniques, such as, UV-Vis, PDA, MS, and NMR to obtain higher sensitivity and detection capabilities of the phytoconstituents for quality control of herbal medicines (Noviana et al., 2022). The hyphenated chromatographic techniques along with chemometric analysis often offer excellent approach for evaluation of quality as well as efficacy of medicinal plants. In RP-HPLC technique, the chemical nature of the stationary phase, i.e. the column is relatively non-polar, in comparison to the mobile phase. The column most widely used is C18 column, which is better known as the octadecylsilane column. The mobile phase containing a mixture of two solvents (Walter et al., 2005). One of the solvent is an aqueous phase, consisting of water (HPLC grade), whose PH has been adjusted with an orthophosphoric acid or glacial acetic acid to a desired PH and another organic solvent, such as Methanol or Acetonitrile. The elution may be isocratic or gradient. The sample is injected to the column through a loop or Rheodyne injector, whose volume is 20 µl. After sample injection, the sample solution is pushed through the column at a high pressure (500-3000 psi). At this high pressure, the complex mixture of different compounds gets separated in to distinct bands. In general laboratory set-ups, we use UV-Vis, PDA, Refractive index detectors for the detection of phytoconstituents. In this set-up, it is possible to record the chromatogram at different wavelength simultaneously. The separated bands, detected by the detector, appear as distinct, symmetric peaks in the chromatogram. The retention time of a standard marker's chromatogram and in the extract's chromatogram are compared, and by comparing the peak areas of these two (through a regression analysis), the amount of marker compound in the sample may be calculated. The workflow of typical HPLC analysis is described in Figure 4.2. In this chapter, HPTLC

and RP-HPLC based standardization of *A.* paniculata, P. *nigrum*, and *E. officinalis* was performed with the marker compound. The methods were further validated based on following parameters such as linearity, specificity, accuracy, precision, LOD, LOQ and robustness as per ICH guidelines.

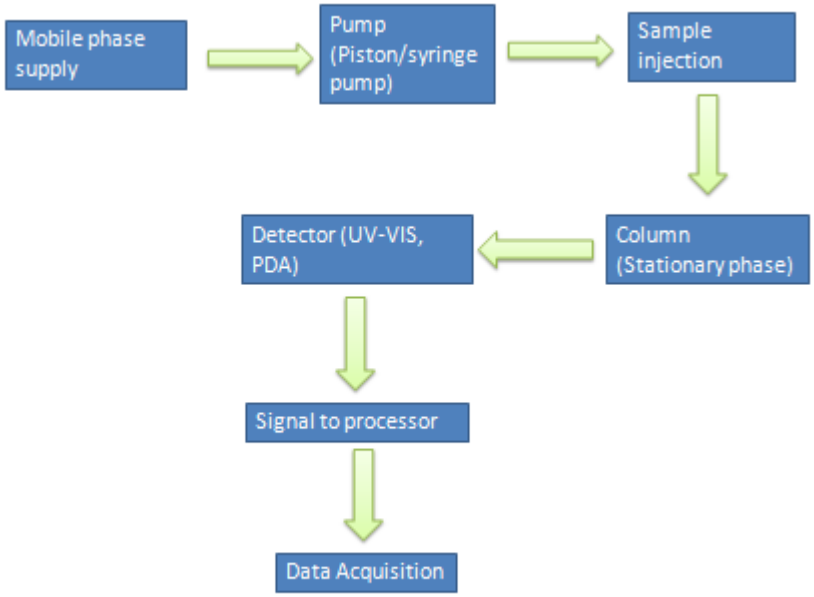


Figure 4.2. Workflow of HPLC analysis

4.2. HPTLC study of individual plants

4.2.1. *Andrographis paniculata*

4.2.1.1. Equipments and reagents

Equipments

Item used	Details
TLC plates	Silica Gel 60F_{254} plates, Merck
Automatic sample applicator used	CAMAG Linomat V
Photodocumentation chamber	CAMAG Reprostar 3

Densitometric scanner used	CAMAG TLC scanner 3
Software	WINCATS software
100 µl syringe	HAMILTON, Switzerland
Whatman's Syringe filter	NYL 0.45µ
Digital balance	Shimadzu
Auto pipettes	Eppendorf and Accupipet
Lamp	D2 and W lamp

Reagents, chemicals and solvents

Reagents	Brand
Chloroform, Methanol, Ethyl acetate, Formic acid	Merck
Water	MilliQ
Eppendorf tube	Tarsone

4.2.1.2. Preparation of standard solution and sample solution

A standard stock solution of andrographolide was prepared by dissolving 10 mg of andrographolide in 10 mL of methanol to make a 1 mg/mL solution. 10 mg of each of the leave extract was weighed and dissolved in 1 mL of methanol to prepare a 10 mg/mL extract. Each standard and extract solution was vortexed until completely dissolved and filtered through a 0.45 µm syringe filter (Millipore, Burlington, MA, USA). Linearity of the response generation standards was determined using a standard curve generated with five different volumes of the standard (andrographolide, 1 mg mL $^{-1}$) ranging from 2 to 10 µL. solutions for

HPTLC analyses were thoroughly vortexed, filtered, and sonicated until no particulate matter was visible in the solution.

4.2.1.3. Chromatographic conditions

The mobile phase was optimized for the HPTLC analysis. A mixture of chloroform:methanol (7:1) used as mobile phase gave the better separation of andrographolide. The external standard calibration curve for andrographolide was prepared with calibration solutions in a concentration range of 200 to 1000 ng mL $^{-1}$. 50 µl of standard solution was then drawn into the CAMAG LINOMAT syringe and placed into the Linomat applicator to obtain the required standard concentration. The same procedure was used for extracts. The plates were then dried and developed with mobile phase in a CAMAG double trough glass chamber. The developed plate was scanned at 254 nm wavelength for andrographolide by using densitometric HPTLC scanner III (Camag, Switzerland). The data was integrated by the WinCATS software and amount of the marker compound was calculated.

4.2.1.4. Method validation

The HPTLC method was performed according to the ICH guideline recommendations for parameters such as linearity, specificity, accuracy, precision, LOD, LOQ and robustness.

Linearity: A prepared stock standard solution was diluted to obtain a linearity standard solution containing gallic acid (1 mg mL^{-1}) at concentrations ranging from 200 to 1000 ng/spot. Each step was performed three times.The linearity of the response prepared standard was determined using a calibration curve. The

standard deviation (SD), coefficient of determination (r^2) were estimated to determine the method linearity.

Specificity: Specificity were checked according to ICH guidelines to minimize errors due to sample contamination. Peak purity was confirmed by multivariate analysis by comparing retention times and peak areas of standard compounds with extracts and fractions.

LOD and LOQ: LOD and LOQ were estimated by setting linear equations for standard deviation (σ) and slope (S) using the formulas LOD=3:1 σ/S and LOQ=10:1 σ/S . where σ = standard deviation S = slope of the calibration curve.

Accuracy: The accuracy of the method was measured by the recovery of gallic acid in plant extracts and fractions. Spiked the test in triplicate with three known amounts of standard compounds before applying to the plate. Analysis was performed under ambient conditions to determine overall average yields. The resulting marker mean value was taken as the actual value for calculating spike recovery.

Precision: Interday and intraday precision were measured three times on the same day using the same method for different concentrations of gallic acid. The application was run six times to determine the reproducibility of the process.

Robustness: Robustness of the method was measured by analyzing samples under various experimental conditions. To investigate the effect on retention time, we varied flow rate, mobile phase composition, detection wavelength, and column temperature.

4.2.1.5. Results

The developed method was validated for specificity, accuracy and robustness to quantify andrographolide abundance in AP1a, AP1b, AP1c, AP1d, AP1e, AP1f, AP1g, AP1h, AP1i, and APj. It was found to be accurate, reproducible and well below the linear range. The HPTLC method showed that the andrographolide present in AP1a, AP1b, AP1c, AP1d, AP1e, AP1f, AP1g, AP1h, AP1i, and AP1j

extracts were 2.13% (w/w), 2.51% (w/w), 1.01% (w/w) , 1.25% (w/w), 2.15% (w/w), 1.91% (w/w), 2.32% (w/w), 2.46% (w/w), 1.85% (w/w), and 1.71% (w/w) with an Rf of 0.30. Regression and analytical parameters of the method are presented in Table 4.1. The results of HPTLC analysis are shown in Table 4.2. The calibration curve for andrographolide was Y = 11775.084X + 2215.381 and presented in Figure 4.3. The HPTLC data of Intra-day and Inter-day precision studies of the extract are shown in Table 4.3. The results of recovery study are presented in Table 4.4. HPTLC chromatograms of the standard andrographolide and 10 extracts have been shown in Figure 4.4. The plate photo under 254 nm are presented in Figure 4.5. The study confirmed that andrographolide present in AP1b (East Medinipur) extract is highest (2.51% w/w) whereas lowest amount of was reported in AP1c (South 24 Parganas) extract (1.01% w/w).

Table 4.1. Regression and analytical parameters for the determination of andrographolide in *A. paniculata* by HPTLC method

Parameters	**HPTLC**
Calibration range	200-1000 ng/spot
Slope	0.6351
Intercept	0.9098
Regression equation (Y)	11775.084X + 2215.381
Correlation coefficient	0.9998
LOD	0.935 µg/spot
LOQ	6.136 µg/spot

Table 4.2. Andrographolide content estimated by HPTLC study

Serial no	Sample	Voucher specimen No	Yield (w/w %)	HPTLC (w/w %)
1	AP1a	SNPS/JU/2019/1516	25.6	2.13
2	AP1b	SNPS/JU/2019/1517	26.8	2.51
3	AP1c	SNPS/JU/2019/1518	30.3	1.01
4	AP1d	SNPS/JU/2019/1519	27.1	1.25
5	AP1e	SNPS/JU/2019/1520	29.6	2.15
6	AP1f	SNPS/JU/2019/1521	25.2	1.91
7	AP1g	SNPS/JU/2019/1522	28.4	2.32
8	AP1h	SNPS/JU/2019/1523	29.5	2.46
9	AP1i	SNPS/JU/2019/1524	23.2	1.85
10	AP1j	SNPS/JU/2019/1525	25.6	1.71

Table 4.3. Intra-day and Inter-day precision studies of the extract by HPTLC

Marker compound	Precision of HPTLC study (n=6)		
	Amount of andrographolide applied (ng per spot)	Intra-day %RSD	Inter-day %RSD
Andrographolide	200	0.94	0.96
	400	0.96	0.98

Table 4.4. Recovery studies of the extract by HPTLC

Marker compound	Recovery by HPTLC study (n=6)			
	Amount added (ng)	Average recovery (%)	Mean±SD %	%RSD
Andrographolide	0	96.6		
	200	97.3	96.8±1.45	1.37
	400	98.7		
	600	99.4		

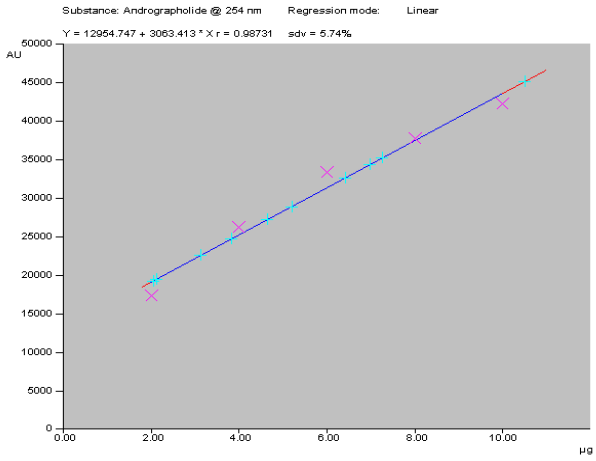
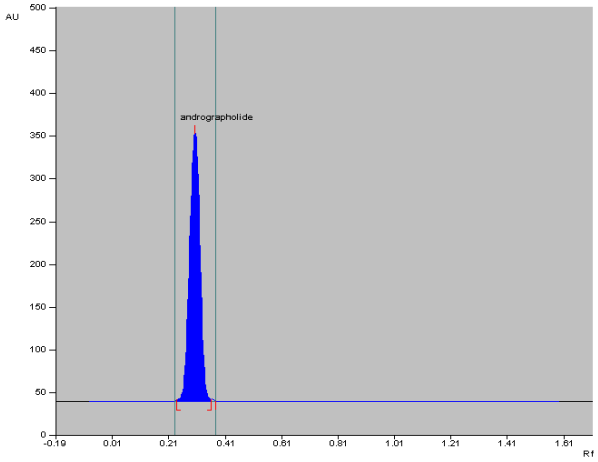


Figure 4.3. Calibration curve of andrographolide



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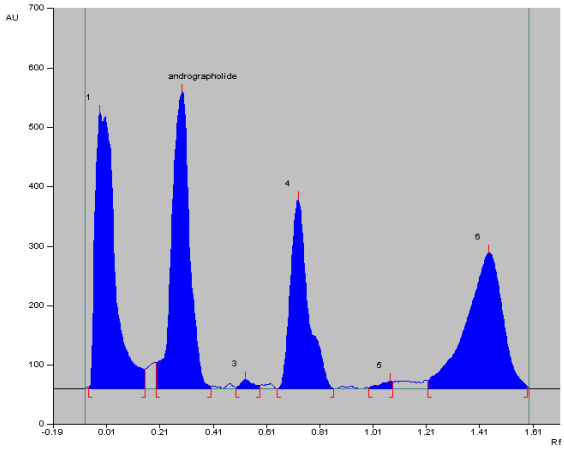
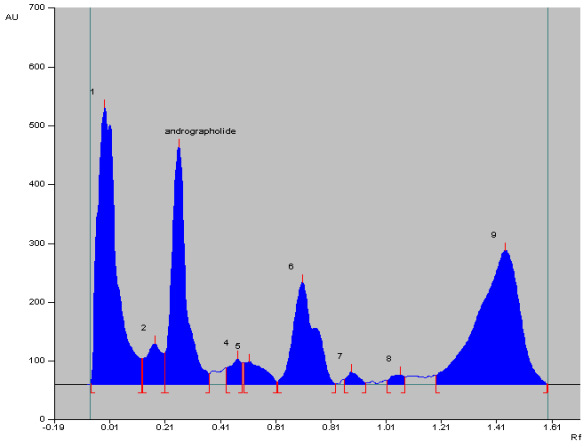


(B)



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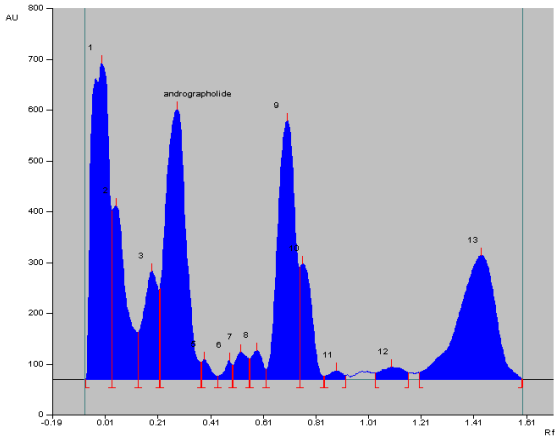
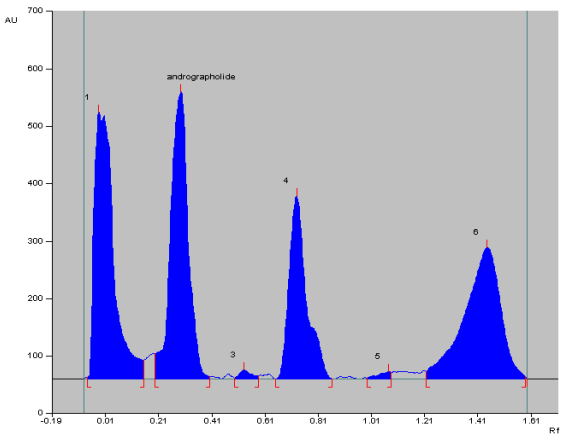
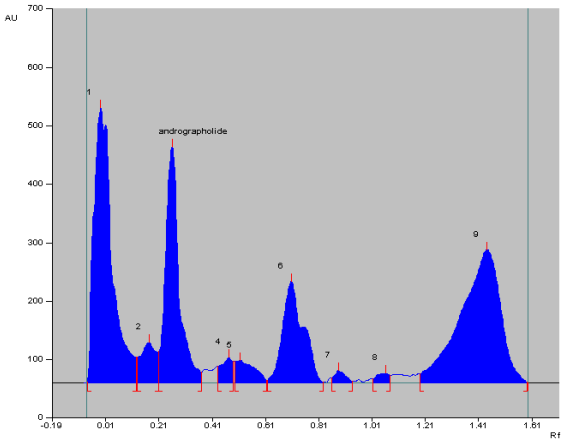


(D)


AU
andrographolide
Rf

(E)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andrographolide
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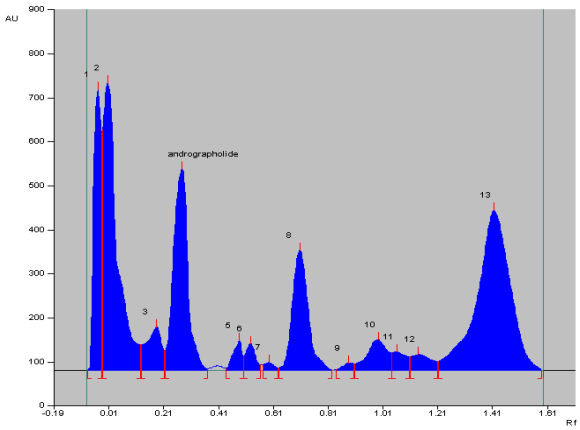
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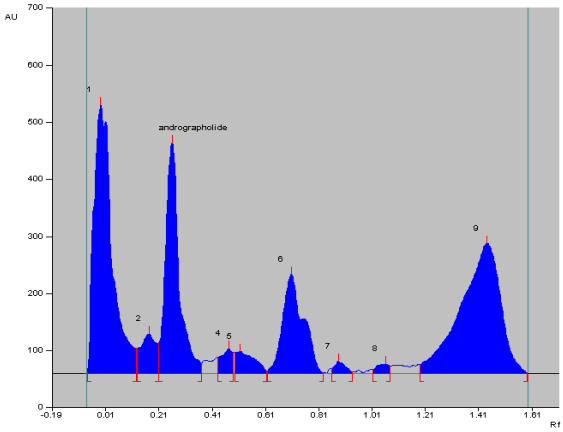
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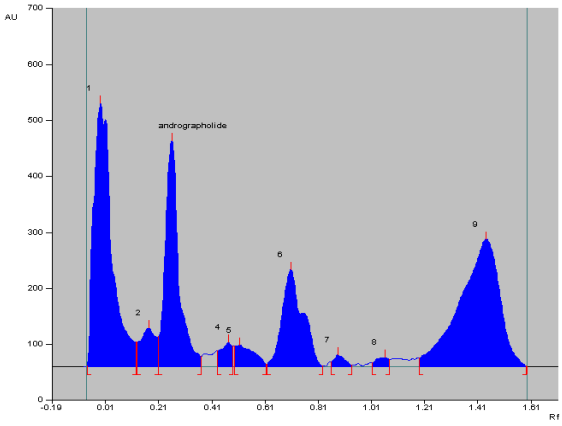
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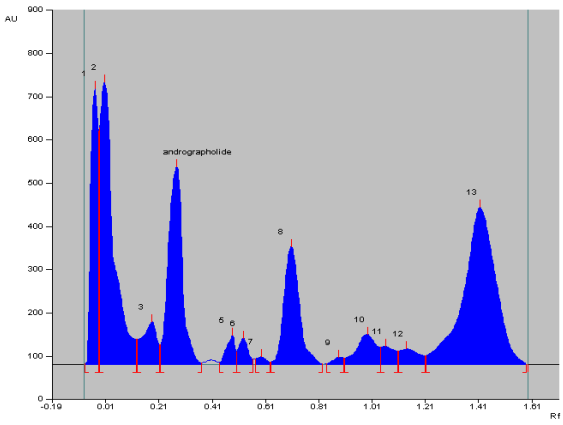
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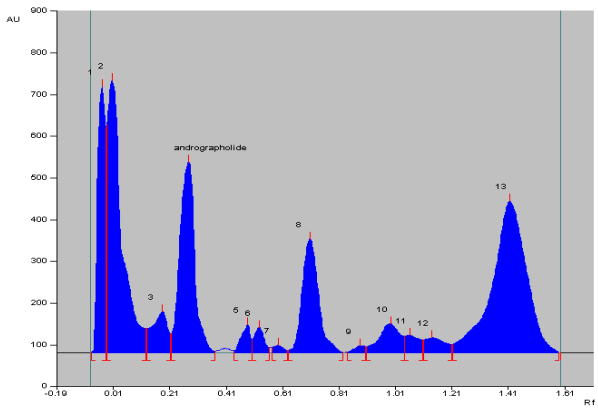


(K)

Figure 4.4. (A) HPTLC chromatogram of Andrographolide standard (B) HPTLC chromatogram of AP1a extract; (C) HPTLC chromatogram of AP1b extract; (D) HPTLC chromatogram of AP1c extract; (E) HPTLC chromatogram of AP1d extract; (F) HPTLC chromatogram of AP1e extract; (G) HPTLC chromatogram of AP1f; (H) HPTLC chromatogram of AP1g; (H) HPTLC chromatogram of AP1h; (I) HPTLC chromatogram of AP1i; (J) HPTLC chromatogram of AP1j; (K) HPTLC chromatogram of AP1k.

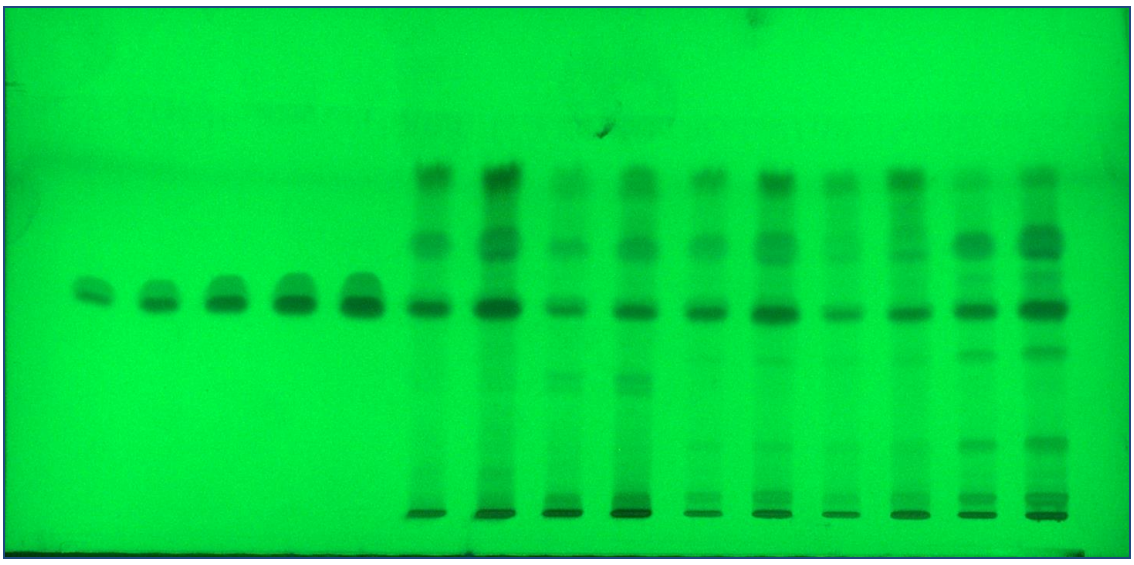


Figure 4.5. Photo-documentation at 254 nm of *Andrographis paniculata* extracts and standard andrographolide

4.2.2. *Piper nigrum*

4.2.2.1. Equipments and reagents

The equipments used were same as described in section 4.2.1.1.

4.2.2.2. Preparation of standard solution and sample solution

Preparation protocol of standard and sample solutions were same as described in section 4.2.1.2. In this study, piperine was used as a standard.

4.2.2.3. Chromatographic conditions

The chromatographic conditions were same as described in section 4.2.1.3.

4.2.2.4. Method validation

Different parameters which are recommended by ICH guidelines for HPTLC method validation were described in 4.2.1.4.

4.2.2.5. Results

Chromatograms were developed with a mobile phase consists of toluene and ethyl acetate in a 3:2 (v/v) ratio. The calibration curve (Figure 4.6.) is linear in the range of 200–1000 ng per point with a correlation coefficient (r^2) of 0.99077, indicating a good linear dependence of peak area on concentration. The regression and analysis parameters for this method are shown in Table 4.5. The HPTLC chromatogram of piperine with an Rf value of 0.63 is shown in Figure 4.7.A, and the chromatograms of *P. nigrum* extracts are shown in Figures 4.7.B-K. The maximum piperine content has been found in PN7 (4.76% w/w), and the minimum content has been found in PN9 (3.67% w/w). Piperine content estimated by the study has been presented in Table 4.6. These mean areas and Rf values were determined in both the daily accuracy study and the daily accuracy study and are presented in Table 4.7. Intraday and intraday assay accuracy and

precision for piperine were estimated at LQC, MQC, and HQC. Each experiment was repeated six times and recoveries were found to range from 99.69 to 99.93%. A low %RSD value indicates high accuracy of the method. The results of recovery studies are shown in Table 4.8. We found that the LOD varied from 611.20 ng per spot to 612.15 ng per spot and the LOQ varied from 1778.12 ng per spot to 1779.21 ng per spot. Robustness test %RSD was less than 2% in all cases.

Table 4.5. Regression and analytical parameters for the determination of piperine in *P. nigrum* by HPTLC method

Parameters	**HPTLC**
Calibration range	200-1000 ng/spot
Slope	0.5398
Intercept	0.99053
Regression equation (Y)	y = 997.3+796.5x
Correlation coefficient	0.99077
LOD	611.20-612.15 ng/spot
LOQ	1778.12-1779.21 ng/spot

Table 4.6. Piperine content estimated by HPTLC study

Sample	Voucher Specimen Number	Yield (% w/w)	Piperine content (% w/w)
*PN*1	SNPS/JU/2019/1506	25.3	3.96
*PN*2	SNPS/JU/2019/1507	29.2	3.78
*PN*3	SNPS/JU/2019/1508	24.3	3.73
*PN*4	SNPS/JU/2019/1509	26.8	3.86
*PN*5	SNPS/JU/2019/1510	27.1	3.94
PN6	SNPS/JU/2019/1511	23.4	3.98
PN7	SNPS/JU/2019/1512	26.1	4.76
PN8	SNPS/JU/2019/1513	29.7	3.89
PN9	SNPS/JU/2019/1514	25.9	3.67
PN10	SNPS/JU/2019/1515	26.7	3.81

Table 4.7. Intra-day & inter-day precision studies of the extract of *Piper nigrum*

Marker compound	Amount of piperine applied (ng per spot)	Intra-day %RSD	Inter-day%RSD
Piperine	200	0.71	0.69
	400	0.53	0.46

Table 4.8. Recovery studies of the extract of *Piper nigrum*

Marker compound	Amount added (ng)	Average recovery (%)	Mean±SD (%)	%RSD
Piperine	0	99.93	98.3±1.56	0.13
	200	99.81		
	400	99.84		
	600	99.69		

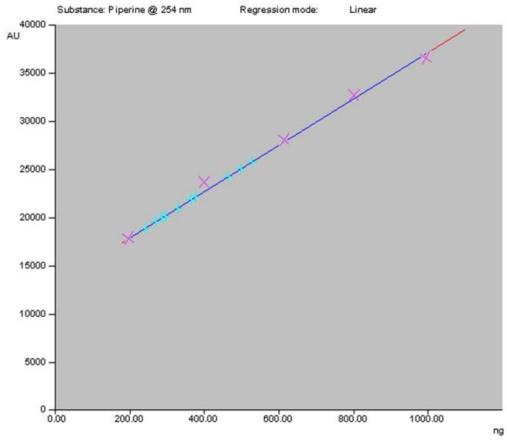
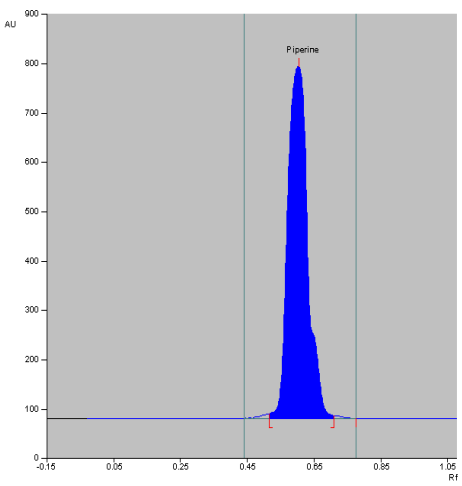
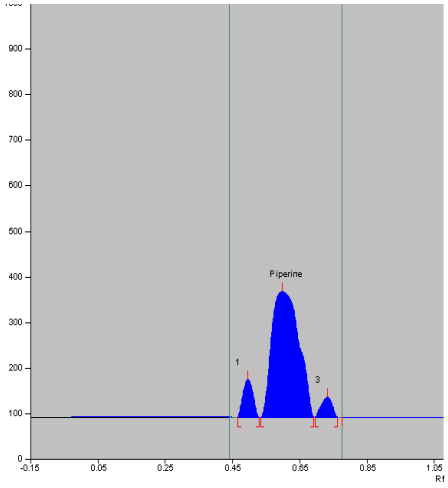


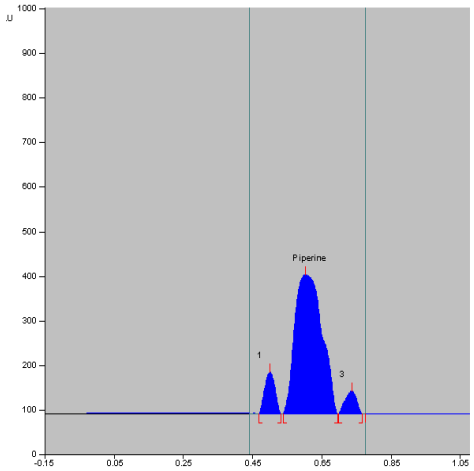
Figure 4.6. Calibration curve of Piperine



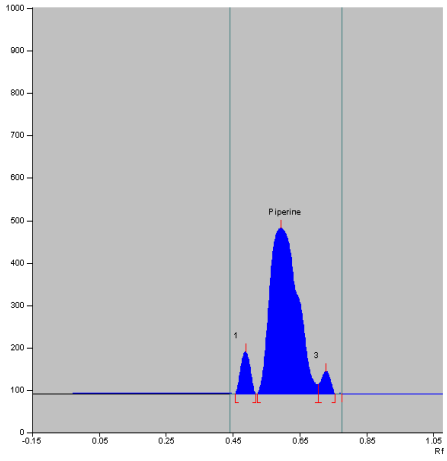
(A)



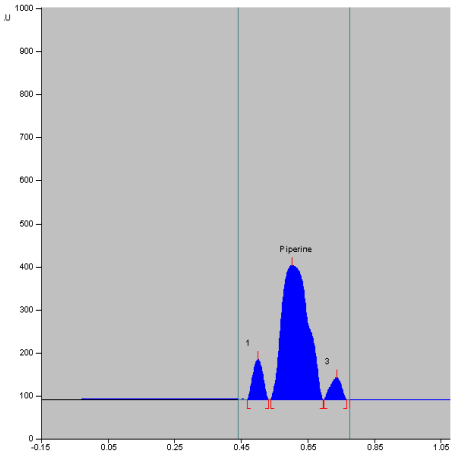
(B)


Piperine
1
3

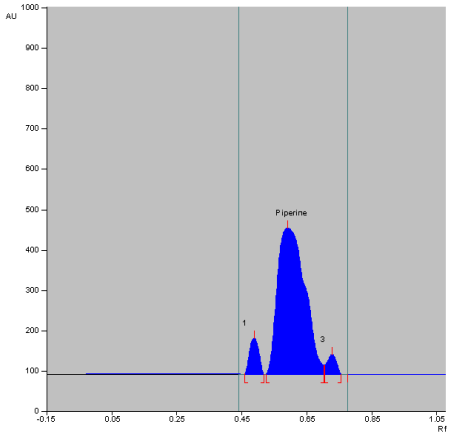
(C)


Piperine
1
3
Rf

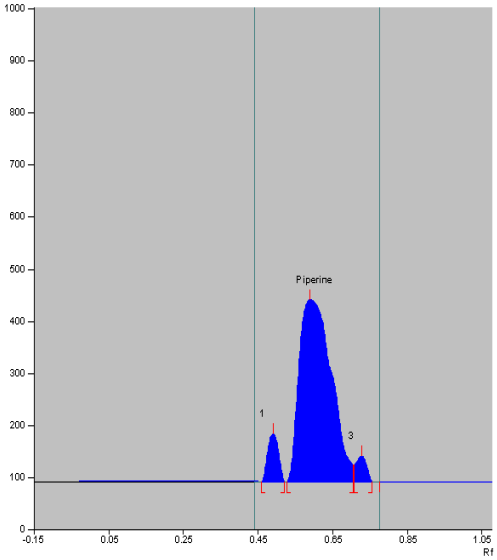
(D)


Piperine
1
3

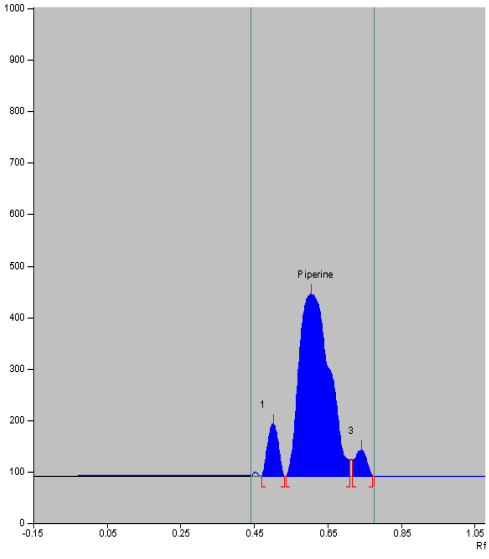
(E)


AU
Piperine
1
3
Rf

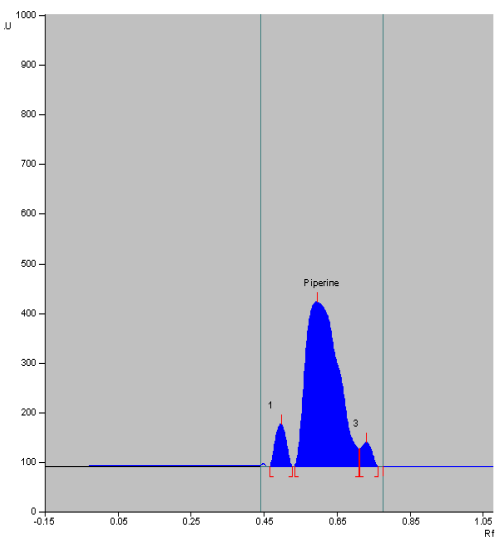
(F)


Piperine
1
3
Rf

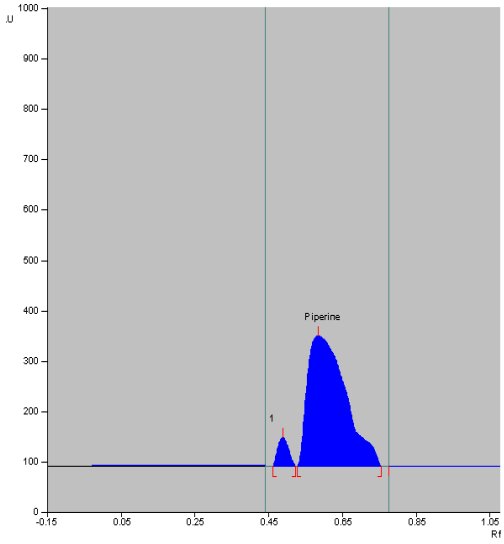
(G)


Piperine
1
3
Rf

(H)



(I)



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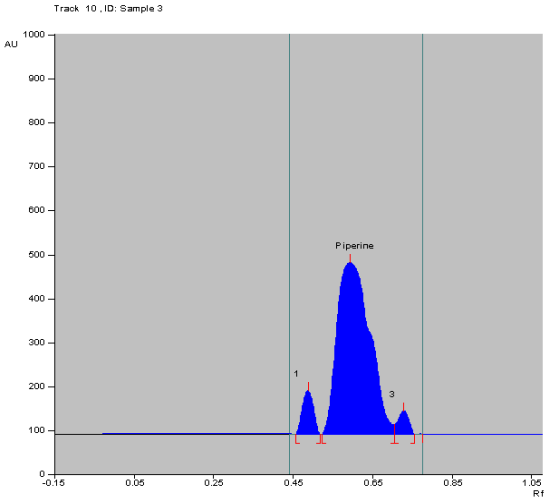


(K)

Figure 4.7. (A) HPTLC chromatogram of Piperine standard (B) HPTLC chromatogram of PN1 extract; (C) HPTLC chromatogram of PN2 extract; (D) HPTLC chromatogram of PN3 extract; (E) HPTLC chromatogram of PN4 extract; (F) HPTLC chromatogram of PN5 extract; (G) HPTLC chromatogram of PN6; (H) HPTLC chromatogram of PN7; (I) HPTLC chromatogram of PN8; (J) HPTLC chromatogram of PN9; (K) HPTLC chromatogram of PN10.

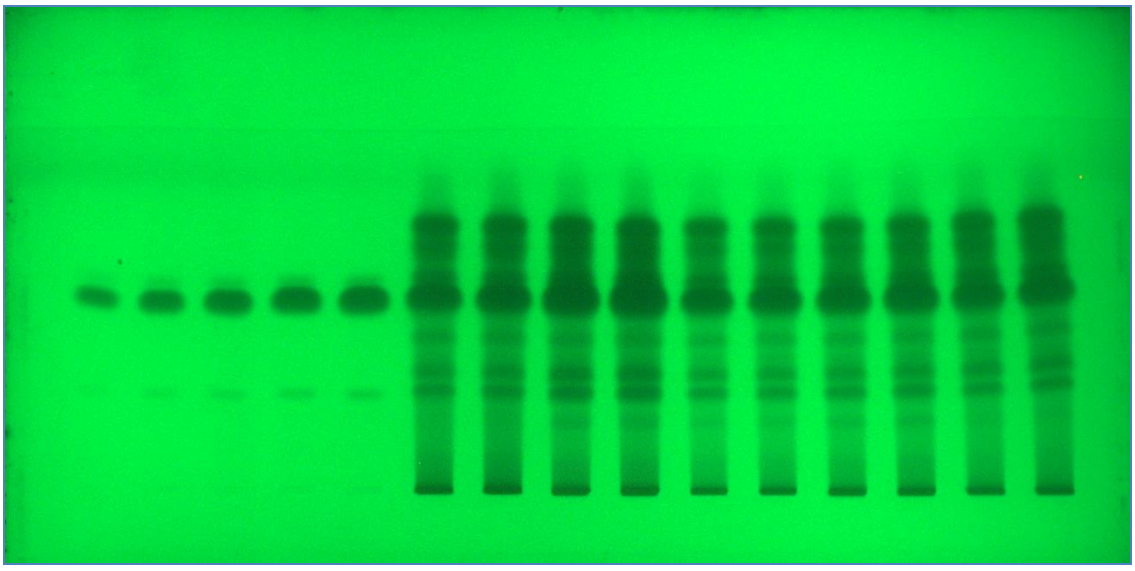


Figure 4.8. Photo-documentation at 254 nm of *Piper nigrum* extracts and standard Piperine

4.2.3. *Emblica officinalis*

4.2.3.1. Equipments and reagents

The equipments used were same as described in section 4.2.1.1.

4.2.3.2. Preparation of standard solution and sample solution

Preparation protocol of standard and sample solutions were same as described in section 4.2.1.2. In this study, gallic acid was used as a standard.

4.2.3.3. Chromatographic conditions

The chromatographic conditions were same as described in section 4.2.1.3.

4.2.3.4. Method validation

Different parameters which are recommended by ICH guidelines for HPTLC method validation were described in 4.2.1.4.

4.2.3.5. Results

Chromatograms were developed with a mobile phase consists of toluene:ethyl acetate:glacial acetic acid:formic acid (20:45:20:05, v/v/v/v). As shown in Figure 4.9, the calibration curve is linear with a correlation coefficient (r^2) of 0.99645 between 200 and 1000 ng/spot, indicating a good linear dependence of peak area on concentration. HPTLC chromatograms of standard gallic acid and all samples with an Rf value of 0.69 are shown in Figure 4.10.A–U. The presence of gallic acid

in plant extracts was confirmed by the corresponding Rf values. Linear regression data for HPTLC analysis are shown in Table 4.9. A photograph of the plate below 254 nm is shown in Figure 4.11. The % (w/w) yield for each sample was calculated and shown in Table 4.10. The highest gallic acid content was found in EO04 (6.13% w/w) and the lowest content in EO05 (3.98% w/w). Table 4.11 shows the daily and daily accuracy studies. The results of recovery studies are shown in Table 4.12. Accuracy and precision of Intraday and intraday of gallic acid were determined at LQC, MQC and HQC. Each experiment was repeated six times and recoveries were found to range from 99.96 to 99.99%. A low %RSD value indicates high accuracy of the method. LODs ranged from 1.563 to 1.786 µg/spot with LOQs from 5.104 to 5.136 µg/spot.

Table 4.9. Linear regression data for calibration plot of gallic acid using the proposed HPTLC method

Parameters	**HPTLC**
Wavelength	254 nm
Calibration range	200-1000 ng/spot
Slope	0.9781
Intercept	1.4890
Regression equation	y = 996.4+729.6x
Correlation coefficient (r^2)	0.99645
Retention factor (R_F)	0.69
LOD	1.786 µg/spot
LOQ	5.136 µg/spot

Table 4.10. Gallic acid content estimated by the HPTLC study

Sample	Voucher specimen number	Yield (% *w/w*)	Gallic acid content (% *w/w*)
EO0 1	SNPS/JU/2020/1526	30.5	5.61
*EO0*2	SNPS/JU/2020/1527	26.1	4.79
*EO0*3	SNPS/JU/2020/1528	29.5	5.54
*EO0*4	SNPS/JU/2020/1529	32.2	6.13
*EO0*5	SNPS/JU/2020/1530	25.3	3.98
*EO0*6	SNPS/JU/2020/1531	31.6	4.77
EO07	SNPS/JU/2020/1532	29.3	6.02
EO08	SNPS/JU/2020/1533	27.1	5.98
EO09	SNPS/JU/2020/1534	25.1	4.79
EO10	SNPS/JU/2020/1535	22.8	4.59
EO11	SNPS/JU/2020/1536	22.6	6.07
EO12	SNPS/JU/2020/1537	26.7	5.39
EO13	SNPS/JU/2020/1538	28.3	5.86
EO14	SNPS/JU/2020/1539	26.8	5.36
EO15	SNPS/JU/2020/1540	27.6	4.97
EO16	SNPS/JU/2020/1541	21.9	5.81
EO17	SNPS/JU/2020/1542	29.4	4.68
EO18	SNPS/JU/2020/1543	30.5	4.86
EO19	SNPS/JU/2020/1544	22.6	5.73
EO20	SNPS/JU/2020/1545	30.9	4.31

Table 4.11. Intra-day and inter-day precision studies of the extract of *Emblica officinalis* (n=6)

Marker compound	Amount of gallic acid applied (ng/spot)	Intra-day, % *RSD*	Inter-day, % *RSD*
Gallic acid	200	0.96	0.98
	400	0.89	0.93

Table 4.12. Recovery studies of the extract of *Emblica officinalis* (n=6)

Marker compound	**Amount added (ng)**	**Theoretic-ally expected amount (ng/spot)**	**Experiment-ally obtained amount (ng/spot)**	**Average recovery (%)**	**Mean ±SD (%)**	***%RSD***
Gallic acid	000	6130.18	6130.18	99.99±1.25		
	200	6330.00	6330.78	99.98±0.89	99.93±1.35	1.07
	400	6530.00	6530.66	99.96±1.97		
	600	6730.00	6730.08	99.98±2.89		

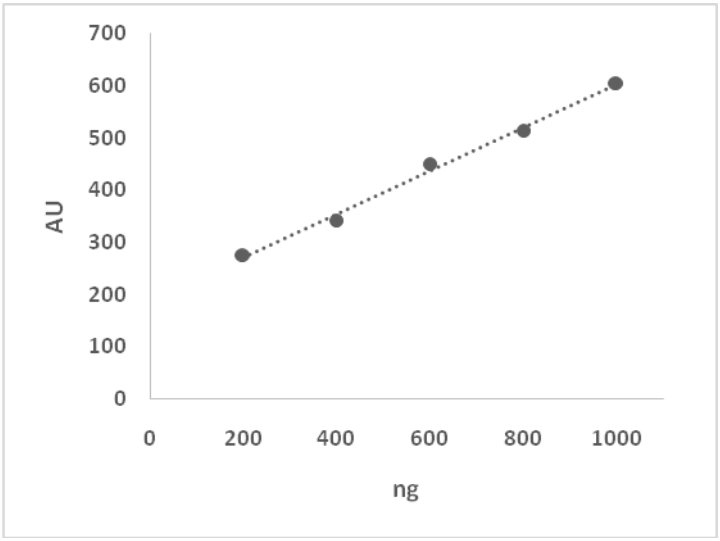
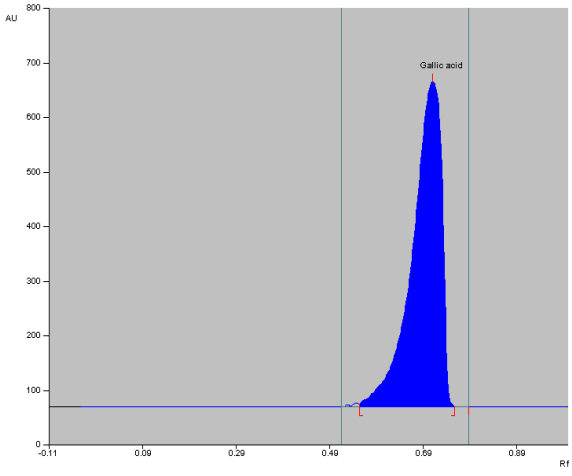


Figure 4.9. Calibration curve of gallic acid



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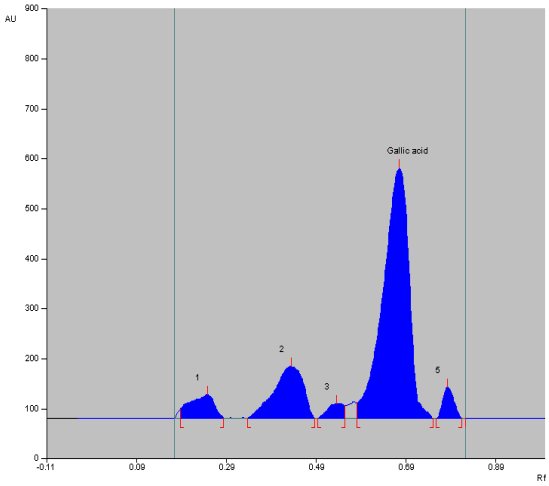

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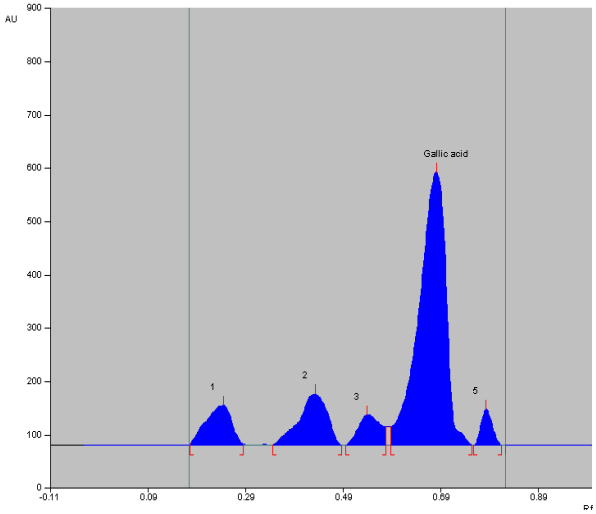

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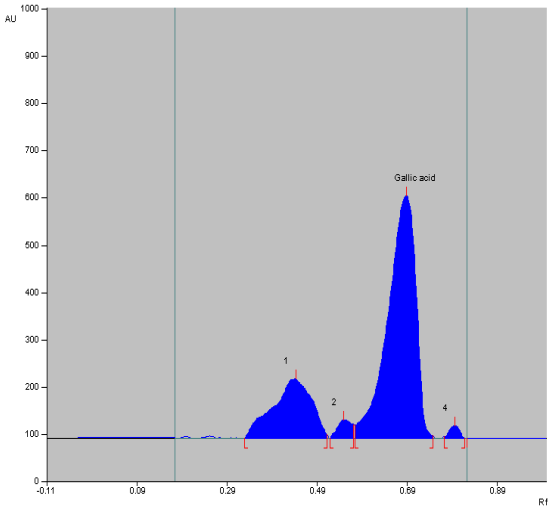

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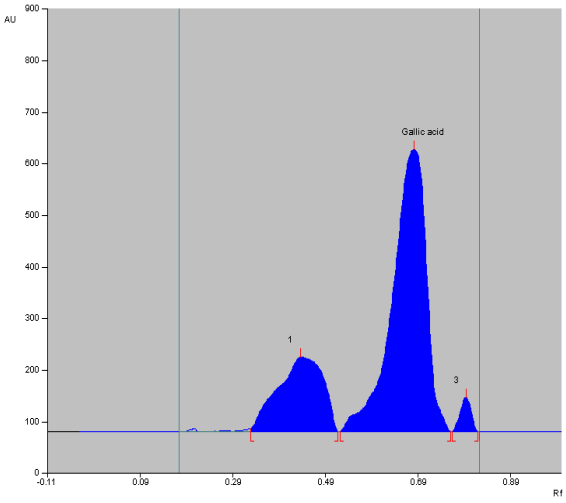
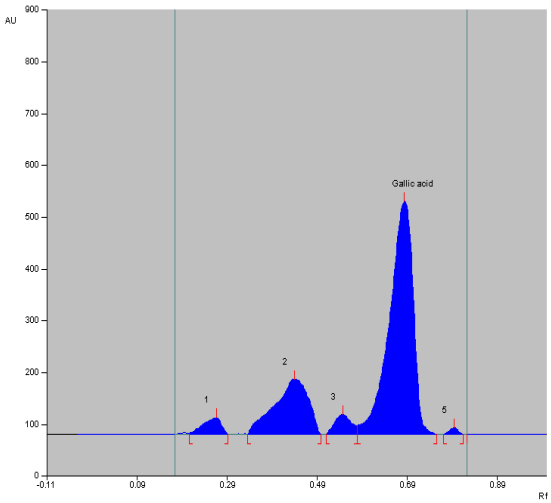
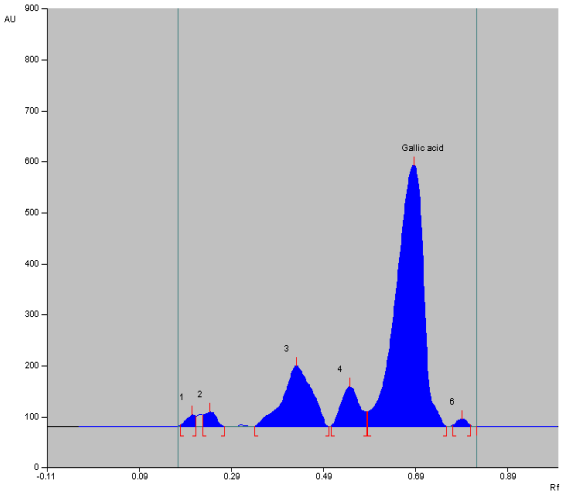

AU
Gallic acid
Rf

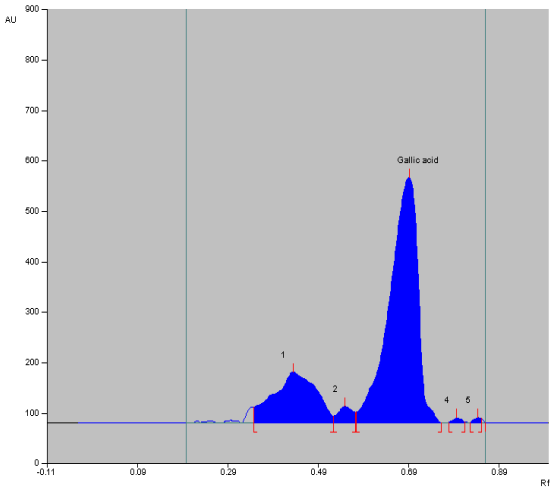
(E)


AU
Gallic acid
Rf

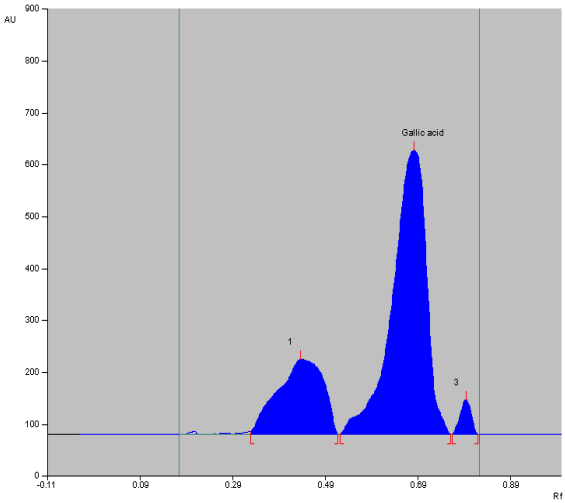
(F)


AU
Gallic acid
Rf

(G)


AU
Gallic acid
Rf

(H)


AU
Gallic acid
Rf

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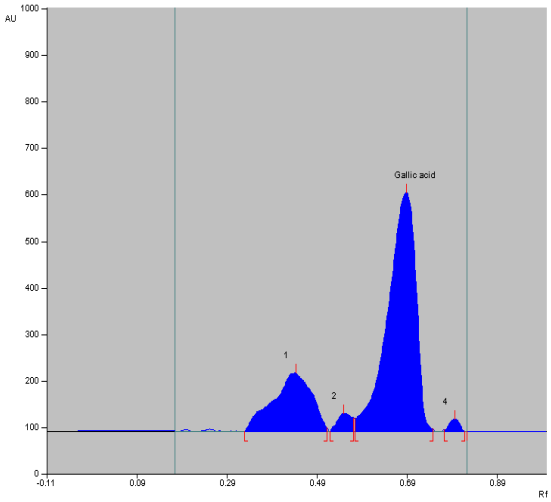
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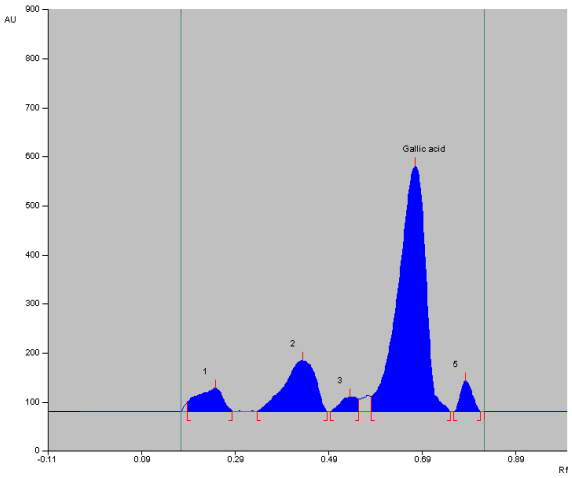
Rf

(J)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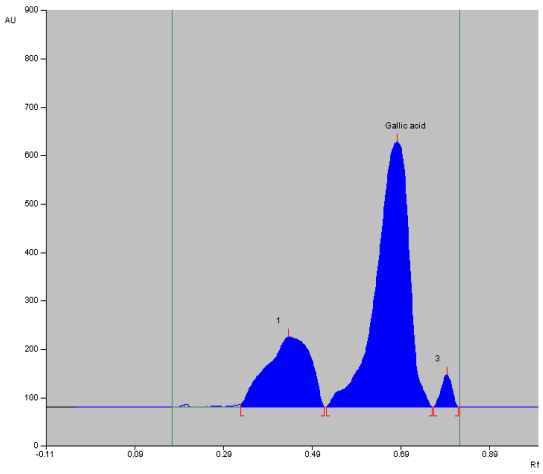
Rf

(K)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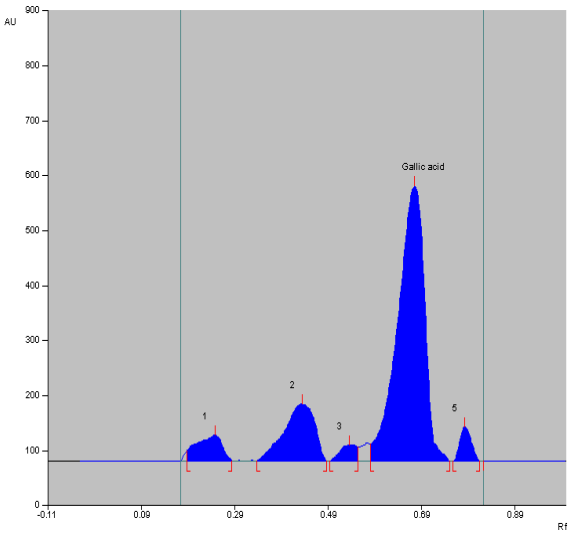
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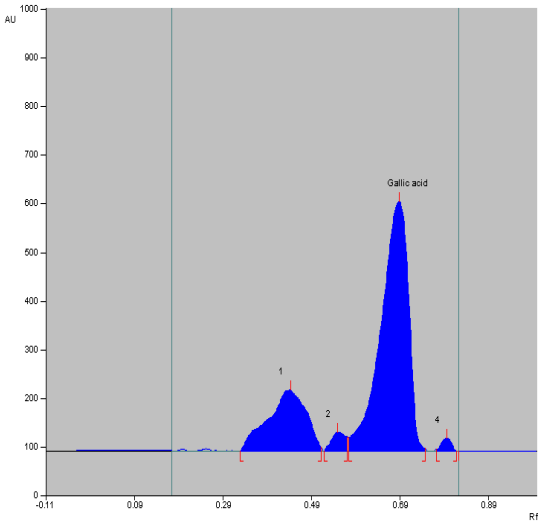
Gallic acid
Rf

(M)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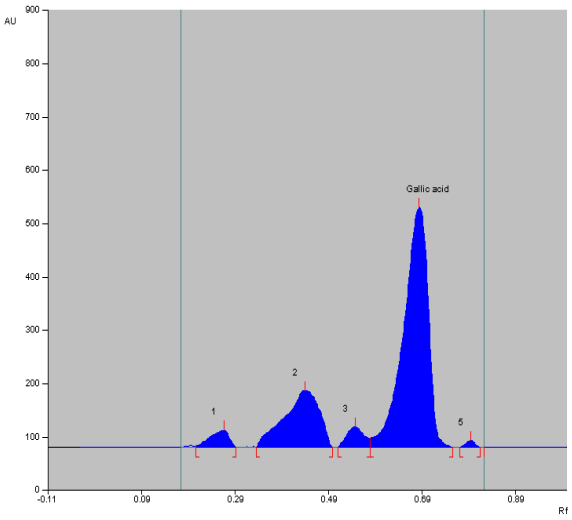
Gallic acid
Rf

(N)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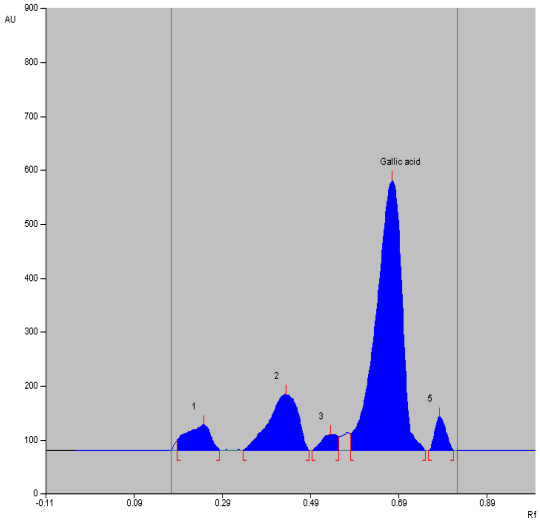
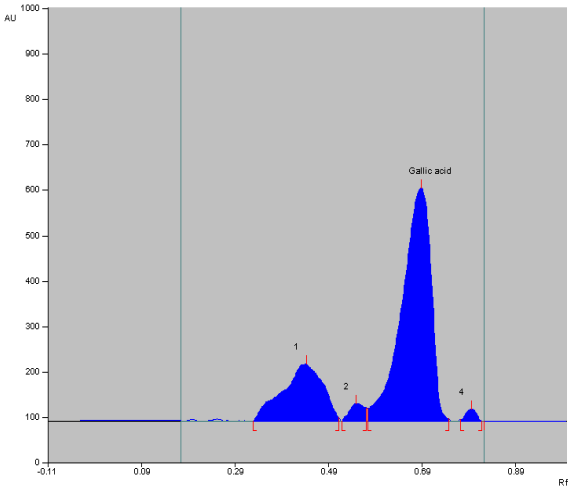
Gallic acid
Rf

(O)


AU
Gallic acid
Rf

(P)



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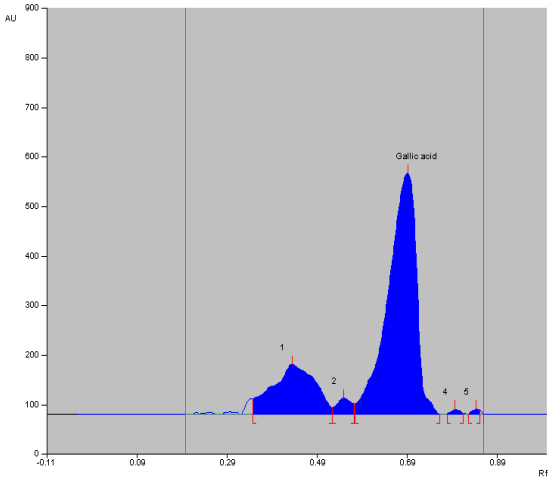

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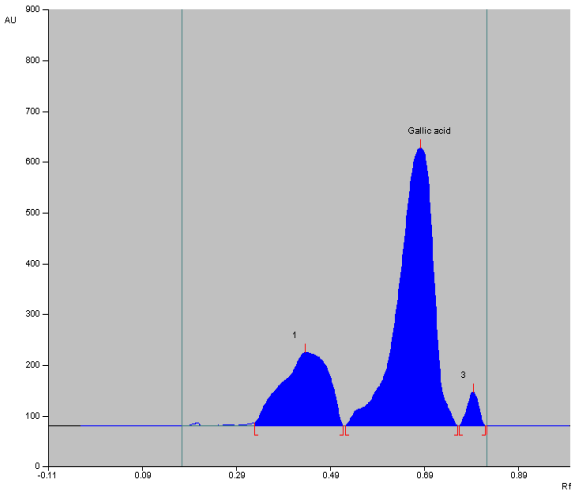

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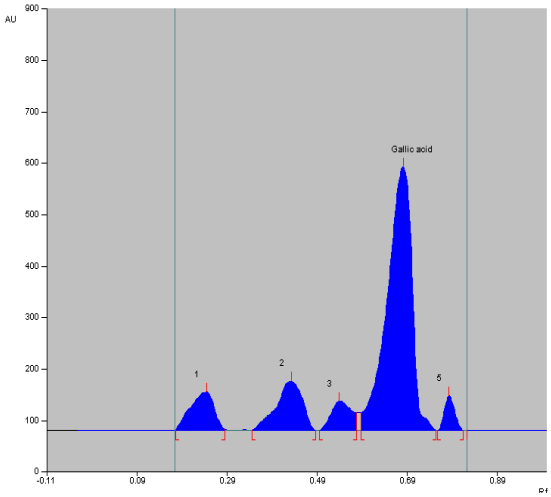
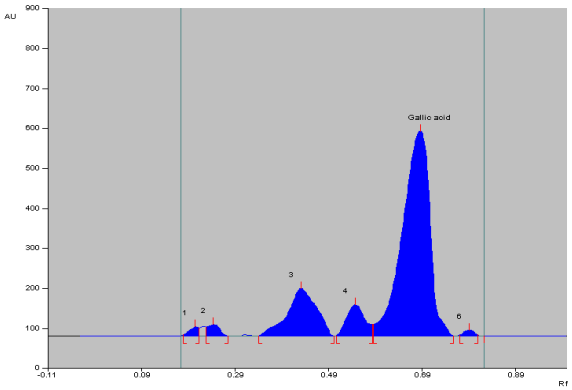


(T)



(U)

Figure 4.10. (A) HPTLC chromatogram of Gallic acid standard (B) HPTLC chromatogram of EO01extract; (C) HPTLC chromatogram of EO02 extract; (D) HPTLC chromatogram of EO03 extract; (E) HPTLC chromatogram of EO04 extract;

(F) HPTLC chromatogram of EO05 extract; (G) HPTLC chromatogram of EO06; (H) HPTLC chromatogram of EO07; (I) HPTLC chromatogram of EO08; (J) HPTLC chromatogram of EO09; (K) HPTLC chromatogram of EO10; (L) HPTLC chromatogram of EO11 extract; (M) HPTLC chromatogram of EO12 extract; (N) HPTLC chromatogram of EO13 extract; (O) HPTLC chromatogram of EO14 extract; (P) HPTLC chromatogram of EO15 extract; (Q) HPTLC chromatogram of EO16 extract; (R) HPTLC chromatogram of EO17 extract; (S) HPTLC chromatogram of EO18 extract; (T) HPTLC chromatogram of EO19 extract; (U) HPTLC chromatogram of EO20 extract.

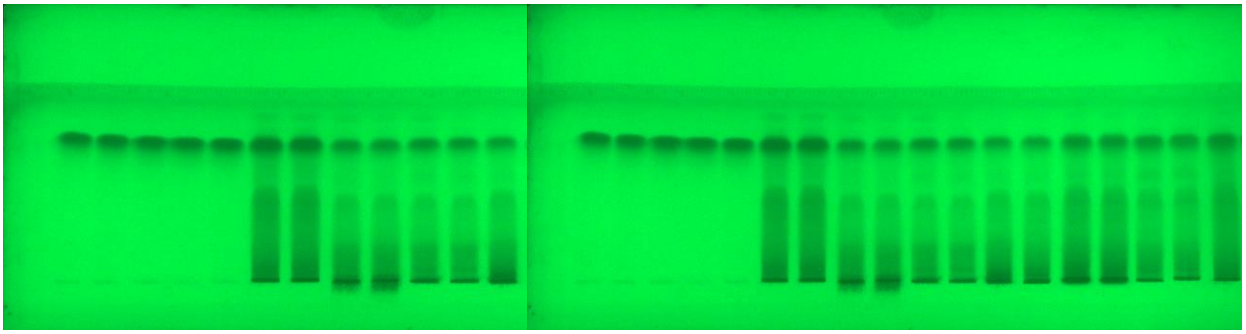


Figure 4.11. Photo-documentation at 254 nm of *Emblica officinalis* extracts and standard gallic acid.

4.3. RP-HPLC Standardization of individual plants

4.3.1. *Andrographis paniculata*

4.3.1.1. Equipments, reagents, solvents and other accessories used

Equipments used

Name of the Equipment	Model used	Manufacturer
Dual piston reciprocating pump	LC-20AD	Shimadzu, Kyoto, Japan
Octadecylsilane RP-HPLC column (250x4.6 nm, 5 µm particle size	Xterra-C18	Waters, India
PDA (Photo diode array detector)	SPD-M20A	Shimadzu, Kyoto, Japan
Software for data handling	LC Solution	Shimadzu, Kyoto, Japan

Name of the Equipment	Model used	Manufacturer
Sample injector	Rheodyne injector (20 µl)	

Solvents and other accessories used

Name of the solvent/Accessories	Company
HPLC grade Methanol and Glacial acetic acid	Finar chemicals, India
HPLC grade water	Milli-Q Water
Micropipettes and Micro-tips	Eppendorff and Accupippette
Membrane filters (0.22 µ and 0.45 µ)	Millipore
Sample injection syringe	Hamilton Microliters syringe (Switzerland)
pH meter	Orion 3-star, Thermo Scientific

4.3.1.2. Preparation of standard solution and sample solution

The andrographolide standard stock solution was prepared by dissolving 1 mg andrographolide in 1 ml methanol to give a 1 mg/ml solution. 10 mg of each leaf extract was weighed and dissolved in 1 ml of methanol to prepare a 10 mg/ml extract. Each standard and extraction solution was vortexed until completely dissolved and filtered through a 0.45 µm syringe filter (Millipore, Burlington, MA, USA). Linearity of response generation standards was determined using a standard curve generated with five different volumes of standard

(andrographolide, 1 mg mL^{-1}) ranging from 2 to 10 µl. Solutions for HPLC analysis were thoroughly vortexed, filtered and sonicated until no suspended particles were visible in solution.

4.3.1.3. Chromatographic conditions

The RP-HPLC system (Waters, USA) consists of an LC-30AD pump, a UV-Vis detector with a 3-line degasser (400 µl volume), and a Rheodyne 7725i injector with a 20 µl loop. Isocratic mobile phase consists of methanol:water:acetic acid (60:40:1, v/v) was used. Solvent A - Methanol, Solvent B - Water (1% acetic acid). The pH of solvent B was adjusted to 3.5 using 1% (v/v) glacial acetic acid. The mobile phase was filtered through a 0.45 mm pore size membrane filter (Millipore) and then sonicated to degas the solvent. Separation was done on a C18 column (5 µm particle size, 250 x 4.6 mm). The column temperature 25°C and the injection volume was 20 µl. Total run time was 10 minutes. The same procedure was used for extracts. The flow rate was set at 0.8 mL min^{-1} and monitored at a wavelength of 254 nm. Andrographolide exhibits a strong peak at 254 nm, excellent resolution and maximum absorbance.

4.3.1.4. Construction of calibration curve

Linearity of standards prepared in reactions was determined using a standard curve generated with 5 different volumes of standards (andrographolide: 1 mg mL^{-1}) ranging from 100 to 500 µg mL^{-1} it was done. A good correlation was found between concentration and peak area, yielding a coefficient of determinant (r^2) > 0.99. A standard curve was obtained by plotting mean peak area (y-axis) versus concentration (x-axis).

4.3.1.5. Method validation

Validation of the HPLC method was performed according to the International Conference on Harmonization (ICH, 1996, 2005) guidelines and FDA guidelines (1994) as recommended. Define detection, precision, precision, robustness, LOD and LOQ.

4.3.1.6. Results

The correlation coefficient was found to be >0.99 from the standard curve, confirming that the data are close to the line of best fit. The regression equation is Y = 2.5874 X + 0.998. LOD and LOQ were estimated to be 1.40 and 7.05 µg/mL, respectively, reflecting the high sensitivity of the method. Various regression and analysis parameters are shown in Table 4.13. Recoveries ranged from 99.04 to 99.30%, demonstrating the accuracy of the method. The results of recovery studies are shown in Table 4.15. The method was highly reproducible as the RSD (%) for intra-day and inter-day precision was < 2%. Table 4.14 shows the daily and daily accuracy studies. The andrographolide content of various extracts is shown in Table 4.16. From the average of six determinations of test concentrations, the theoretical plate number (preferred >2000), capacity factor (preferred 2-10), and tailing factor (preferred <1.5) were determined to be 29087, 7.92, and 1.35 of each decision. Chromatograms of typical andrographolide and Andrographis paniculata extract are shown in Figure 4.12.

Table 4.13. Regression and analytical parameters for the determination of andrographolide in *A. paniculata* by RP-HPLC

Parameters	**RP-HPLC**
Calibration range	100-500 µg mL $^{-1}$
Slope	0.0315
Intercept	0.4871
Regression equation (Y)	2.5874X + 0.998
Correlation coefficient	0.9998
LOD	1.40 µg mL $^{-1}$
LOQ	7.05 µg mL $^{-1}$

Table 4.14. Intra-day and Inter-day precision studies of the extract by RP-HPLC

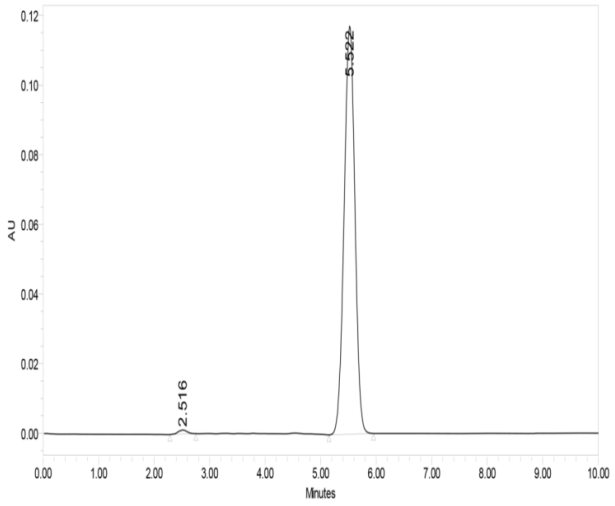
Marker compound	**Precision of RP-HPLC study (n=6)**		
	Amount of andrographolide applied (ng per spot)	**Intra-day %RSD**	**Inter-day %RSD**
Andrographolide	200	0.91	0.95
	400	0.88	0.92

Table 4.15. Recovery studies of the extract by RP-HPLC

Marker compound	**Recovery by RP-HPLC study (n=6)**			
	Amount added (ng)	**Average recovery (%)**	**Mean±SD %**	**%RSD**
Andrographolide	0	99.08		
	200	99.27	99.91±0.85	1.03
	400	99.04		
	600	99.30		

Table 4.16. Content of Andrographolide estimated by the RP-HPLC study

Serial no	Sample	Voucher specimen number	HPLC (w/w %)
1	AP1a	SNPS/JU/2019/1516	2.71
2	AP1b	SNPS/JU/2019/1517	3.19
3	AP1c	SNPS/JU/2019/1518	1.83
4	AP1d	SNPS/JU/2019/1519	1.73
5	AP1e	SNPS/JU/2019/1520	2.94
6	AP1f	SNPS/JU/2019/1521	2.98
7	AP1g	SNPS/JU/2019/1522	1.95
8	AP1h	SNPS/JU/2019/1523	2.59
9	AP1i	SNPS/JU/2019/1524	2.35
10	AP1j	SNPS/JU/2019/1525	3.05



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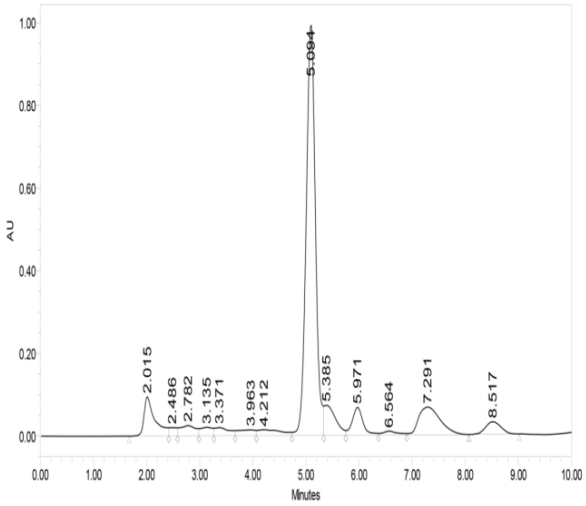

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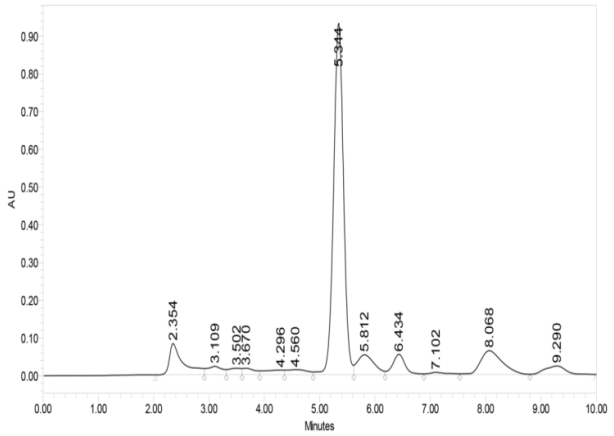
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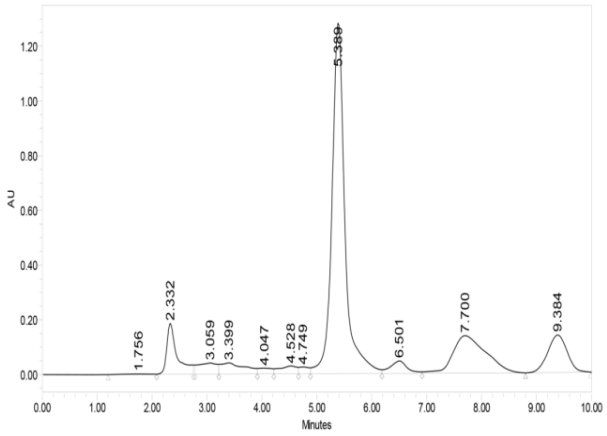
9.290

(C)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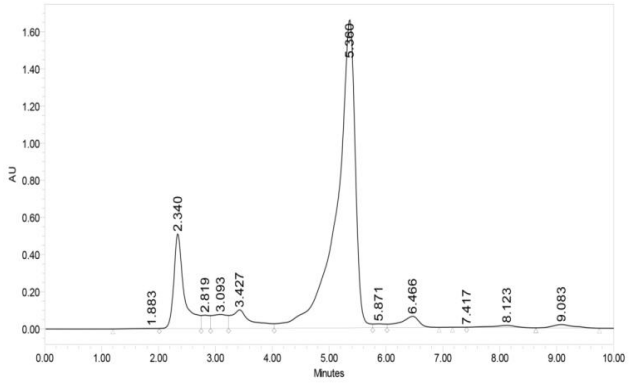
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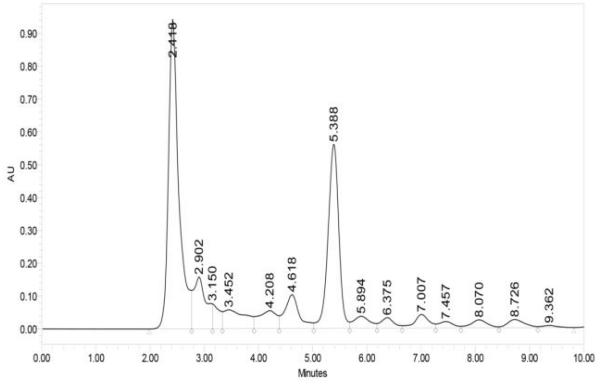
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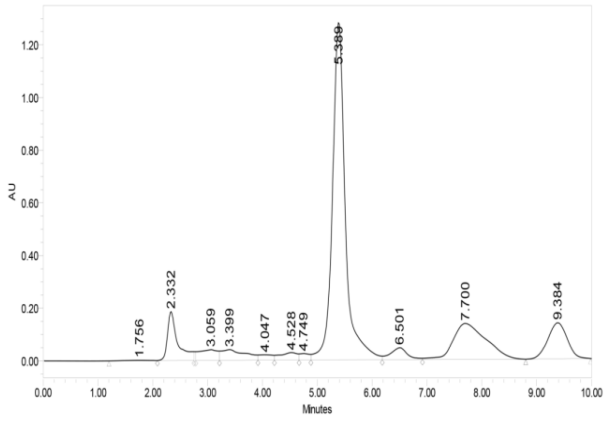
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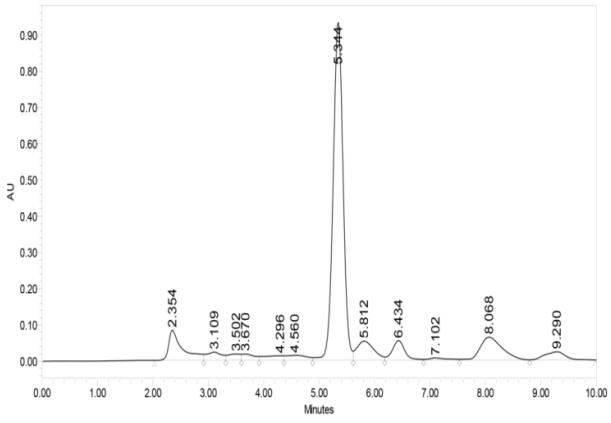
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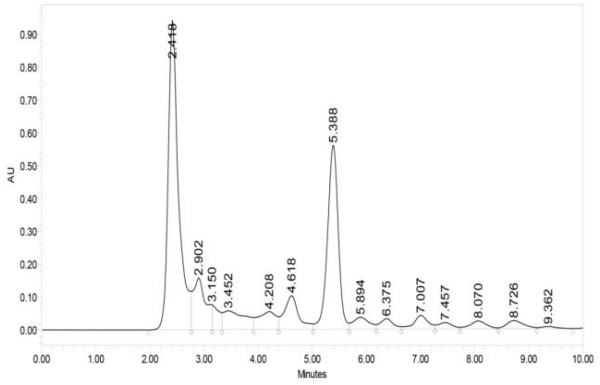

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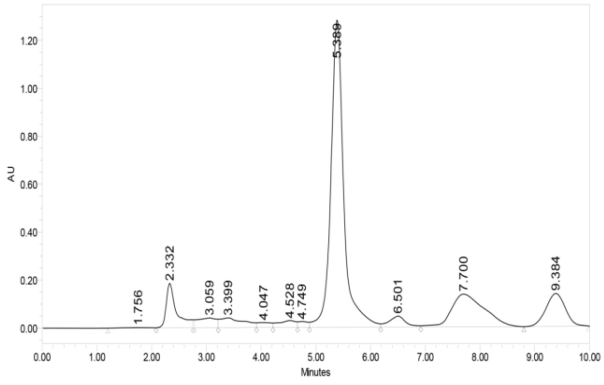

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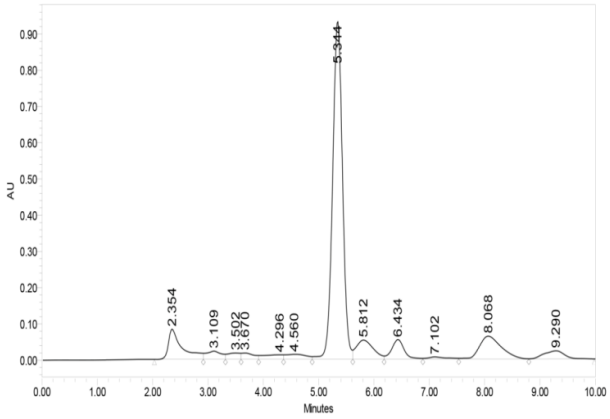

(K)

Figure 4.12. (A) HPLC chromatogram of andrographolide standard; (B) HPLC chromatogram of AP1a extract; (C) HPLC chromatogram of AP1b extract; (D) HPLC chromatogram of AP1c extract; (E) HPLC chromatogram of AP1d extract; (F) HPLC chromatogram of AP1e extract; (G) HPLC chromatogram of AP1f extract; (H) HPLC chromatogram of AP1g extract; (I) HPLC chromatogram of AP1h extract; (J) HPLC chromatogram of AP1i extract; (K) HPLC chromatogram of AP1j extract.

4.3.2. *Piper nigrum*

4.3.2.1. Equipments and reagents

The equipment and reagents used are the same as described in Section 4.3.1.1.

4.3.2.2. Preparation of standard solution and sample solution

The standard and sample solutions preparation were carried out in the same manner as described in section 4.3.1.2. In this study, piperine was used as standard.

4.3.2.3. Chromatographic conditions

The RP-HPLC system (Shimadzu LC, Japan) consists of an LC-30AD pump, an SPD-M20A photodiode array (PDA) detector with a 3-line degasser (400 µL capacity), and a 20 µL Rheodyne 7725i injector with 20 µL loop. A Milli-Q water purification system (Bedford, MA, USA) fitted with a 0.22 mm Millipak Express filter and an Eyela (Tokyo, Japan) rotary vacuum evaporator was used. An isocratic mobile phase consists of methanol:Milli-Q water:acetic acid (75:24:1, v/v/v) was used. Solvent A - Methanol, Solvent B - Water (1% acetic acid). The pH of solvent B was adjusted to 3.5 using 1% (v/v) glacial acetic acid. After the mobile phase was filtered through a 0.45 µm pore size membrane filter (Millipore), the solvent was degassed by sonication, and the sample was filtered using a syringe filter (NYL 0.45 µm) to extract the solution. Separation was performed on a C18 column (5 µm particle size, 250 x 4.6 inches). The column temperature was 25°C and the injection volume was 20 µl. The total run time was set 15 minutes and the same procedure was used for extraction.

4.3.2.4. Construction of calibration curve

The calibration curve construction was done in the same way as section 4.3.1.4.

4.3.2.5. Method validation

Method validation were same as mentioned in section 4.3.1.5.

4.3.2.6. Results

Correlation coefficient was found to be >0.99, the regression equation is Y = 999.8 + 886.6x. LOD and LOQ were estimated to be 1.05 and 5.39 µg/mL, respectively, reflecting the high sensitivity of the method. Various regression and analysis parameters are shown in Table 4.17. Recoveries ranged from 99.06 to 99.67%, demonstrating the accuracy of the study. The results of the recovery study are shown in Table 4.19. The method was highly reproducible as the RSD (%) for intra-day and inter-day precision was < 2%. Table 4.18 shows the daily and daily accuracy studies. The piperine content of various extracts is shown in Table 4.20. From the average of six determinations of test concentrations, the theoretical plate number (preferred >2000), capacity factor (preferred 2-10), and tailing factor (preferred <1.5) were determined to be 29087, 7.92, and 1.35 of each decision. Chromatograms of standard extracts from piperine and *Piper nigrum* are shown in Figure 4.13.

Table 4.17. Regression and analytical parameters for the determination of piperine in *P. nigrum* by RP-HPLC

Parameters	**RP-HPLC**
Calibration range	100-500 µg mL^{-1}
Slope	0.0816
Intercept	0.9876
Regression equation (Y)	999.8 + 886.6x
Correlation coefficient	0.9998
LOD	1.05 µg mL^{-1}
LOQ	5.39 µg mL^{-1}

Table 4.18. Intra-day and Inter-day precision studies of the extract by RP-HPLC

Marker compound	Precision of RP-HPLC study (n=6)		
	Amount of Piperine applied (ng per spot)	Intra-day %RSD	Inter-day %RSD
Piperine	200	0.87	0.92
	400	0.89	0.95

Table 4.19. Recovery studies of the extract by RP-HPLC

Marker compound	Recovery by RP-HPLC study (n=6)			
	Amount added (ng)	Average recovery (%)	Mean±SD %	%RSD
Piperine	0	99.06		
	200	99.35	99.93±0.91	1.04
	400	99.29		
	600	99.67		

Table 4.20. Content of Piperine estimated by the RP-HPLC study

Sample	Voucher Specimen Number	Piperine content (% w/w)
*PN*1	SNPS/JU/2019/1506	4.05
*PN*2	SNPS/JU/2019/1507	3.81
*PN*3	SNPS/JU/2019/1508	3.74
*PN*4	SNPS/JU/2019/1509	3.91
*PN*5	SNPS/JU/2019/1510	3.98
PN6	SNPS/JU/2019/1511	3.99
PN7	SNPS/JU/2019/1512	4.83
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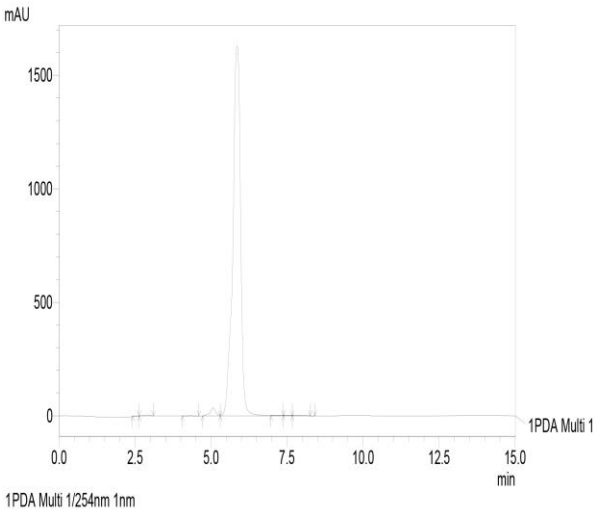
PN9	SNPS/JU/2019/1514	3.76
PN10	SNPS/JU/2019/1515	3.87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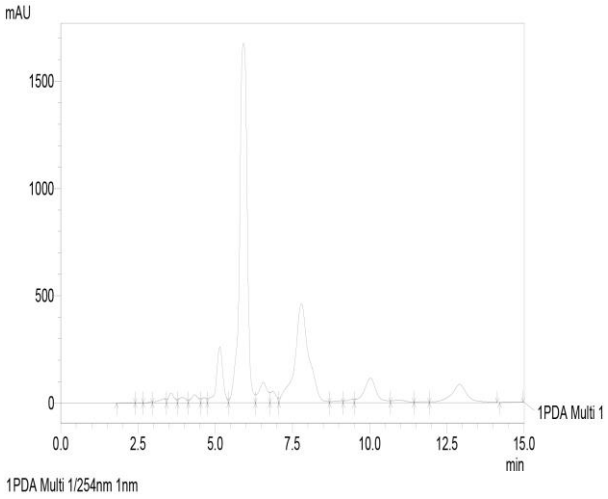
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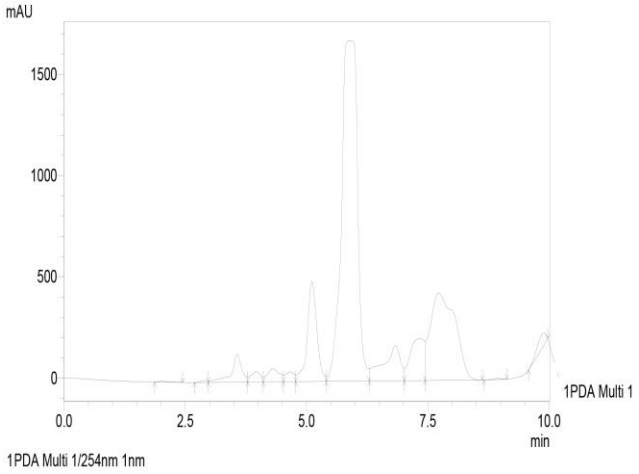
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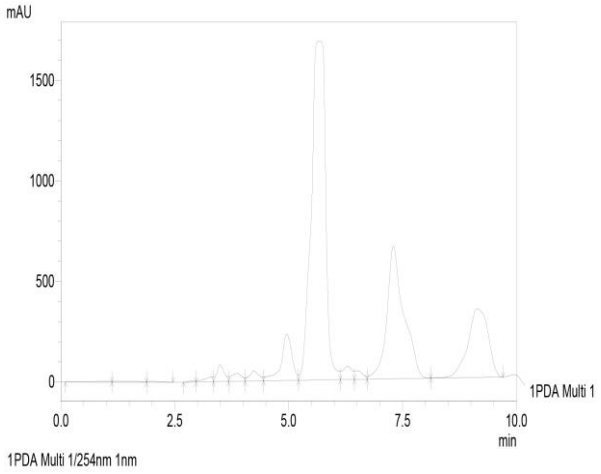
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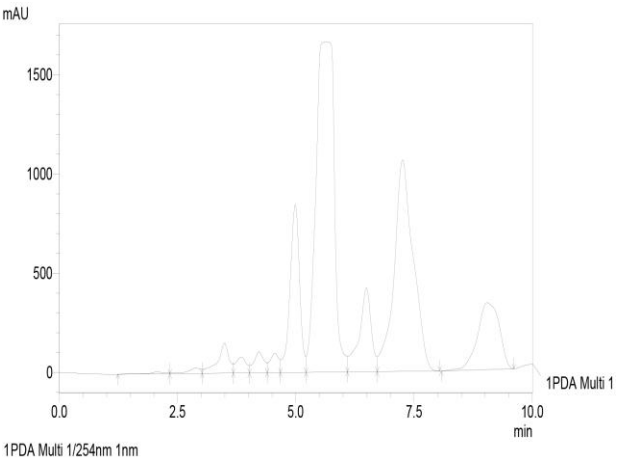
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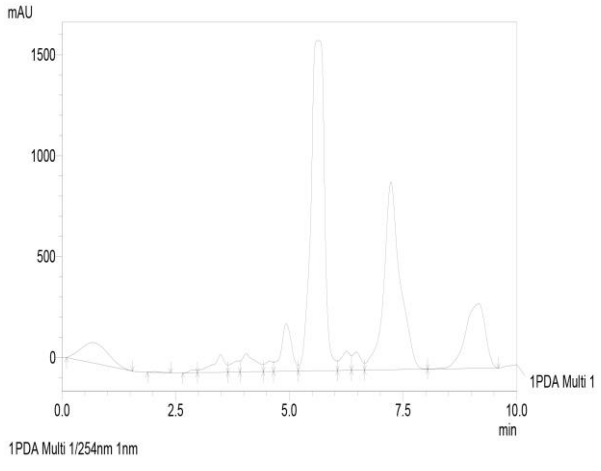
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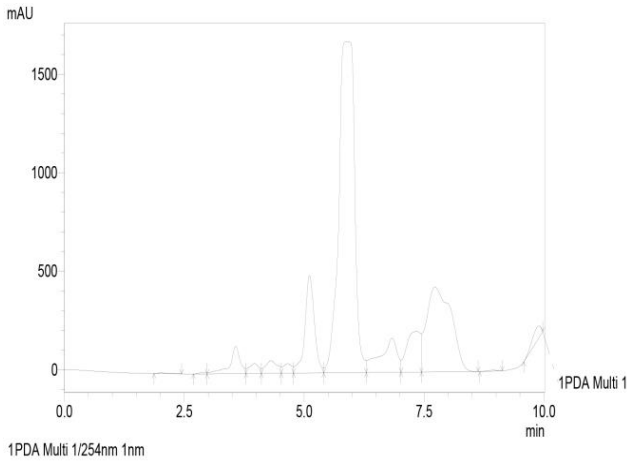

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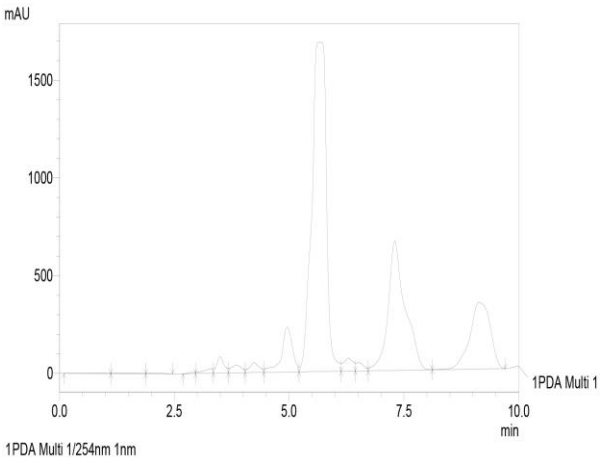

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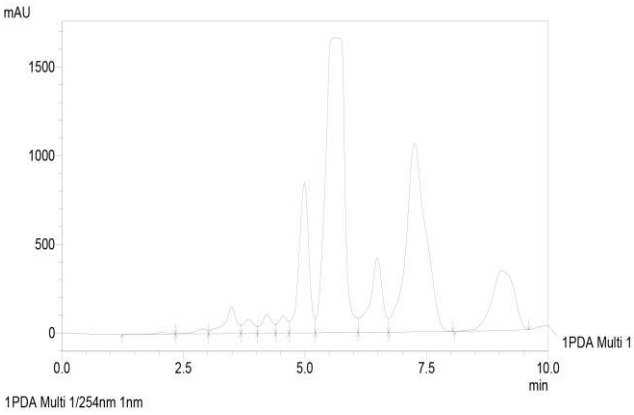

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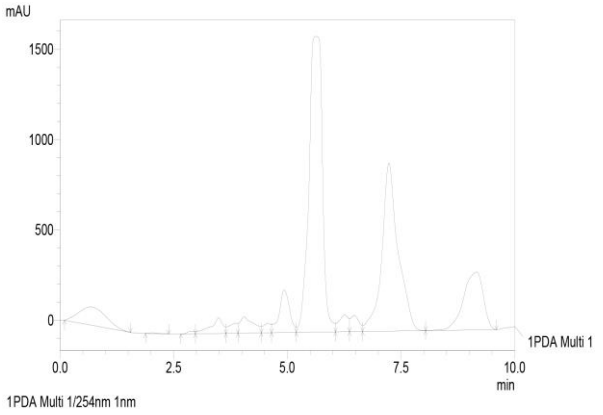

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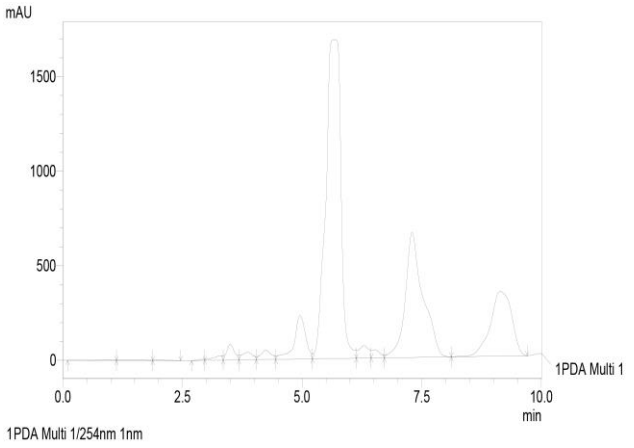

(K)

Figure 4.13. (A) HPLC chromatogram of piperine standard; (B) HPLC

chromatogram of PN1 extract; (C) HPLC chromatogram of PN2 extract; (D) HPLC chromatogram of PN3 extract; (E) HPLC chromatogram of PN4 extract; (F) HPLC chromatogram of PN5 extract; (G) HPLC chromatogram of PN6 extract; (H) HPLC chromatogram of PN7 extract; (I) HPLC chromatogram of PN8 extract; (J) HPLC chromatogram of PN9 extract; (K) HPLC chromatogram of PN10 extract.

4.3.3. *Emblica officinalis*

4.3.3.1. Equipments and reagents

The equipments and reagents used were same as described in section 4.3.1.1.

4.3.3.2. Preparation of standard solution and sample solution

The standard and sample solutions preparation were carried out in the same manner as described in section 4.3.1.2. In this study, gallic acid was used as standard.

4.3.3.3. Chromatographic conditions

The RP-HPLC system (Shimadzu LC, Japan) consists of LC-30AD pump, an SPD-M20A Photodiode array (PDA) detector equipped with a 3-line degasser (400 µL capacity) and a 20 µL Rheodyne 7725i injector was configured. A Milli-Q water purification system (Bedford, MA, USA) fitted with a 0.22 mm Millipak Express filter and an Eyela (Tokyo, Japan) rotary vacuum evaporator was used. An isocratic mobile phase consisting of methanol:water:acetic acid (76:23:1, v/v/v) was used. Solvent A - Methanol, Solvent B - water (1% acetic acid). The pH of the solvent B was adjusted to 3.5 with 1% (v/v) glacial acetic acid.The mobile phase was filtered through 0.45 µm pore size (Millipore) membrane filter followed by

sonication to degas the solvent and syringe filters (NYL 0.45 µm) were used for the filtration of the sample and extract solution. The separation was carried out on a C18 column (5µm particle size, 250 × 4.6").The temperature of the column was kept at 25°C and the injection volume was 20 µL. The total run time was set at 10 minutes.The same method was applied for the extract. The flow rate was adjusted at 0.8 mL/min and monitored at a wavelength of 254 nm. Quantitative estimation was performed in the Empower 2 software program using an external calibration method.

4.3.3.4. Construction of calibration curve

The calibration curve construction was done in the same way as section 4.3.1.4.

4.3.3.5. Method validation

Method validation were same as described in section 4.3.1.5.

4.3.3.6. Results

The correlation coefficient was found to be > 0.99 from, confirming that the data are close to the line of best fit. The regression equation is Y = 9993.5 + 830.4x. LOD and LOQ were estimated to be 1.62 and 4.156 µg/mL respectively. Different regression and analytical parameters have been presented in Table 4.21. The % recovery was found to be 99.01-99.81%,that is indicates accuracy of the study. The results of the recovery study are shows in Table 4.23. The method was highly repeatable, this is because RSD (%) for intra-day and inter-day precision is <2%. Inter-day and intra-day precision study are presented in Table 4.22. Gallic acid content of different extracts are shown in Table 4.24. From the average of six determinations of test concentrations, the theoretical plate number (preferred >2000), capacity factor (preferred 2-10), and tailing factor (preferred <1.5) were

determined to be 29087, 7.92, and 1.35 of each decision. The chromatograms of standard gallic acid and *Emblica officinalis* extracts are presented in Figure 4.14.

Table 4.21. Linear regression data for calibration plot of gallic acid using the proposed RP-HPLC method

Parameters	**RP-HPLC**
Calibration range	100-500 µg mL $^{-1}$
Slope	0.4298
Intercept	0.9983
Regression equation (Y)	9993.5 + 830.4x
Correlation coefficient	0.99081
LOD	1.62 µg mL $^{-1}$
LOQ	4.156 µg mL $^{-1}$

Table 4.22. Intra-day and Inter-day precision studies of the extract by RP-HPLC

Marker compound	**Precision of RP-HPLC study (n=6)**		
	Amount of Gallic acid applied (ng per spot)	**Intra-day %RSD**	**Inter-day %RSD**
Gallic acid	200	0.91	0.96
	400	0.93	0.98

Table 4.23. Recovery studies of the extract by RP-HPLC

Marker compound	**Recovery by RP-HPLC study (n=6)**			
	Amount added (ng)	**Average recovery (%)**	**Mean±SD %**	**%RSD**
Gallic acid	0	99.01		
	200	99.53	99.95±0.89	1.09
	400	99.81		
	600	99.13		

Table 4.24. Gallic acid content estimated by the RP-HPLC study

Sample	Voucher specimen number	Gallic acid content (% *w/w*)
EO0 1	SNPS/JU/2020/1526	5.79
*EO0*2	SNPS/JU/2020/1527	4.98
*EO0*3	SNPS/JU/2020/1528	6.16
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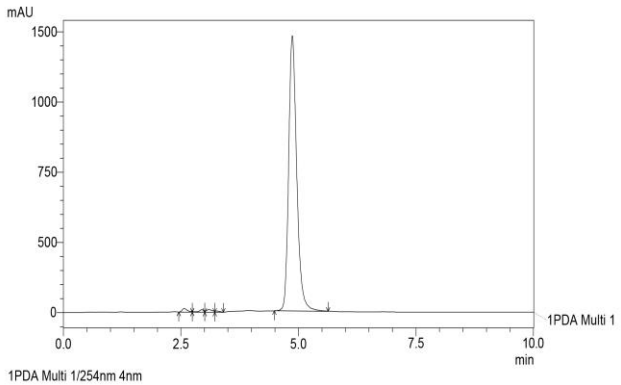

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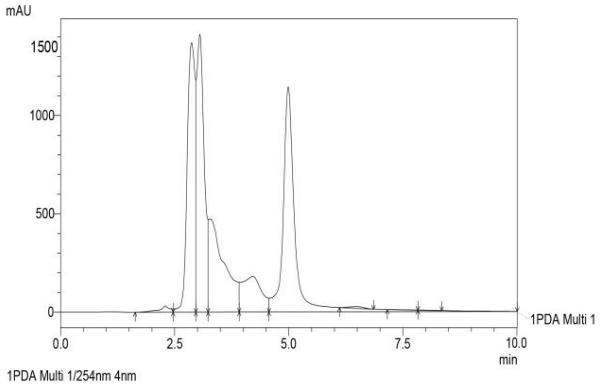

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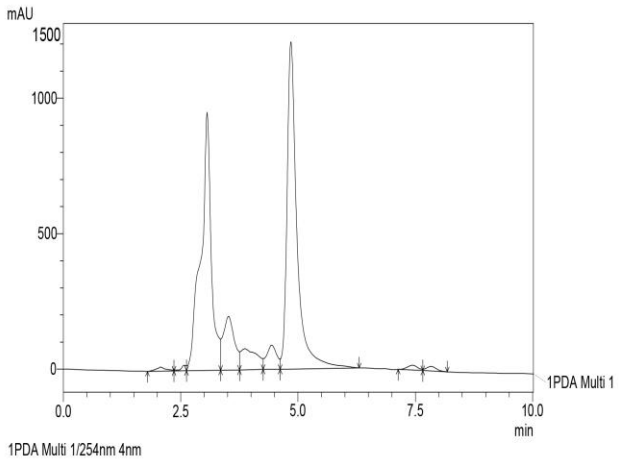
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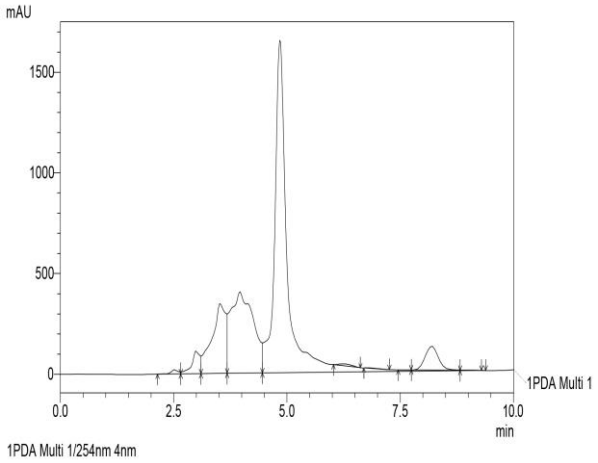
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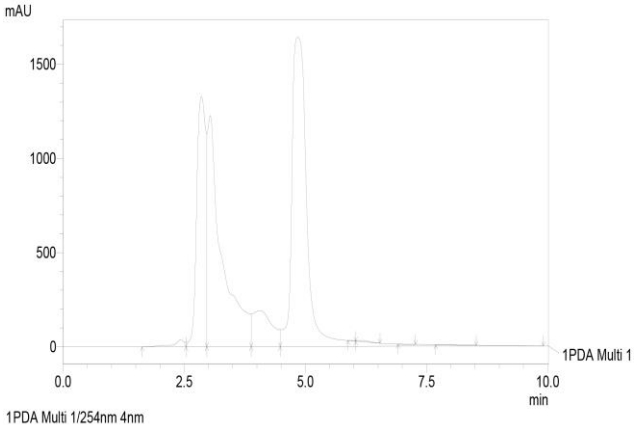
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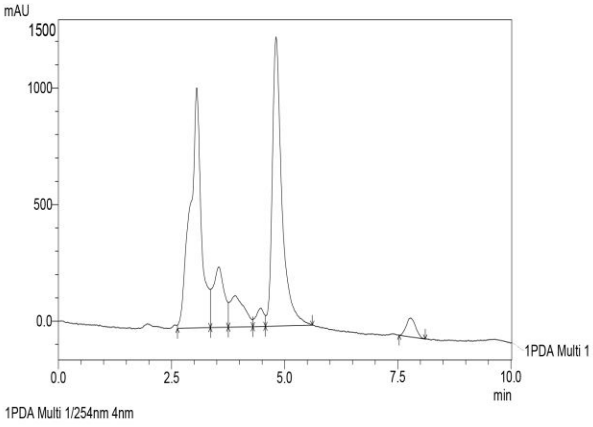
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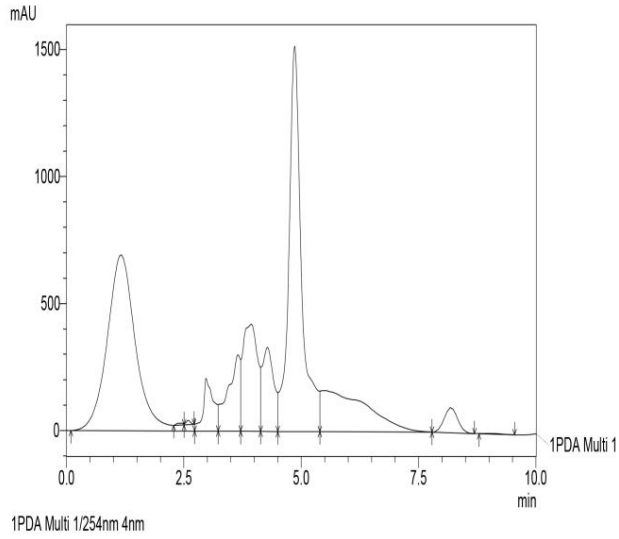
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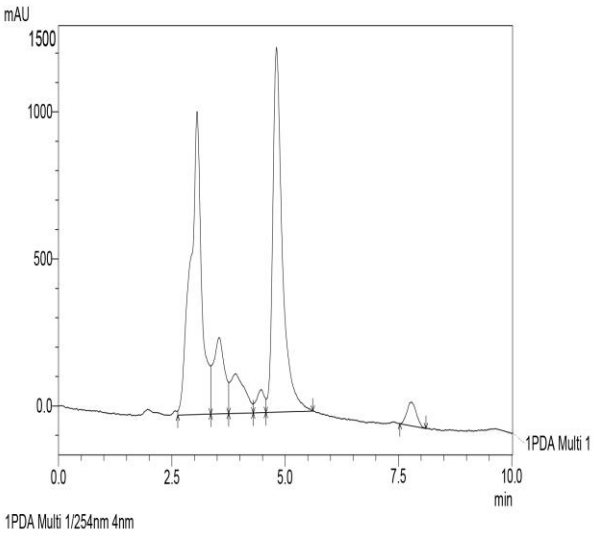
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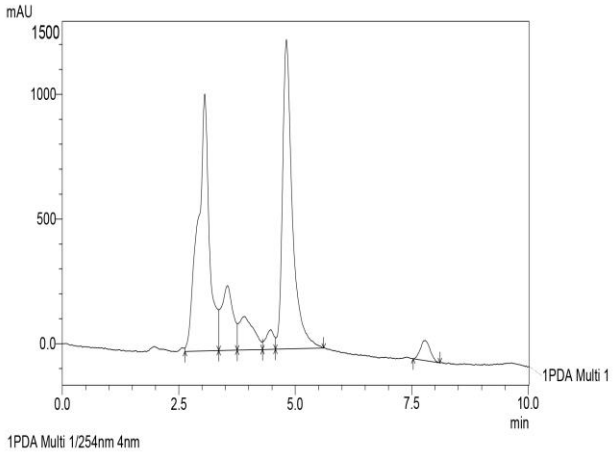
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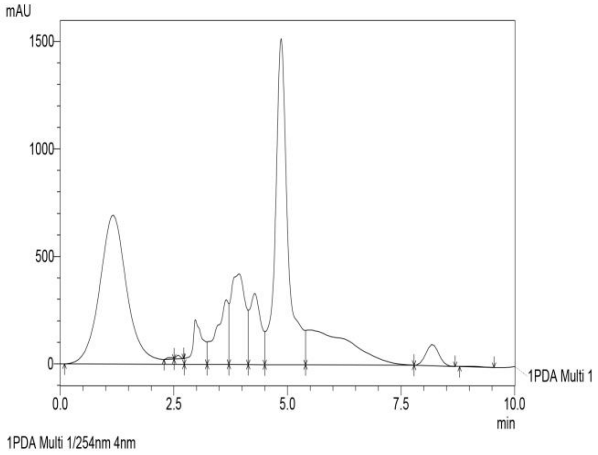
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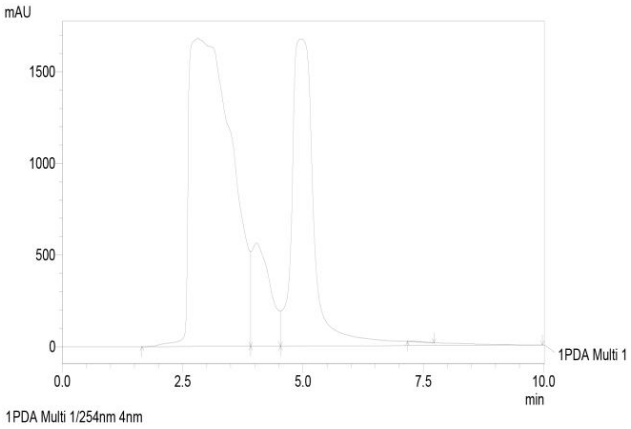
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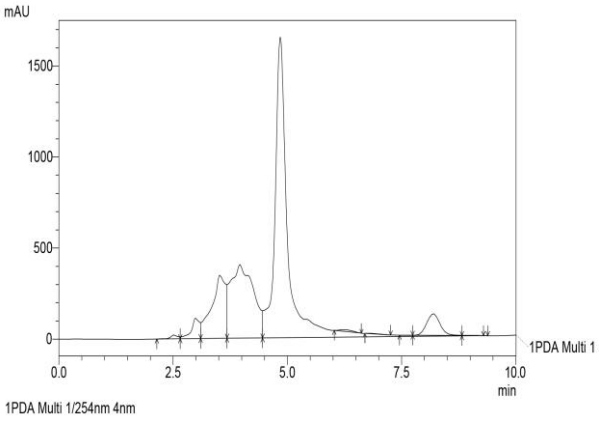
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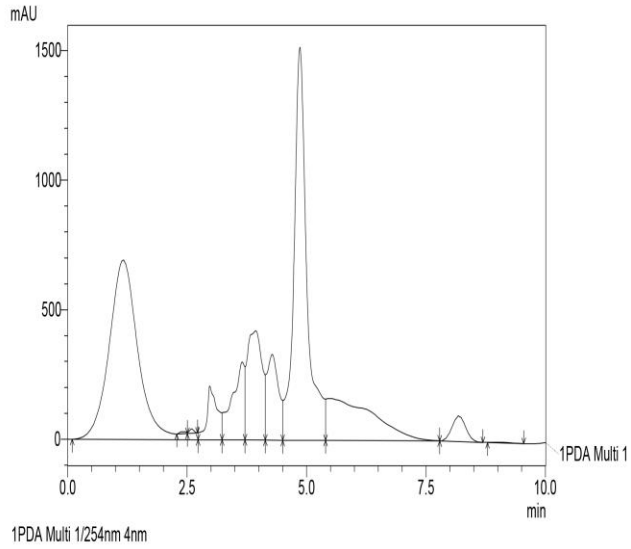
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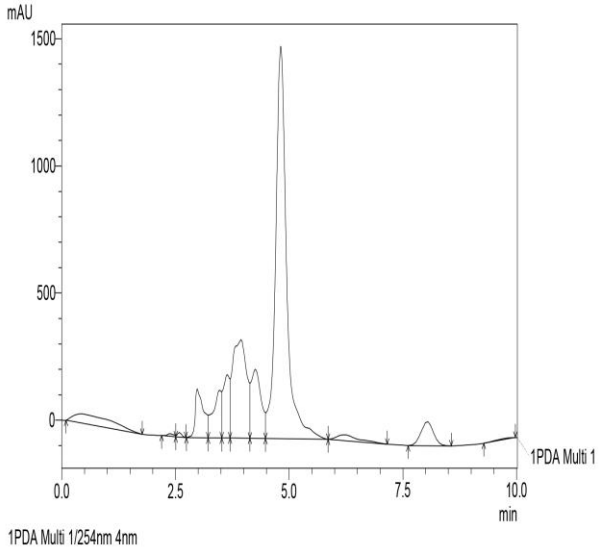
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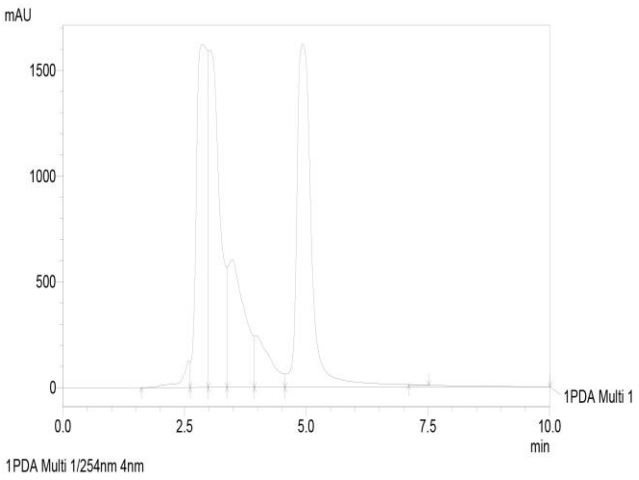
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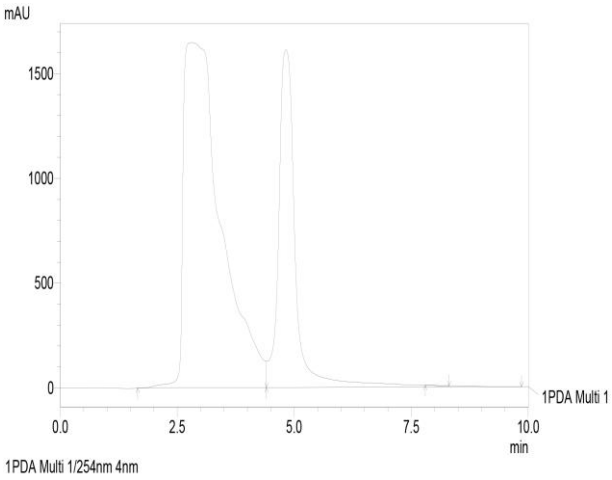
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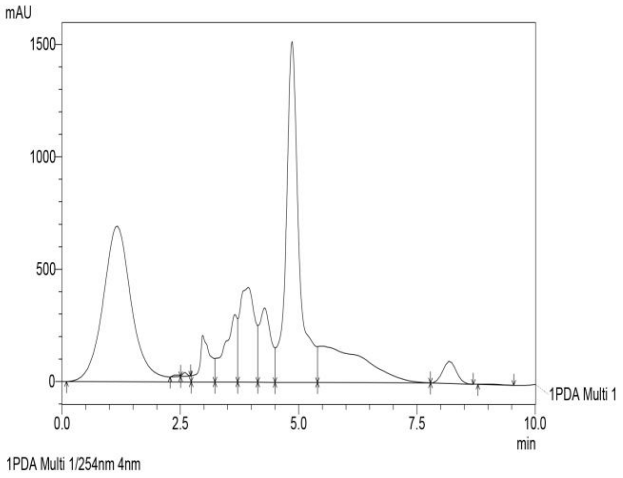
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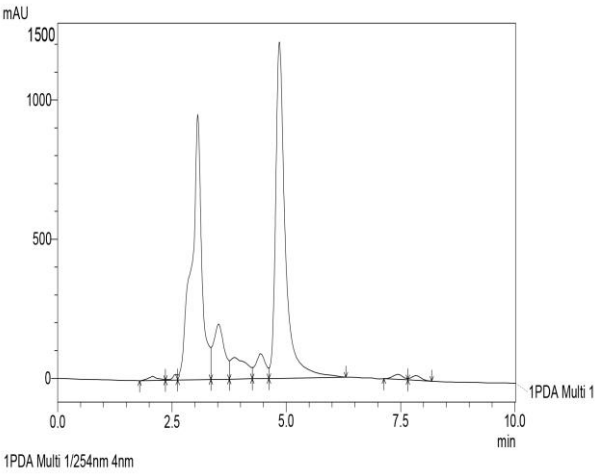
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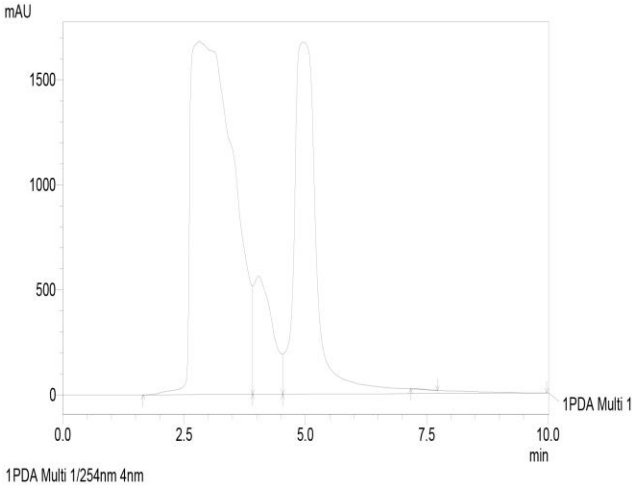
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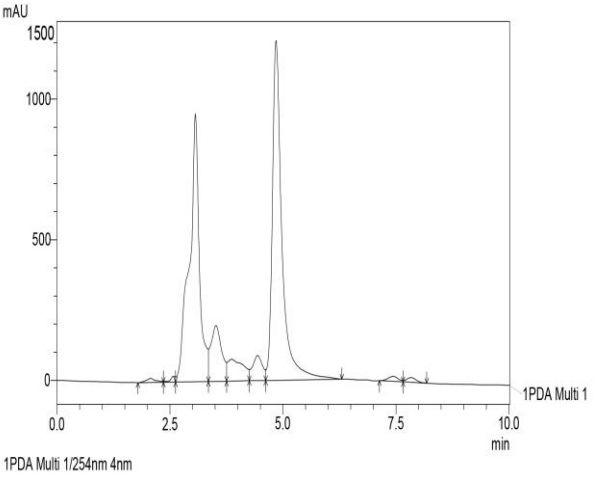
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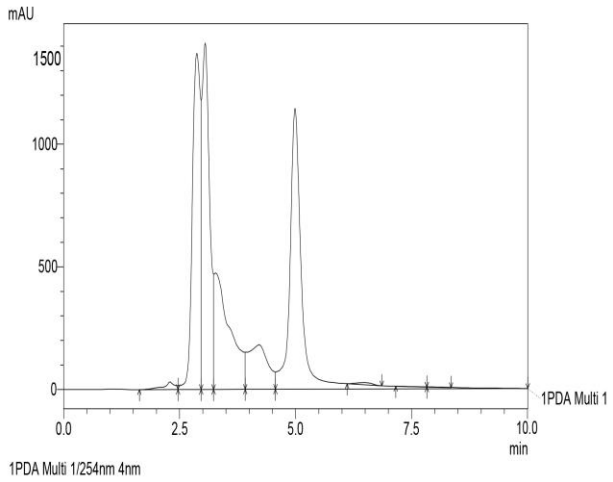

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Figure 4.14. (A) HPLC chromatogram of Gallic acid standard (B) HPLC chromatogram of EO01extract; (C) HPLC chromatogram of EO02 extract; (D) HPLC chromatogram of EO03 extract; (E) HPLC chromatogram of EO04 extract; (F) HPLC chromatogram of EO05 extract; (G) HPLC chromatogram of EO06; (H) HPLC chromatogram of EO07; (I) HPLC chromatogram of EO08; (J) HPLC chromatogram of EO09; (K) HPLC chromatogram of EO10; (L) HPLC chromatogram of EO11 extract; (M) HPLC chromatogram of EO12 extract; (N) HPLC chromatogram of EO13 extract; (O) HPLC chromatogram of EO14 extract; (P) HPLC chromatogram of EO15 extract; (Q) HPLC chromatogram of EO16 extract; (R) HPLC chromatogram of EO17 extract; (S) HPLC chromatogram of EO18 extract; (T) HPLC chromatogram of EO19 extract; (U) HPLC chromatogram of EO20 extract.

Publications:

1. Quantification of piperine in different varieties of *Piper nigrum* by a validated high-performance thin-layer chromatography–densitometry method. **Shibu Narayan Jana**, Dilip Sing, Subhadip Banerjee, Pallab Kanti Haldar, Barun

Dasgupta, Amit Kar, Nanaocha Sharma, Rajib Bandyopadhayay, Pulok K Mukherjee, JPC–Journal of Planar Chromatography–Modern TLC, 34, 2021, 521-530.

2. Quantification and Standardization of andrographolide in *Andrographis paniculata samples* by validated RP-HPLC and HPTLC methods. **Shibu Narayan Jana**, Subhadip Banerjee, Sayan Biswas, Dilip Sing, Amit Kar, Rajib Bandyopadhayay, Pallab K Haldar, Nanaocha Sharma and Pulok K. Mukherjee, Journal of Chromatographic Sciences, Oxford University Press. (**Accepted, "in press"**).

3. Quality evaluation and quantification of gallic acid in different varieties of *Emblica officinalis* by a validated high performance thin-layer chromatography-densitomety method. **Shibu Narayan Jana**, Amit Kar, Barun Dasgupta, Subhadip Banerjee, Pallab Kanti Haldar, Rajib Bandyopadhyay, and Pulok K. Mukherjee, Journal of Planar Chromatography. ("Under review").

Presentations:

1. Participated and presented an Oral presentation at 5th Convention Society for Ethnopharmacology, India (SFE 2018, Sept 7-8) at Jadavpur University, Kolkata "Quality evaluation and standardization of Piperine in *Piper nigrum* samples using RP-HPLC and NIRs" **Shibu Narayan Jana**, Dilip Sing, Barun Dasgupta, Subhadip Banerjee, Rajib Bandyopadhyay, and Pulok K. Mukherjee.

2. Participated and presented a poster at 7th International Congress of Society for Ethnopharmacology, India (SFEC 2020, Feb 15-17) at Jamia Hamdard, New Delhi "HPLC and HPTLC method for quantification and standardization of andrographolide in *Andrographis paniculata* extract" **Shibu Narayan Jana**, Dilip Sing, Rajib Bandyopadhyay, Subhadip Banerjee, and Pulok K. Mukherjee.

Chapter 5

Near Infra-red spectroscopic technique for quality evaluation of *E. officinalis*

5.1. NIRs in medicinal plants analysis

5.1.1. NIRs study of *Emblica officinalis*

5.1.1.1. Experimental set up of Near Infrared Spectroscopy

5.1.1.2. Standard Normal Variate (SNV)

5.1.1.3. Multiplicative Scatter Correction (MSC)

5.1.1.4. Derivative Algorithm

5.1.1.5. Principle Component Analysis (PCA)

5.1.1.6. Linear Discriminant Analysis

5.1.1.7. Model evaluation

5.1.2. Results

5.1. NIRs in medicinal plants analysis

Medicinal plants and its products are commonly used for different pharmacological interventions. Various pharmaceutical industry is initiating plant screening programs through high-throughput techniques to find new biologically active molecule with diverse medicinal and pharmacological activities. Near Infrared (NIR) spectroscopy is a attractive analytical tool in medicinal research at current time, which is mentioned in the European Pharmacopoeia since 1997 (Huck, 2015). The principles of the NIR method differ from the convetional analytical techniques, such as GC, mass spectroscopy, HPLC, or HPTLC which are traditionally used in quality evaluation and validation purposes of medicinal plants (Beć et al., 2021). This approach is used to estimate quality related parameters such as starch, amylose, protein, moisture, glycosides, polyphenolic compounds in medicinal plants. This technique can provide specific, simultaneous, rapid, non-destructive and accurate estimation of phytochemically active molecule of different medicinal plants and its products. In near-infrared spectroscopy, molecules are excited in the wavelength range of 750-2500 nm. Harmonics and combinations can be found in the NIR range (Huck, 2016).

5.1.1. NIRs study of *Emblica officinalis*

5.1.1.1. Experimental set up of Near Infrared Spectroscopy

A DWARF-Star NIR spectrometer (StellarNet Inc., USA) coupled to the RFX-3D upward diffuse reflectance accessory was used to acquire diffuse reflectance spectra *of Emblica officinalis* samples. The scan range was 900-1700 nm and RS50 was used as calibration standard. Samples were placed on standard 1 mm thick quartz plates on the RFX-3D. The RFX-3D integrates a 5W halogen lamp and 3 fiber connectors to the spectrometer, each arranged 120° in a circle, eliminating

the need to rotate coarse particles or non-uniform samples. Each sample was scanned 16 times with an integration time of 300 ms and the averaged spectra were used for analysis. The signal-to-noise ratio is set to 4000:1, the wavenumber accuracy is within ±0.01 cm^{-1}, and the resolution is set to 2.5 nm resolution. The temperature was maintained at about 25°C during the experiment.

In the Stellarnet spectrometer used in study, data points were collected from 900 to 1700.5 nm with the gap of 1.75 nm. So each spectrum had (1700.5-900)/1.75 = 459 points. Eight replicates were performed for each sample. Therefore, a total of 459×20×8 spectral data points were obtained for 20 samples. The effect of NIR spectroscopy can be assigned to shifts in the absorption bands in the first, second and third overtone regions, possibly due to differences in the amount of compounds exhibiting these bonds in *Emblica officinalis*. Therefore, we can speculate that *Emblica officinalis* powders with different substrates showed differences in chemical composition detected by NIR spectroscopy.

5.1.1.2. Standard Normal Variate (SNV)

pretreated spectral data obtained from the NIR instrument with the *Emblica officinalis* samples were analyzed. SNV is a method for the correction of scatter. Standard Normal Variate (SNV) method used to normalization of the spectra.

5.1.1.3. Multiplicative Scatter Correction (MSC)

MSC is another important method for correcting stray light. In this method, the degree of spectral variability across the data set is corrected for the spectrum of the blank sample. MSC requires a reference spectrum. The reference spectrum is ideally a spectrum without scattering effects.

5.1.1.4. Derivative Algorithm

Derivatives are used to find solutions for peak overlaps and to remove fixed and linear baseline drifts within samples. First and second derivatives are the most commonly used higher derivatives.

5.1.1.5. Principle Component Analysis (PCA)

Most widely used variable reduction is principal component analysis (PCA). PCA is a mathematical approach that decomposes spectral data into orthogonal components that approximates the original data with a linear combination. Classical PCA was used to visualize the multivariate data through scores and loadings. This was carried out to investigate the relationships between the *Emblica officinalis* samples and spectra acquired using the spectroscopy systems.

5.1.1.6. Linear Discriminant Analysis

The samples were visualized in a low-dimensional hyperplane using linear discriminant analysis. LDA maximizes the separation between samples. Only the first two her axes were considered in the data visualization. Class information was used to represent the data. PCA and LDA were implemented in Matlab V10.0 (Mathworks Co., USA).

5.1.1.7. Model validation

The performance of the final model was evaluated according to the root mean squared error of calibration (RMSEC).

5.1.2. Results

This study evaluates the feasibility of using near infra-red spectroscopy (NIRs) as a rapid and environmentally friendly approach for validation and quantification of the gallic acid in *Emblica officinalis* powders. Gallic acid has many possible conformers depending on the orientations of its three OH and COOH groups. In this study, gallic acid forms very stable complexes with iron (Chelating agent). The complex starts at pH=3 and continues up to pH=9. Phenolic hydroxyl vibrations were at 1282 and 1212 cm^{-1} . The near-infrared spectrum of gallic acid shows a

sharp absorption band of the carboxyl group -COOH at 1664 cm^{-1}. Absorption bands at 1428 cm ⁻1 and 864 cm ⁻1 for δOH.

5.1.2.1. Principal Component Analysis

A PCA model was used to predict gallic acid concentrations in *Emblica officinalis* samples. A PCA model was prepared using the sample's preprocessed spectra from NIR spectroscopy. The optimal number of factors or latent variables used in the PCA model was chosen based on the minimum predicted squared error of calibration (RMSEC). The first and second principal components (PC) account for 91.83% and 6.91% of the total variance, respectively.

5.1.2.2. Partial Least Square Regression Analysis

The PLSR model was used to predict gallic acid concentrations in *Emblica officinalis* samples similar to the PCA model. The optimal number of factors used in the PLSR model was chosen based on the minimum root mean squared error of calibration (RMSEC) predicted by the cross-validation process. The model has a low RMSE value (< 0.503) and a high coefficient of determination for the calibration data (> 0.993). The PLSR scatterplots for the calibration and prediction sets with the SNV pretreatment method are shown in Figure 5.1, and the prediction results for the five samples are shown in Table 5.1.

5.1.2.3. Estimation

Prediction was carried out with external dataset of 20 number of *Emblica officinalis* samples. For predicting the external dataset, PCA and PLSR were subjected to pre processing technique. The prediction results reflect a PCA RMSE of 0.91 and a prediction coefficient of determination of 0.975 for eight latent variables. Similarly, the PLSR has an RMSEP of 0.737, a predictive R-squared of 0.985, and four latent variables.The best prediction parameters of gallic acid in

Emblica officinalis were obtained as correlation coefficient of prediction (Rp^2) = 0.91, RMSEP = 0.568 mg/mL and RPD = 5.3 (Table 2).

Table 5.1. Test results of five *Emblica officinalis* samples

Reference gallic acid (% *w/w*)	**Estimated gallic acid (% w/w)**	**Deviation**	**% of accuracy**	**Average accuracy**	**RMSEP**	**R^2**	**RPD**
3.98	4.93	±0.94	89.26				
4.13	5.03	±0.87	91.06				
5.02	6.26	±1.27	87.03	89.2	0.568	0.91	5.3
6.83	7.67	±0.78	89.76				
7.18	7.98	±0.81	90.23				

Note: RMSEP, Root mean square error of prediction; R^2, Correlation factor; RPD, Residual predictive deviation.

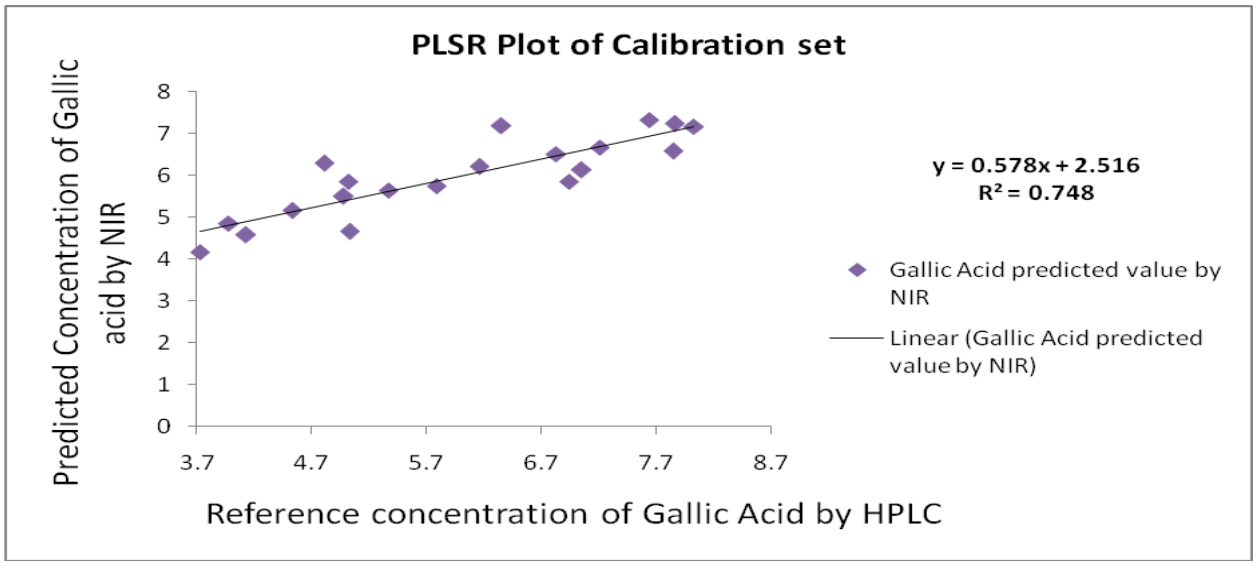

Figure 5.1. Scatter diagram of calibration set by partial least squares regression using pre processing of standard normal variate.

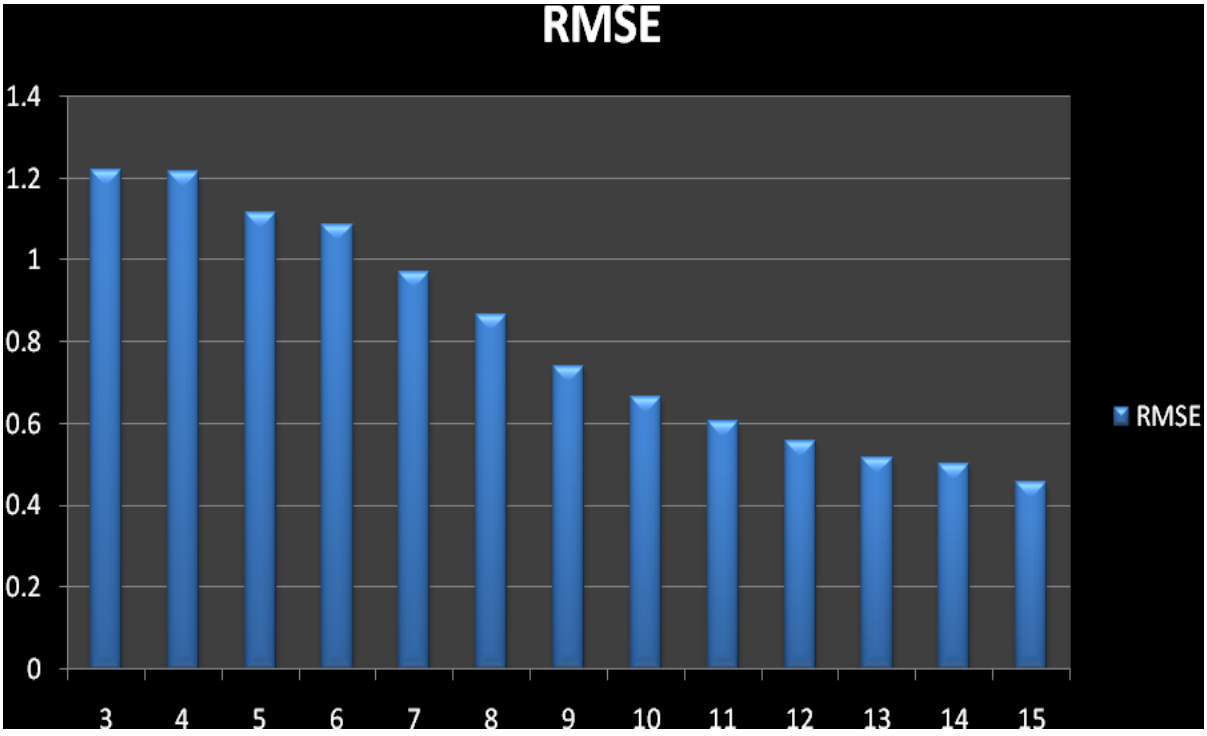

Figure 5.2. Root mean square error vs number components for calibration set.

Publication:

Estimation of gallic acid in *Emblica officinalis* samples using Near infra-red spectroscopy and Reverse-phase high performance liquid chromatography. **Shibu Narayan Jana**, Dilip Sing, Subhadip Banerjee, Rajib Bandyopadhyay, and Pulok K. Mukherjee, Phytochemical analysis. (Communicated).

Presentation:

Participated and presented an Oral presentation at 9th convention Society for Ethnopharmacology, India (SFE 2022, Sept 23-24) at Jadavpur University, Kolkata "Estimation of gallic acid in *Emblica officinalis* samples using Near infra-red spectroscopy" **Shibu Narayan Jana**, Amit Kar, Dilip Sing, Barun Dasgupta, Rajib Bandyopadhayay, Pallab K Haldar, and Pulok K. Mukherjee.

Chapter 6

Evaluation of Antidiabetic activity of *P. nigrum* and *E. officinalis*

6.1. Role of natural products in Diabetes mellitus

6.1.1. Antidiabetic activity of *Piper nigrum*

6.1.1.1. Materials and Methods

6.1.1.2. Results

6.1.2. Antidiabetic activity of Emblica officinalis

6.1.2.1. Materials and Methods

6.1.2.2. Results

6.1. Role of Natural products in Diabetes mellitus

There are several chemical and natural substances that help in control hyperglycaemic condition. At present scenario, the pharmacological interventions with natural products started being used to control diabetic and hyperglycaemic conditions (Kooti et al., 2016). 800 medicinal plants are used for cure and treatment of diabetes mellitus (Patel et al., 2012). Phenolic compounds and anthocyanins are potent hypoglycaemic (Mutha et al., 2021). Natural products for the treatment of type 2 diabetes target the same key pathophysiological mechanisms as synthetic drugs. It also prevents complications of hyperlipidemia and diabetes, maintains weight balance, and improves health (Magkos et al., 2009). Natural antidiabetic substances such as resveratrol, curcumin, tannins, lignans, anthocyanins, epigallocate chin gallate, quercetin, naringin, rutin, and kaempferol and plant fruits, vegetables like garlic, green tea, blackcurrant, bilberry, strawberry, sesame oil, and carrot. Different biological interventions are developed to treat type-2 diabetes mellitus, attention to herbs or herbal formulations in the treatment of type-2 diabetes mellitus is growing. Medicinal plant is mainly used to treat type-2 diabetes mellitus through its anti-inflammatory, anti-oxidation, hypolipidaemic, and hypoglycaemic properties (Wen et al., 2019).

6.1.1. Antidiabetic activity of *Piper nigrum*

6.1.1.1. Materials and Methods

6.1.1.1.1. Plant Materials

Samples of *Piper nigrum* (dried fruit) were obtained from Spices Board India, Ministry of Industry and Trade, Government. from india. Plant product was

identified and certified by Dr. S. Rajan, Field Botanist, Medicinal Plant Collection Unit, Ooty, Tamil Nadu, Government. from india. Specimen copies of five samples (PN1-PN5, sample number SNPS/JU/2021/1535-39) was kept at the School of Natural Products Studies, Jadapur University, Kolkata, India for future reference.

6.1.1.1.2. Extraction of plant materials

Piper nigrum fruit samples (100 g each sample) were ground to produce a moderately coarse powder using a mechanical mill and subsequently extracted by soxation using hydroalcoholic (70:30). The filtrate consists of the extract was collected and concentrated at 45 °C in a rotary vacuum evaporator (Tokyo, Japan), and the concentrated extract was freeze-dried.

6.1.1.1.2. Chemicals

Streptozotocin (STZ) (Sigma-Aldrich, Himedia) and metformin (Himedia) were used. HPLC grade solvents like methanol, acetic acid, chloroform and analytical grade reagents were used.

6.1.1.1.3. Animals

Six to eight week old male Wistar albino rats (180 ± 20 g) were obtained from M/S Chakraborty Enterprise, Kolkata. CPCSEA Accession No. 1443/PO/Br/s/11/CPCSEA. Animals were maintained at 25±2°C. and 40-45% relative humidity with alternating 12 hour light/night cycles. Animals had free access to pelleted diet (India, Mumbai, Hindustan liver) and water. The experimental protocol was approved by the Institutional Animal Ethics Committee (JU/IAEC-22/18).

6.1.1.1.4. Acute Oral Toxicity Studies

Toxicity studies conducted on male Wistar albino rats based on OECD guidelines 420 for acute oral toxicity-acute toxic class method. Male Wistar albino rats weighing 180 ± 20 g were used for this study. Extract suspended in water

containing 2% w/v Tween 80 at doses of 5, 50, 300 and 2000 mg/kg body weight. Fasted healthy rats (n=6) were orally administered overnight. The treated animals were observed mortality and general behavior for 14 days. No deaths were observed by the end of the study.

6.1.1.1.5. Induction of Diabetes

Streptozotocin was dissolved in citrate buffer (0.01 M, pH 4.5). Diabetes was induced in overnight fasted rats weighing (180 ± 20 g) by a single intraperitoneal injection of STZ (60 mg/kg body weight). Diabetes was confirmed by elevated glucose levels measured after 72 hours. Animals with blood glucose levels above 250 mg/dL were classified as hyperglycemic in the study.

6.1.1.1.6. Experimental Design

Group I. Normal control (2% Tween 80; 1 mL/kg body weight).

Group II. Diabetic (STZ) control rats given sterile water.

Group III. Diabetic animals treated with PN1 200 mg/kg body weight.

Group IV. Diabetic animals treated with PN1 400 mg/kg body weight.

Group V. Diabetic animals treated with PN2 200 mg/kg body weight.

Group VI. Diabetic animals treated with PN2 400 mg/kg body weight.

Group VII. Diabetic animals treated with PN3 200 mg/kg body weight.

Group VIII. Diabetic animals treated with PN3 400 mg/kg body weight.

Group IX. Diabetic animals treated with PN4 200 mg/kg body weight.

Group X. Diabetic animals treated with PN4 400 mg/kg body weight.

Group XI. Diabetic animals treated with PN5 200 mg/kg body weight.

Group XII. Diabetic animals treated with PN5 400 mg/kg body weight.

Group XIII. STD drug; diabetic animals treated with 0.5 mg/kg body weight of metformin.

Plant extracts, metformin, and vehicle, were administered via the nasogastric tube for 30 days. Assessments were performed for all groups on 0^{th}, 14^{th}, 21^{th}, and 28^{th} day of the experimental protocol.

6.1.1.1.7. Estimation of body weights

The body weights of the animals were measured on 0^{th}, 14^{th}, 21^{th} and 28^{th} day with a weighing balance.

6.1.1.1.8. Oral glucose tolerance test (OGTT)

On day 15^{th} of treatment, an oral glucose tolerance test was measured in overnight fasted normal and drug-treated rats.

6.1.1.1.9. Fasting blood glucose level estimations

Fasting blood glucose levels were estimated using a one-touch glucometer. For this evaluation, animals were bled from the tail vein and evaluated on days 0, 14, 21, and 28.

6.1.1.1.10. Lipid profile estimations

Lipid profiles such as total cholesterol, triglycerides and HDL were estimated using standard kit methods (Span Diagnostic Ltd. India). Blood for performing of lipid profile studies was collected from the retro-orbital plexus. Serum was separated from plasma by centrifugation at 1500 rpm for 10 minutes. The sera obtained in this way were used for further study.

6.1.1.1.11. Biochemical analysis

On day 14th, blood was collected from the retro-orbital plexus to measure SGPT, SGOT and ALP. All animals in various groups were killed by decapitation on day 28 and liver, kidney and pancreas were separated. The isolated tissue was thoroughly washed with 0.9% w/v saline. A 10% homogenate of tissue was then prepared using Tris buffer (pH 7.4). Tissue homogenates were then used to determine TBARS and GSH. Substances that react with thiobarbiturates were analyzed in liver and kidney using the method of Fraga et al. (1981), expressed as mmol 100 g $^{-}$1 tissue. Reduced glutathione is measured using the method outlined by Moron and GSH activity is expressed as mg 100 g $^{-}$1 tissue.

6.1.1.1.12. Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis of all data was performed according to one-way analysis of variance (ANOVA) and Dunnett's t-test. A value of $P < 0.001$ was considered highly statistically significant and $P < 0.05$ was considered significant.

6.1.1.2. Results

6.1.1.2.1. Acute Oral Toxicity Studies

Acute oral toxicity studies have shown that *Piper nigrum* fruit extract is safe up to 2000 mg/kg body weight. No fatal or toxic reactions were observed by the end of the study.

6.1.1.2.2. Effect of *P. nigrum* on body weight

The basal body weights of the animals were considered statistically equivalent and are summarized in Table 6.1. Induction of diabetes caused significant changes in the body weight of rats during the experimental period compared to the normal control group. Final body weight in the normal control group was 250.89 ± 5.7 g, which was significantly ($p<0.001$) reduced to 135.39 ± 3.7 g in the diabetic control group. This decreased body weight was significantly increased by treatment with *P. nigrum* and metformin. *P. nigrum* and metformin caused a significant ($p<0.001$) reduction in body weight compared to normal controls. This weight loss with *P. nigrum* and metformin was greater than that observed in diabetic controls.

6.1.1.2.3. Effect of *P. nigrum* on oral glucose tolerance test

In an oral glucose tolerance test, glucose-loaded test animals showed a significant increase in blood glucose levels after 30 minutes. *P. nigrum* and metformin significantly ($p<0.001$) suppressed the rise in blood glucose levels at 60 and 120 minutes compared to diabetic controls. The effects of *P. nigrum* on OGTT are shown in Table 6.2.

6.1.1.2.4. Effect of *P. nigrum* on Fasting blood glucose levels

Basal serum glucose levels in rats were considered statistically equivalent and are summarized in Table 6.3. There was a significant increase in glucose levels in the

diabetic control group during the experimental period. At the end of the experimental period, serum glucose levels in the diabetic control group increased significantly from 69.05 mg/dL to 415.8 mg/dL compared to the normal control group ($p<0.001$). Administration of various *P. nigrum* extracts at doses of 200 and 400 mg kg^{-1} body weight to STZ-induced diabetic rats significantly reduced blood glucose levels compared to STZ controls.

6.1.1.2.5. Effect of *P. nigrum* on lipid profile

Mean serum total cholesterol (TC) levels in the normal control group were 55.96 ± 2.28 mg/dL and significantly increased to 162.01 ± 0.35 mg/dL in the diabetic control group ($p<0.001$) (Table 6.4). This elevated serum TC level was significantly reduced by treatment with *Piper nigrum* and metformin. Diabetes significantly ($p<0.001$) reduced serum HDL cholesterol levels from 53.71 ± 1.1 mg/dL to 16.56 ± 0.6 mg/dL compared to normal controls (Table 6.4). Treatment with *P. nigrum* and metformin resulted in a significant increase in serum HDL-cholesterol levels compared with the diabetic control group. Mean serum triglyceride levels in the normal control group were 95.88 ± 0.6 mg/dL and significantly increased to 205.01 ± 0.3 mg/dL in the diabetic control group ($p<0.001$) (Table 6.4.). This elevated serum triglyceride level is associated with *P. nigram* and metformin.

6.1.1.2.6. Effect of *P. nigrum* on biochemical parameters and In vivo antioxidant enzymes

As shown in Table 6.5, biochemical enzymes (SGPT, SGOT, and ALP) showed significant ($p<0.05$) improvement compared to the diabetic group. Thiobarbiturate-reactive substances (TBARS) levels were found to be significantly

increased in diabetic controls compared to normal controls ($p<0.001$). The diabetic control group treated with *P. nigrum* extract showed a significant decrease in TBARS levels compared to the diabetic control group ($p<0.05$) and significantly decreased GSH levels compared to the diabetic control group ($p<0.001$) (Table 6.6.).

Table 6.1. Effect of *Piper nigrum* on body weight (Mean ± SEM, n=6).

Group	Treatment	Body weight (gms)	
		Initial	Final
I.	Normal control	190.7 ± 3.5	250.89 ± 5.7
II.	STZ (60 mg/kg)	195.9 ± 2.89	$135.39 \pm 3.7^{***}$
III.	STZ + PN1 (200 mg/kg)	200.5 ± 4.2	206.4 ± 6.3^{aa}
IV.	STZ + PN1 (400 mg/kg)	198.3 ± 2.9	211.5 ± 3.5^{aa}
V.	STZ + PN2 (200 mg/kg)	193.8 ± 2.8	198.5 ± 3.7
VI.	STZ + PN2 (400 mg/kg)	198.7 ± 2.7	203 ± 5.3^{aa}
VII.	STZ + PN3 (200 mg/kg)	197.5 ± 5.2	205 ± 3.8^{aa}
VIII.	STZ + PN3 (400 mg/kg)	199.8 ± 2.9	206.3 ± 1.9^{aa}
IX.	STZ + PN4 (200 mg/kg)	205.3 ± 3.7	211.5 ± 5.8^{aa}
X.	STZ + PN4 (400 mg/kg)	198.5 ± 4.8	208.3 ± 5.7^{aa}
XI.	STZ + PN5 (200 mg/kg)	196 ± 4.5	201.5 ± 6.9^{aa}
XII.	STZ + PN5 (400 mg/kg)	194 ± 2.7	209.7 ± 3.6^{aa}
XIII.	STD drug	201.3 ± 5.3	236.6 ± 6.9^{aaa}

Probability values of $^{***}p < 0.001$ compared with normal control group. Probability values of $^{aaa}p < 0.001$ and $^{aa}p < 0.05$ were compared with STZ control group.

Table 6.2. Effect of *Piper nigrum* on Oral glucose tolerance test (Mean ± SEM, n=6).

Group	Treatment	Blood glucose (mg/dL)			
		0 min	30 min	60 min	120 min
I.	Normal control	70.2 ± 7.4	145.3 ± 6.2	135 ± 8.4	120.2 ± 7.6
II.	STZ (60 mg/kg)	345 ± 1.1***	398.7 ± 5.3***	401 ± 6.1***	399 ± 8.8***
III.	STZ + PN1 (200 mg/kg)	227 ± 1.5^{aa}	298.2 ± 1.4^{aa}	205.3 ± 0.9^{aa}	198 ± 2.5^{aaa}
IV.	STZ + PN1 (400 mg/kg)	225.7 ± 9.7^{aa}	283 ± 6.1^{aa}	203.8 ± 5.7^{aa}	183 ± 6.7^{aaa}
V.	STZ + PN2 (200 mg/kg)	235.3 ± 6.9	288 ± 5.8	262 ± 7.3	235.3 ± 7.4
VI.	STZ + PN2 (400 mg/kg)	231.2 ± 1.3	291 ±14.3	255 ± 8.3	240 ± 9.3
VII.	STZ + PN3 (200 mg/kg)	198.5 ± 5.3^{aa}	245 ± 6.2^{aa}	218.3 ± 6.5^{aa}	201 ± 7.6^{aa}
VIII.	STZ + PN3 (400 mg/kg)	215 ± 1.7^{aa}	256 ± 3.9^{aa}	235 ± 8.4^{aa}	213 ± 7.5^{aa}
IX.	STZ + PN4 (200 mg/kg)	265 ± 6.9	315.7 ± 8.4	316 ± 7.1	308 ± 7.5
X.	STZ + PN4 (400 mg/kg)	254.3 ± 1.6	311 ± 1.4	308.5 ± 1.8	305 ± 2.5
XI.	STZ + PN5 (200 mg/kg)	199.8 ± 1.1^{aaa}	235 ± 0.9^{aa}	201.3 ± 1.2^{aa}	185 ± 2.3^{aaa}
XII.	STZ + PN5 (400 mg/kg)	188.3 ± 5.1^{aaa}	227 ± 6.9^{aa}	191 ± 7.1^{aa}	176 ± 5.7^{aaa}
XIII.	STD drug	163 ± 2.3^{aaa}	217 ± 5.4^{aaa}	184 ± 4.8^{aaa}	163 ± 5.3^{aaa}

Probability values of $^{***}p < 0.001$ compared with normal control group. Probability values of $^{aaa}p < 0.001$ and $^{aa}p < 0.05$ were compared with STZ control group.

Table 6.3. Effect of *Piper nigrum* on fasting blood glucose level (Mean ± SEM, n=6).

Group	Treatment	Fasting blood glucose level (mg/dL)			
		Day 0	Day 14	Day 21	Day 28
I.	Normal control	69.05 ± 7.9	78.35 ± 0.5	77.39 ± 1.4	77.05 ± 3.1
II.	STZ (60 mg/kg)	298 ± 6.8***	360.1 ± 1.3***	375.8 ± 1.1***	415.8 ± 1.1***
III.	STZ + PN1 (200 mg/kg)	292.5 ± 11.4	235.7 ± 7.3	198 ± 10.7^{aa}	145.8 ± 6.3^{aaa}
IV.	STZ + PN1 (400 mg/kg)	299.3 ± 8.6	230.8 ± 8.5	189.7 ± 16.8^{aa}	131.3 ± 6.3^{aaa}
V.	STZ + PN2 (200 mg/kg)	287.2 ± 13.2	216.7 ± 4.6	199.7 ± 7.6	175.3 ± 6.3
VI.	STZ + PN2 (400 mg/kg)	289.3 ± 1.6	216.7 ± 6.6	199.7 ± 7.6	175.3 ± 13.2
VII.	STZ + PN3 (200 mg/kg)	293.7 ± 8.6	210.7 ± 10.3	185.16 ± 9.9	161 ± 8.6
VIII.	STZ + PN3 (400 mg/kg)	289.5 ± 7.9	203.7 ± 6.8	181.2 ± 16.8^{aa}	159.3 ± 5.3^{aa}
IX.	STZ + PN4 (200 mg/kg)	285 ± 11.2	201.09 ± 13.9	179.33 ± 1.1	153 ± 6.3^{aa}
X.	STZ + PN4 (400 mg/kg)	289.39 ± 1.7	198.4 ± 1.2^{aa}	169 ± 11.5^{aa}	151.7 ± 7.7^{aa}
XI.	STZ + PN5 (200 mg/kg)	296.7 ± 7.7	199.5 ± 6.9^{aa}	155.2 ± 1.6^{aa}	135 ± 9.9^{aaa}
XII.	STZ + PN5 (400 mg/kg)	293.5 ± 10.2	195.4 ± 3.9^{aa}	147.2 ± 1.2^{aa}	133.5 ± 1.1^{aaa}
XIII.	STD drug	289.3 ± 7.9	179.8 ± 1.5^{aaa}	137.5 ± 10.7^{aaa}	110.5 ± 4.2^{aaa}

Probability values of $^{***}p < 0.001$ compared with normal control group. Probability values of $^{aaa}p < 0.001$ and $^{aa}p < 0.05$ were compared with STZ control group on corresponding day.

Table 6.4. Effect of *Piper nigrum* on levels of lipid parameter (Mean ± SEM, n=6).

Group	Treatment	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL (mg/dL)
I.	Normal control	55.96 ± 2.28	95.88 ± 0.6	53.71 ± 1.1
II.	STZ (60 mg/kg)	162.01 ± 0.35***	205.01 ± 0.3***	16.56 ± 0.6***
III.	STZ + PN1 (200 mg/kg)	116.23 ± 1.25^{aa}	125.84 ± 1.1^{aa}	30.51 ± 0.4^{aa}
IV.	STZ + PN1 (400 mg/kg)	110.76 ± 0.5^{aa}	123.09 ± 0.6^{aa}	31.37 ± 0.5^{aa}
V.	STZ + PN2 (200 mg/kg)	101.31 ± 0.6^{aa}	110.81 ± 0.4^{aa}	25.91 ± 1.1^{aa}
VI.	STZ + PN2 (400 mg/kg)	99.67 ± 0.9^{aa}	108.01 ± 0.3^{aa}	26.09 ± 1.89^{aa}
VII.	STZ + PN3 (200 mg/kg)	76.03 ± 1.2^{aaa}	185.35 ± 0.5	41.07 ± 0.9^{aaa}
VIII.	STZ + PN3 (400 mg/kg)	75.14 ± 0.3^{aaa}	180.08 ± 0.3	42.80 ± 0.6^{aaa}
IX.	STZ + PN4 (200 mg/kg)	135.04 ± 0.9^{aa}	122.79 ± 0.17^{aa}	26.1 ± 0.5^{aa}
X.	STZ + PN4 (400 mg/kg)	133.83 ± 0.3^{aa}	117.09 ± 0.17^{aa}	27.3 ± 0.7^{aa}
XI.	STZ + PN5 (200 mg/kg)	98.36 ± 1.07^{aa}	129.87 ± 0.5^{aa}	29.90 ± 1.4^{aa}
XII.	STZ + PN5 (400 mg/kg)	96.87 ± 0.3^{aa}	125.31 ± 0.8^{aa}	31.01 ± 1.2^{aa}
XIII.	STD drug	67.89 ± 1.7^{aaa}	120.91 ± 1.2^{aa}	46.73 ± 0.3^{aaa}

Probability values of $^{***}p < 0.001$ compared with normal control group. Probability values of $^{aaa}p < 0.001$ and $^{aa}p < 0.05$ were compared with STZ control group.

Table 6.5. Effect of *Piper nigrum* on levels of serum liver enzymes ((Mean ± SEM, n=6).

Group	Treatment	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)
I.	Normal control	26.16 ± 1.16	30.05 ± 0.37	12.89 ± 0.23
II.	STZ (60 mg/kg)	35.89 ± 1.98***	41.01 ± 0.27***	28.03 ± 0.08***
III.	STZ + PN1 (200 mg/kg)	32.13 ± 3.67	39.78 ± 0.71	23.15 ± 0.53
IV.	STZ + PN1 (400 mg/kg)	31.79 ± 3.16	39.09 ± 1.95	23.09 ± 0.73
V.	STZ + PN2 (200 mg/kg)	33.10 ± 3.17	40.06 ± 0.47	20.78 ± 0.33aa
VI.	STZ + PN2 (400 mg/kg)	32.11 ± 1.81	40.01 ± 0.37	20.13 ± 0.64aa
VII.	STZ + PN3 (200 mg/kg)	30.98 ± 2.35	39.30 ± 0.23	22.05 ± 0.85aa
VIII.	STZ + PN3 (400 mg/kg)	30.13 ± 1.75	39.07 ± 0.30	21.89 ± 0.42aa
IX.	STZ + PN4 (200 mg/kg)	33.16 ± 1.86	38.01 ± 0.27	24.06 ± 0.64
X.	STZ + PN4 (400 mg/kg)	32.76 ± 1.16	38.79 ± 0.45	24.01 ± 0.35
XI.	STZ + PN5 (200 mg/kg)	33.78 ± 3.22	38.16 ± 0.44	23.71 ± 0.78aa
XII.	STZ + PN5 (400 mg/kg)	32.16 ± 1.96	38.07 ± 0.6	23.08 ± 0.87aa
XIII.	STD drug	28.03 ± 2.86aaa	34.15 ± 0.47aaa	15.01 ± 0.65aaa

Probability values of $^{***}p < 0.001$ compared with normal control group. Probability values of $^{aaa}p < 0.001$ and $^{aa}p < 0.05$ were compared with STZ control group.

Table 6.6. Effect of *Piper nigrum* on levels of oxidative stress marker (TBARs) and antioxidant enzyme (Glutathione) ((Mean ± SEM, n=6).

Group	Treatment	TBARs	Glutathione
I.	Normal control	0.93 ± 0.18	46.89 ± 2.31
II.	STZ (60 mg/kg)	1.98 ± 0.78***	24.79 ± 1.51***
III.	STZ + PN1 (200 mg/kg)	1.69 ± 0.13	35.31 ± 2.79aa
IV.	STZ + PN1 (400 mg/kg)	1.61 ± 0.79aa	37.71 ± 3.09
V.	STZ + PN2 (200 mg/kg)	1.58 ± 0.35aa	39.03 ± 1.36
VI.	STZ + PN2 (400 mg/kg)	1.56 ± 0.81aa	38.05 ± 1.96
VII.	STZ + PN3 (200 mg/kg)	1.67 ± 1.17	32.06 ± 1.46aa
VIII.	STZ + PN3 (400 mg/kg)	1.63 ± 0.10	30.16 ± 1.86aa
IX.	STZ + PN4 (200 mg/kg)	1.53 ± 0.62aa	31.79 ± 2.91aa
X.	STZ + PN4 (400 mg/kg)	1.51 ± 0.14aa	30.01 ± 1.98aa
XI.	STZ + PN5 (200 mg/kg)	1.66 ± 0.08	29.76 ± 2.63aa
XII.	STZ + PN5 (400 mg/kg)	1.63 ± 0.29	28.76 ± 1.96aa
XIII.	STD drug	1.07 ± 0.61aaa	42.98 ± 1.29aaa

Probability values of ***p < 0.001 compared with normal control group. Probability values of aaap < 0.001 and aap < 0.05 were compared with STZ control group.

6.1.2. Antidiabetic activity of *Emblica officinalis*

6.1.2.1. Materials and Methods

6.1.2.1.1. *Collection and authentication of Plant Materials*

Emblica officinalis fruits were collected from different outlets of Hamdard Nagar, New Delhi, India, based on morphology, price and quality. Plant material was identified and certified by Dr. S. Rajan, Field Botanist, Medicinal Plant Collection Unit, Ooty, Tamil Nadu, Government. from india. The specimen (SNPS/JU/2020/1526-32) was kept at the School of Natural Products Studies, Jadapur University, Kolkata, India.

6.1.2.1.2. Extraction of Plant Materials

Samples of *Emblica officinalis* fruit (100 g each sample) were ground using a mechanical grinder to produce a moderately coarse powder, followed by soxation

extraction using hydroalcoholic (70:30). The filtrate containing the extract was collected and concentrated at 45 °C in a rotary vacuum evaporator (Tokyo, Japan), and the concentrated extract was freeze-dried. The gallic acid content (w/w) of each sample was estimated by RP-HPLC and HPTLC studies and described in Chapter 4. Out of seven samples, the maximum amount of gallic acid content has been found in SNPS/JU/2020/1532 i.e., EO07 (6.94% w/w) by RP-HPLC study. This *E. officinalis* sample (i.e., EO07) has been subjected to in-vivo antidiabetic study in male Wistar albino rats.

6.1.2.1.3. Chemicals and Reagents

Gallic acid (>98%) supplied by SRL Pvt. Ltd. (Taroja, Maharashtra, India). Micropipettes and microtips obtained from Eppendorf and Accupipets from Tarsons Products Pvt. GmbH. (Kolkata, India). Streptozotocin (STZ) (Sigma-Aldrich, Himedia) and metformin (Himedia) were used. A 0.45 µm pore size membrane filter (Millipore, Burlington, MA, USA) was used for mobile phase filtration and a Whatman syringe filter (nylon; 0.45 µm) was used for sample filtration, defaults were used. Methanol (AR grade), toluene, and ethyl acetate were purchased from Merck Ltd. (Mumbai, India) Purchased.

6.1.2.1.4. Animals

Six to eight week old male Wistar albino rats (180 ± 20 g) were obtained from M/S Chakraborty Enterprise, Kolkata. CPCSEA Accession No. 1443/PO/Br/s/11/CPCSEA. Animals were maintained at 25±2° C. and 40-45% relative humidity with alternating 12 hour light/dark cycles. Animals had free access to pelleted diet (India, Mumbai, Hindustan liver) and water. The experimental protocol was approved by the Institutional Animal Ethics Committee (JU/IAEC-22/18).

6.1.2.1.5. Acute Oral Toxicity Studies

Toxicity studies conducted on male Wistar albino rats based on OECD guidelines 420 for acute oral toxicity-acute toxic class method. Male Wistar albino rats weighing 180 ± 20 g were used for this study. Extract suspended in water containing 2% w/v Tween 80 at doses of 5, 50, 300 and 2000 mg/kg body weight. Fasted healthy rats (n=6) were orally administered overnight. Mortality and general behavior of treated animals were observed for 14 days. No deaths were observed by the end of the study.

6.1.2.1.6. Induction of Diabetes

Streptozotocin was dissolved in citrate buffer (0.01 M, pH 4.5). Diabetes mellitus was induced in overnight fasted rats weighing (180 ± 20 g) by a single intraperitoneal injection of STZ (60 mg/kg body weight). Diabetes was confirmed by elevated glucose levels measured after 72 hours. Animals with blood glucose levels above 250 mg/dL were classified as hyperglycemic in the study.

6.1.2.1.7. Experimental Design

Group I. Normal control (2% tween 80; 1 mL/kg body weight).

Group II. Diabetic (STZ) control rats receiving sterile water.

Group III. Diabetic animals treated with *E. officinalis* extract 200 mg/kg body weight.

Group IV. Diabetic animals treated with *E. officinalis* extract 400 mg/kg body weight.

Group V. STD drug; diabetic animals treated with 0.5 mg/kg body weight of metformin.

Plant extracts, metformin and vehicle, were administered via the nasogastric tube for 30 days. Assessments were done for all groups on 0^{th}, 14^{th}, 21^{th}, and 28^{th} day of the experimental protocol.

6.1.2.1.8. Estimation of body weights

The body weights of the animals were measured on 0^{th}, 14^{th}, 21^{th} and 28^{th} day with a weighing balance.

6.1.2.1.9. Oral glucose tolerance test

On day 15^{th} of treatment, OGTT was performed in overnight fasted normal and drug-treated rats.

6.1.2.1.10. Fasting blood glucose level estimations

Fasting blood glucose levels were estimated using a one-touch glucometer. For this evaluation, animals were bled from the tail vein and evaluated on days 0, 14, 21, and 28.

6.1.2.1.11. Lipid profile estimations

Lipid profiles such as total cholesterol, triglycerides and HDL were estimated using standard kit methods (Span Diagnostic Ltd. India). Blood for performing lipid profile studies was drawn from the retro-orbital plexus. Serum was separated from plasma by centrifugation at 1500 rpm for 10 minutes. The sera obtained in this way were used for further study .

6.1.2.1.12. Biochemical analysis

On day 14th, blood was collected from the retroorbital plexus to measure SGPT, SGOT and ALP. All animals in different groups were killed by decapitation on day 28 and liver, kidney and pancreas were separated. The isolated tissue was thoroughly washed with 0.9% w/v saline. A 10% His homogenate of tissue was then prepared using Tris buffer (pH 7.4).

Tissue homogenates were then used to determine TBARS and GSH. Substances that react with thiobarbiturates were measured in liver and kidney using the method of *Fraga et al.* (1981), expressed as mmol 100 g $^{-}$1 tissue. Reduced glutathione is measured using the method outlined by Moron and GSH activity is expressed as mg 100 g $^{-}$1 tissue.

6.1.2.1.13. Statistical Analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis of all data was performed according to one-way analysis of variance (ANOVA) and Dunnett's t-test. A value of $P < 0.001$ was considered highly statistically significant and $P < 0.05$ was considered significant.

6.1.2.2. Results

6.1.2.2.1. Acute Oral Toxicity Studies

In acute oral toxicity studies, *Emblica officinalis* fruit extract was shown to be safe up to 2000 mg/kg body weight. No lethality or toxic reactions were observed at the end of the study.

6.1.2.2.2. Effect of *E. officinalis* on body weight

Basal body weight of animals was considered to be statistically significant and summarized in Table 6.7. Induction of diabetes caused significant changes in body

Body weight of rats during the experimental period compared to normal controls. Final body weight in the normal control group was 255.84 ± 6.7 g, which was significantly ($p<0.001$) reduced to 136.49 ± 8.7 g in the diabetic control group. This decreased body weight was significantly increased by treatment with *E. officinalis* and metformin. *E. officinalis* and metformin produced a significant ($p<0.001$) reduction in body weight compared to normal controls. Weight loss with *E. officinalis* and metformin was greater than that observed in diabetic controls.

6.1.2.2.3. Effect of *E. officinalis* on oral glucose tolerance test

In an oral glucose tolerance test, glucose-loaded test animals showed a significant increase in blood glucose levels after 30 minutes. *E. Officinalis* and metformin significantly ($p<0.001$) suppressed rising blood glucose levels at 60 and 120 minutes compared to diabetic controls. The impact of *E. officinalis* on his OGTT is shown in Table 6.8.

6.1.2.2.4. Effect of *E. officinalis* on Fasting blood glucose levels

Basal serum glucose levels in rats were considered statistically significant and are summarized in Table 6.9. There was a significant increase in serum glucose levels in the diabetic control group during the experimental period. At the end of the experimental period, serum glucose levels in the diabetic control group increased significantly from 79.06 ± 3.5 mg/dL to 417.3 ± 4.1 mg/dL compared to the normal control group ($p<0.001$). Administration of different *E. officinalis* extracts at doses of 200 and 400 mg kg^{-1} body weight to STZ-induced diabetic rats significantly decreased blood sugar levels compared to STZ controls.

6.1.2.2.5. Effect of on *E. officinalis* lipid profile

Mean serum total cholesterol (TC) levels in the normal control group were 56.96 ± 4.2 mg/dL and significantly increased to 163.01 ± 0.55 mg/dL in the diabetic control group ($p<0.001$) (Table 6.10.). This elevated serum TC level was significantly reduced by treatment with *Emblica officinalis* and metformin. Diabetes significantly ($p<0.001$) reduced serum HDL cholesterol levels from 54.81 ± 5.1 mg/dL to 17.86 ± 0.9 mg/dL compared to normal controls (Table 6.10.).Treatment with *E. officinalis* and metformin resulted in a significant increase in serum HDL cholesterol levels compared to diabetic controls. Mean serum triglyceride levels in the normal control group were 94.83 ± 1.6 mg/dl and significantly increased to 209.01 ± 0.8 mg/dl in the diabetic control group ($p<0.001$) (Table 6.10.). This elevated serum triglyceride level was significantly reduced by treatment with *E. officinalis* and metformin.

6.1.2.2.6. Effect of *E. officinalis* on biochemical parameters and In vivo antioxidant enzymes

As shown in Table 6.11, biochemical enzymes (SGPT, SGOT, and ALP) showed significant ($p<0.001$) improvement compared to the diabetic group. Thiobarbiturate-reactive substances (TBARS) levels were found to be significantly increased in diabetic controls compared to normal controls ($p<0.001$). The diabetic control group treated with E. officinalis extract showed a significant decrease in TBARS levels compared to the diabetic control group ($p<0.001$) and a significant decrease in GSH levels compared to the diabetic control group increased ($p<0.001$) (Table 6.12.).

Table 6.7. Effect of *Emblica officinalis* on body weight (Mean ± SEM, n=6).

Group	Treatment	Body weight (gms)	
		Initial	Final
I.	Normal control	189.3 ± 4.5	255.84 ± 6.7
II.	STZ (60 mg/kg)	191.6 ± 3.79	136.49 ± 8.7***
III.	STZ + EO (200 mg/kg)	199.5 ± 2.2	204.8 ± 5.3aa
IV.	STZ + EO (400 mg/kg)	197.3 ± 3.9	210.5 ± 2.5aa
V.	STD drug	198.3 ± 6.3	231.4 ± 7.9aaa

Probability values of ***p < 0.001 compared with normal control group. Probability values of aaap < 0.001 and aap < 0.05 were compared with STZ control group.

Table 6.8. Effect of Emblica officinalis on Oral glucose tolerance test (Mean ± SEM, n=6).

Group	Treatment	Fasting blood glucose level (mg/dL)			
		0 min	30 min	60 min	120 min
I.	Normal control	75.2 ± 6.4	150.4 ± 5.2	137 ± 8.4	118.2 ± 7.8
II.	STZ (60 mg/kg)	355 ± 1.7***	396.7 ± 7.3***	405 ± 4.1***	400 ± 8.4***
III.	STZ + EO (200 mg/kg)	265 ± 7.4	298.3 ± 6.5aa	269.5 ± 7.6aa	253.1 ± 5.3aa
IV.	STZ + EO (400 mg/kg)	261 ± 7.7	292.1 ± 6.5aa	263.5 ± 6.2aa	245 ± 6.7aa
V.	STD drug	181 ± 9.7aa	231.3 ± 9.6aa	188 ± 8.7aa	175.3 ± 9.2aaa

Probability values of ***p < 0.001 compared with normal control group. Probability values of aaap < 0.001 and aap < 0.05 were compared with STZ control.

Table 6.9. Effect of *Emblica officinalis* on fasting blood glucose level (Mean ± SEM, n=6).

Group	Treatment	Fasting blood glucose level (mg/dL)			
		Day 0	Day 14	Day 21	Day 28
I.	Normal control	71.03 ± 4.9	79.35 ± 0.7	78.7 ± 2.4	79.06 ± 3.5
II.	STZ (60 mg/kg)	335 ± 5.8***	367.1 ± 6.3***	379.9 ± 6.1***	417.3 ± 4.1***
III.	STZ + EO (200 mg/kg)	375.1 ± 2.9	295 ± 2.8^{aa}	209.2 ± 1.5^{aa}	172 ± 2.5^{aaa}
IV.	STZ + EO (400 mg/kg)	368 ± 2.9	266.3 ± 3.8^{aa}	193.2 ± 1.3^{aa}	145.6 ± 3.6^{aaa}
V.	STD drug	289 ± 0.6^{aa}	276.8 ± 2.7^{aa}	153 ± 2.3^{aaa}	117.8 ± 3.8^{aaa}

Probability values of $^{***}p < 0.001$ compared with normal control group. Probability values of $^{aaa}p < 0.001$ and $^{aa}p < 0.05$ were compared with STZ control group on corresponding day.

Table 6.10. Effect of *Emblica officinalis* on levels of lipid parameter (Mean ± SEM, n=6).

Group	Treatment	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL (mg/dL)
I.	Normal control	56.96 ± 4.2	94.83 ± 1.6	54.81 ± 5.1
II.	STZ (60 mg/kg)	163.01 ± 0.55***	209.01 ± 0.8***	17.86 ± 0.9***
III.	STZ + EO (200 mg/kg)	117.13 ± 1.5^{aa}	129.84 ± 1.8^{aa}	29.01 ± 0.5^{aa}
IV.	STZ + EO (400 mg/kg)	109.56 ± 0.3^{aa}	122.08 ± 0.7^{aa}	32.57 ± 0.3^{aa}
V.	STD drug	69.79 ± 1.6^{aaa}	116.81 ± 1.5^{aa}	48.03 ± 0.9^{aaa}

Probability values of ***p < 0.001 compared with normal control group. Probability values of $^{aaa}p < 0.001$ and $^{aa}p < 0.05$ were compared with STZ control group.

Table 6.11. Effect of Emblica officinalis on levels of serum liver enzymes ((Mean ± SEM, n=6).

Group	Treatment	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)
I.	Normal control	26.16 ± 1.16	30.05 ± 0.37	12.89 ± 0.23
II.	STZ (60 mg/kg)	$35.89 \pm 1.98^{***}$	$41.01 \pm 0.27^{***}$	$28.03 \pm 0.08^{***}$
III.	STZ + EO (200 mg/kg)	30.46 ± 4.87^{aaa}	34.78 ± 0.81^{aaa}	20.17 ± 0.55^{aa}
IV.	STZ + EO (400 mg/kg)	29.89 ± 4.13^{aaa}	32.09 ± 2.95^{aaa}	20.09 ± 0.79^{aa}
V.	STD drug	28.05 ± 4.66^{aaa}	34.18 ± 0.67^{aaa}	14.02 ± 0.85^{aaa}

Probability values of $^{***}p < 0.001$ compared with normal control group. Probability values of $^{aaa}p < 0.001$ and $^{aa}p < 0.05$ were compared with STZ control group.

Table 6.12. Effect of *Emblica officinalis* on levels of oxidative stress marker (TBARs) and antioxidant enzyme (Glutathione) ((Mean ± SEM, n=6).

Group	Treatment	TBARs	Glutathione
I.	Normal control	0.93 ± 0.18	46.89 ± 2.31
II.	STZ (60 mg/kg)	$1.98 \pm 0.78^{***}$	$24.79 \pm 1.51^{***}$
III.	STZ + EO (200 mg/kg)	1.46 ± 0.16^{aa}	39.51 ± 2.87^{aaa}
IV.	STZ + EO (400 mg/kg)	1.38 ± 0.81^{aa}	37.71 ± 3.09^{aaa}
V.	STD drug	1.07 ± 0.61^{aaa}	42.98 ± 1.29^{aaa}

Probability values of $^{***}p < 0.001$ compared with normal control group. Probability values of $^{aaa}p < 0.001$ and $^{aa}p < 0.05$ were compared with STZ control group.

Publication:

Marker analysis and Antidiabtic potential of *Piper nigrum* L. seeds. **Shibu Narayan Jana**, Dilip Sing, Subhadip Banerjee, Pallab Kanti Haldar, Barun Dasgupta, Amit Kar, Nanaocha Sharma, Rajib Bandyopadhayay, Pulok K Mukherjee, Orient Pharm Exp Med (Communicated).

Presentation:

Participated and presented a poster at 8th convention Society for Ethnopharmacology, India (SFE 2021, Dec 10) "Marker profiling and antidiabetic potential of *Emblica officinalis*" **Shibu Narayan Jana**, Amit Kar, Barun Dasgupta, Dilip Sing, Rajib Bandyopadhyay, Subhadip Banerjee, Shibu Narayan Jana, and Pulok K. Mukherjee.

Chapter 7

Summary and Conclusion

7.1. Summary

7.2. Conclusion

7.1. Summary

Traditional medicine is broadly used for various human ailments. Standard drugs and formulations are essential for assessing drug quality based on botanical concentration, physicochemical, phytochemical, in vitro and in vivo parameters. For acceptability in modern system of medicine, proper quality evaluation, validation and standardization of medicinal plant and its formulation is mandatory. Proper documentation and validation is very much essential to not only assure the quality, authenticity and efficacy but also to crop up with good safety profiling to evaluate the risk associated with pharmacological and toxicological effects. Quality evaluation and validation of *A. paniculata*, *P. nigrum*, and *E. officinalis* through marker profiling by HPLC, HPTLC, and NIRs techniques as well as evaluation of antidiabetic potential were performed.

In chapter-1, importance of quality evaluation, validation, and standardization of medicinal plant and its product was highlighted. Medicinal plants are considered as a rich sources of phytoconstituents which used in drug and formulation development either pharmacopoeial, non-pharmacopoeial or synthetic drugs. It is very important to maintain quality, safety and efficacy of the medicinal plants and its products to avoid serious health issues. For quality evaluation and standardization of medicinal plants obtained from the extract of plants can be estimated for the phyto-constituents with HPLC and HPTLC techniques. In this study, the basic pathophysiology of diabetes mellitus, and role of medicinal plants in the management of hyperglycaemic condition was highlighted. Medicinal plants can be useful in both preventing or supporting the therapy of hyperglycaemic conditions. There is a need for developing natural products-based hypoglycaemic therapy to minimize the side effects of the synthetic drugs.

Chapter-2, deals with the scope, objectives and plan of work of the study. In the present study, the medicinal plants were collected and extracted with suitable solvents, followed by their phytochemical screening and marker profiling through HPLC, HPTLC and NIRs. *Piper nigrum* and *Emblica officinalis* were evaluated for their antidiabetic potential.

Chapter-3, deals with the profiles, collection, authentication and extraction of the selected medicinal plants, *Andrographis paniculata*, *Piper nigrum*, and *Emblica officinalis*. The morphology, phytoconstituents and the biological activities of the plants have been presented here. These plants are reported to contain several phenolic and flavonoid compounds, like Quercetin, chlorogenic acid, gallic acid, kaemferol etc. The plants are also reported to have antidiabetic and hypolipidaemic activities. The plants were extracted using a hydroalcoholic solvent system of Methanol:Water, 70:30. The extracts were dried and the % yields were calculated.

Chapter 4,5 discusses about the HPTLC, RP-HPLC and NIRs study of the plant extracts. The HPTLC and RP-HPLC finger-printing of the plant extracts were performed using optimized solvent system. In HPTLC and HPLC analysis, marker compounds andrographolide, piperine and gallic acid were quantified in *A. paniculata*, *P. nigrum* and *E. officinalis* extracts, respectively. HPTLC and HPLC studies were performed according to ICH Q2R1 guidelines for linearity, specificity, accuracy, precision, limit of quantitation (LOQ), and limit of detection (LOD).

pre-processed NIR spectra were investigated to quantitatively detect gallic acid in *E. officinalis*. A calibration model based on the NIR spectra of 20 samples of *Emblica officinalis* was constructed for principal component analysis (PCR) and

partial least squares (PLSR). 5 additional samples were used for external validation. The coefficient of determination (R2) for validation and prediction and the mean squared error for calibration were 0.91 and 0.568, respectively.

Chapter 6, deals with in-vivo antidiabetic potential of *P. nigrum* and *E. officinalis* extracts. Oral administration of both extracts at concentrations of 200 and 400 mg/kg body weight. Each day for 28 days showed a significant ($p < 0.001$) decrease in fasting blood sugar compared to diabetic rats. These extracts also significantly ($p < 0.05$) decreased all biochemical parameters (SGOT, SGPT ALP, and lipid profile). Treatment also significantly ($p < 0.05$) increased reduced glutathione.

7.2. Conclusion

According to WHO, 80% of the world population trust on medicinal and herbal products. In India, there are six traditional systems of medicines such as Ayurveda, Yoga & Naturopathy, Unani, Siddha, and Homoeopathy being used for preventing and managing of diseases. Quality control, validation, and standardization are essential operation for safest and effective uses of these products. HPLC and HPTLC methods have a great potential for the identification, quantification, standardization and characterization of medicinal products. Under this study, quantification of andrographolide from *A. paniculata*, piperine from *P. nigrum* and gallic acid from *E. officinalis* samples were performed through RP-HPLC, HPTLC and NIRs methods. These reproducible, accurate, and specific HPLC and HPTLC methods can be useful for quality evaluation and validation of medicinal plants or products/formulation in industrial aspect. The uses of NIR spectroscopy to the medicinal plant extracts analysis was confirmed and demonstrated in combination with chemometric analysis as a suitable method for the detection, quantification and prediction of gallic acid. A handheld short-range NIR instrument serves as a field lab-scale tool for industry to detect and predict gallic acid content in medicinal plants. In this study, *P. nigrum* and *E. officinalis* were evaluated for the antidiabetic potential. Some literature evidences were found for the antidiabetic property of the plant extracts. The results suggest that the hydroalcoholic extracts of both plants may effectively normalize the hyperglycaemic status in STZ induced diabetes at dose dependent manner.

Chapter 8

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