CHAPTER - I

1.1 Research Background

Plants are the foundation of life on Earth, and people have long relied on them for food and medicines. Majority of people in developing countries rely on traditional herbal medicine to address their basic healthcare requirements. The Indian subcontinent has a rich ethnobotanical legacy as a result of its great cultural diversity (Jain, 1991). Throughout human history, medicinal plants have been utilized to cure a variety of illnesses. It is believed that up to four billion people (representing 80% of the world's population) depend on herbal medicinal products as their major source of healthcare, and traditional medical practice including the use of herbs is considered an intrinsic part of the culture in such communities (Mukherjee, 2002; Bodeker *et al*., 2005; Bandaranayake, 2006; Abdala *et al*., 2012). Due to the shown efficacy of medicinal plants in healing specific ailments and claims that it is safe to use, the global consumption of medicinal plants is increasing (Perez Gutierrez & Baez, 2009). The worldwide trade value of these plants surpassed $60 billion in 2006 (Sher, 2013). This tendency is anticipated to grow substantially by 2050 (Lang, 2008). This is due to the growing popularity of herbal medicine and the developing usage of herbs as flavourings in a broad range of foods (Khan *et al*., 2011). Since the dawn of human civilization, medicinal plants have played an important role in medicine and also contribute to the production of pharmaceuticals today (Rastogi *et al*., 2016).

The role of medicinal plants and traditional health systems in resolving the world's health care challenges is growing. As a result of this renewed interest, international research on medicinal plants is flourishing at a significant rate. The majority of emerging countries have incorporated traditional medical practices into their culture (Ghani, 2003). Secondary metabolites (natural compounds) have been the most productive source of prospective therapeutic leads (Mishra & Tiwari, 2011; Rey-Ladino *et al*., 2011; Cragg & Newman, 2005; Haefner, 2003; Butler, 2004).

Since ancient times, people have relied heavily on natural herbs for the treatment and prevention of a wide range of medical conditions. The analysis of the benefits and drawbacks of herbal medicine has led to the development of novel treatments that improve health with few or no negative effects (Yuan, 2016). The wealth of information about natural products has been gradually transformed into a variety of medical systems, such as traditional Indian medicine, European medicine, Japanese Kampo, traditional Chinese medicine, (Mukherjee, 2019) or traditional Arabic and Islamic medicine and folk medicines, which encompass not

only herbal treatments but also pharmaceuticals made from minerals and metals like mercury, gandhaka (purified sulphur oxide), gold, silver, etc., or animals like ivory (Panchkarma) (Elachouri, 2018). Ayurveda is the oldest (6000 BC) and best-organized (still practiced) traditional health care system in India, with preventative and therapeutic procedures as its major components. (Pan *et al*., 2014). Both the Charak Samhita (written between 100 and 500 BC) and the Sushrut Samhita (written between 400 and 200 BC) are considered to be the oldest documented Hindu texts and provide a comprehensive account of the systematic categorization, pharmacological, and therapeutic properties of some 700 different plants. (Sen & Chakraborty, 2015). Indian medicine or herbal medicine is still the primary method of healthcare for 70% of Indians today (Vaidya & Devasagayam, 2007). Ayurvedic doctors used and methodically detailed the effects of a wide variety of drugs, herbs/plants, and even animal urine due to the widespread notion that "everything can be a drug" in Indian culture. There is a lot of information on diseases, treatments, and pharmacies in Ayurveda's extensive literature, which is written in Sanskrit and other Indian languages (Fig. 1.1). The Rig veda, an ancient Indian holy collection of Vedic Sanskrit hymns, and the Atharvaveda, the fourth and final Veda of Hindu literature, include the oldest references to such plants, minerals, and animal products with their utilisation for medical purposes [http://www.indianetzone.com/2/rig veda.htm.]. Modern Ayurvedic practitioners place a great deal of trust in Bhava-Bhava Mishra's Prakasha, the most significant text on herbs/plants (Prasad, 2000; Dev, 1988; Prasad & Narayana, 2007). The Siddha medical system, which has its roots in the period between around 3000 BC and 2000 BC and developed in the southern region of India, is one such example. (Jankiraman, 2020). The Unani method, which was developed by Hippocrates and refined by Galen before being brought to India by Arabs and Persians in the eleventh century. The use of plants as a source of medicine has been passed down through generations and is an essential component of India's health-care system. Most practitioners in Indian systems of medicine develop and disseminate their own ways, necessitating thorough documentation and study.

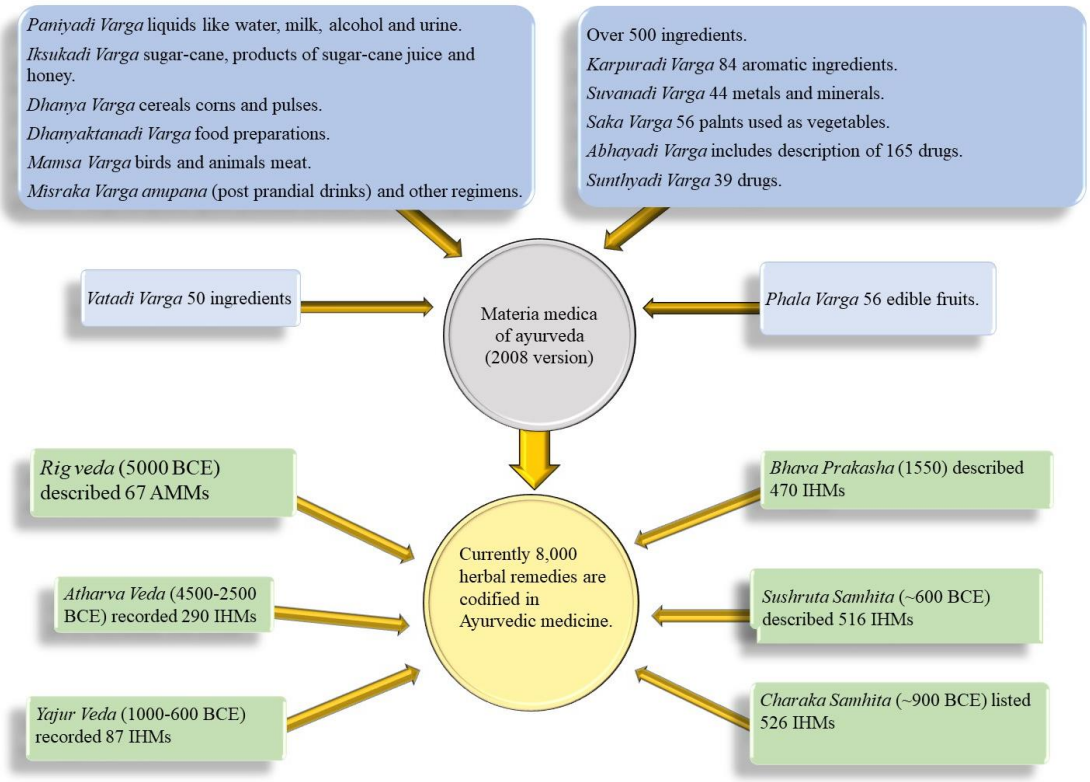


Figure 1.1: Some important texts of Indian herbal medicine

Complementary and alternative treatments (CAMs) are becoming popular in the United Kingdom, the rest of Europe, North America, and Australia. Herbal therapies have also been extensively accepted in many developed nations (Committee on the Use of Complementary, and Alternative Medicine by the American Public, Board on Health Promotion, and Disease Prevention, Institute of Medicine, 2005; Calapai, 2008; Braun *et al*., 2010; Anquez-Traxler, 2011). In reality, although countries like the United Kingdom have a long history of utilising herbal remedies (Nissen, 2010), the practise is common and well-established in a number of other European nations as well (Calapai, 2008). Due to the varied adverse effects of allopathic medications, the lack of curative current therapy for different chronic diseases, microbiological resistance, and the unprecedented expenditures in pharmaceutical research and development (R&D), (Pan *et al*., 2010) the use of herbs has expanded in recent years. Approximately 1,200 new medications have been authorised by the Food and Drug Administration (FDA) of the United States since 1950 (Munos, 2009). Consequently, the use of herbs and herbal products for health reasons has expanded globally during the last four decades, in both developing and developed nations (Humber, 2002). In addition, global pharmaceutical corporations equipped with current science/technology and concepts have started to rediscover herbs as a potential source of novel drug candidates and have refocused their efforts on natural product drug

research and discovery (Seidl, 2002; Li & Zhang, 2008; Corson & Crews, 2007; Schmidt *et.al*., 2007). Medicinal plants are the principal source of innovative pharmaceuticals and healthcare commodities (Ivanova *et al*., 2005). The usage of medicinal plants in industrialised civilizations may be traced back to the extraction and production of various drugs from these plants, as well as traditional folk medicine (Shrikumar, 2007).

The Indian Flora accounts for 11.4% of the world's total recorded plant species, and around 28% of the plant species are endemic (native to a specific location). Angiosperms are the major plant group in India, with a total of 21,849 species, constituting 39.92% of the country's floral diversity (Arisdason & Lakshminarasimhan Biodiversity, http://bsienvis.nic.in/Database/Status_of_Plant_Diversity_in_India_17566.aspx). Currently, the Indian subcontinent is home to over 45,000 species (almost 20% of the world's species), of which ~3,500 are plants with medicinal potential, 500 are employed in the modern Ayurvedic sector, and ~80% are obtained from wild sources (Singh, 2006). About 80% of herbal medicines used in Ayurveda come from the western Himalayas 46% of Unani, and 33% of allopathic systems (Baragi *et al*., 2008); and 50% of medications listed in the British Pharmacopoeia are connected to medicinal plants occurring in this area (Dev, 1997). With the expansion of the market for HMs, several nations have given some guidelines and rules to guarantee the safe use of medications by patients or consumers. As per the WHO, in 2018, around 90% of member states/countries have their own policies for herbal medicine (WHO, 2019). The majority of these countries using national or other pharmacopoeias (often those of the United Kingdom, the United States, and Canada) and Europe) or monographs including herbal medicine, so long as the specifications for acceptable manufacturing methods and techniques assure adherence to manufacturing parameters and set special regulatory requirements for herbal medicines. These guidelines are established and implemented to provide an quality control and safety checks for herbal medicines efficiency, and effectiveness.

Substitute or counterfeit herbal supplements are widespread in the industry. The chemical composition and quantity of bioactive chemicals, even for the same species, may change drastically depending on collection season, locality, and storage conditions. Because of this, herbal extracts or products must undergo chemical standardisation before they may be used in biological, pharmacological, or therapeutic investigations. Standardization of herbal medicines is the process of prescribing a set of intrinsic features, consistent parameters, and definite qualitative and quantitative values that provide guarantee of quality, effectiveness, safety, and repeatability. Herbal medicine's complex composition makes it more difficult to ensure the

quality of herbal goods. The use of various analytical tools like HPLC, equipped with UV, MS, and other detectors, as well as the growing understanding of the bioactive and primary compounds in the majority of commonly used herbs, has made fingerprint chromatograms powerful qualitative and quantitative methods for standardising herbal medicines. The standardisation is essential not only for the quality monitoring of finished herbal products, but also for the collecting and cultivation of species and the optimization of the processing processes. Due to their extensive, synergistic effects on physiological systems and the comparably lower occurrence of side effects compared to synthetic medications, herbal drugs have attracted more attention from society in recent years (Noviana *et al*., 2022). Herbal drugs may not be completely risk-free, however, since some studies have shown negative effects such nephrotoxicity, hepatotoxicity, cardiotoxicity, neurotoxicity, and skin toxicity when herbal drugs were administered. These negative consequences could result from the manipulation of herbal drugs, the presence of harmful pollutants or metabolites like aflatoxins, or both. As a result, herbal drugs authenticity and quality must be verified (Heinrich, 2015). Major quality concerns have been brought up by herbal drugs complexity, which generally consists of several components. The bioactive components of extracts and/or herbal drugs preparations may vary and are often highly complicated. Therefore, the observed bioactivity or pharmacological effects may not always be repeatable if the precise chemical composition is not established precisely and explicitly. It is difficult to ensure high-quality herbal drugs with repeatable quality, effectiveness, and safety. As a result, the availability of proper analytical procedures is of the utmost importance not only for the identification and standardisation of herbal drugs, but also for the detection of impurities and contaminants (Muyumba *et al*., 2021). Analytical method validation is especially crucial in this case since it ensures the reliability of the data and without it, the data's dependability cannot be guaranteed, and the results would be difficult for other scientists to replicate (Indrayanto, 2022). Methods for general standardization of herbal drugs or botanicals have been given by the current compendia and regulatory agencies (EMA, 2011; FDA, 2016; WHO, 2017; Farmakope Herbal Indonesia, 2017; Taiwan Herbal Pharmacopeia, 2019; British Pharmacopoeia, 2020; Hong Kong Chinese Materia Medica Standards, 2020; USP Herbal Medicine Compendium, 2021; USP44-NF39, 2021b, 2021c). Similarly, the procedures for collecting plant specimens have been outlined in USP44-NF39, 2021b. The general standardization parameters described by those official recommendations are macroscopic/ microscopic characterizations, chemical tests, chromatographic fingerprinting, DNA profiling, quantitative determination of certain compounds/markers or a

group of compounds, test for chemical contaminants/microorganisms, and physicochemical tests. The Association of Official Analytical Chemists has established basic guidelines for the development, validation, and standardisation of novel or non-official techniques of analysis for botanicals/herbs and dietary supplements (AOAC, 2019). Only a comprehensive pharmacognostical, phytochemical, and pharmacological inquiry can reveal a medication's action and active ingredients, hence all three elements should be investigated together. As a result, for plant material standardisation and medicinal efficacy; pharmacognostic, phytochemical, and bioactivity approach are necessary.

Pharmacognostic studies are useful for correctly identifying the substances whereas the phytochemical investigations are useful for determining chemical nature, biosynthetic origin, and chemical characterisation. The presence or absence of chemicals affects the quality of raw materials used to make crude medications from medicinal plants. The chemical makeup of plants determines their biological activity, which is influenced by age, environmental factors such as soil conditions and nutrition.

The extraction and characterisation of a number of active phytocompounds from the plants has resulted in the development of a number of medicines with high activity profiles (Mandal *et al*., 2007). It is believed that crude extract from medicinal plants is more biologically active than isolated compounds due to their synergistic effects (Jana & Shekhawat, 2010). Secondary metabolites of plants serve as defence mechanisms against predation by many microorganisms, insects and herbivores (Cowan, 1999). Indeed, commercial and public demand have risen to the point that many medicinal plants are now threatened with extinction or genetic diversity loss (Misra, 2009).

Phytochemistry is the study of plant compounds, particularly secondary metabolites, which are created as a self-defence mechanism against insects, pests, diseases, herbivores, UV radiation, and environmental threats. Drug discovery and the development of innovative therapeutic agents against illnesses require a thorough grasp of phytochemicals. Herbalists' ethno-medical claims are verified through medicinal plant research, with the ultimate goal of identifying active components and standardising crude extracts used in traditional remedies (Sofowora, 1986, 1993).

1.2 Phytochemical characterisation of Taverniera cuneifolia

Nature has always shown to be an exceptional source of plenty of valuable resources. There is no disease for which it does not have a cure. Since time immemorial, humans have relied on natural sources, mostly plants, for nutritional and medical needs. Plants have been utilised for therapeutic reasons by humans throughout history, and they continue to serve as the foundation for many pharmaceuticals in use today (Beutler, 2019).

The plant kingdom is believed to create almost one million metabolites (Afendi *et al*., 2012), however many of them have yet to be discover. All of these specialised metabolites are generated from fundamental metabolite precursors, such as sugars, amino acids, nucleotides, organic acids, and fatty acids, which are crucial for cellular homeostasis and the survival of whole organisms (Fig. 1.2).

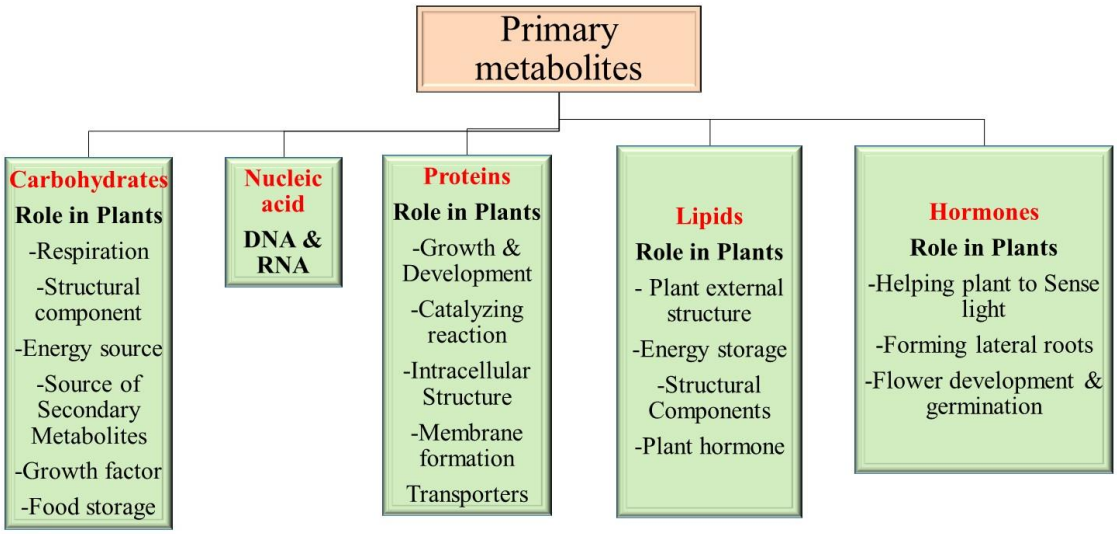


Figure 1.2: Classification of primary metabolites

There are two main types of metabolism in plants: primary (or central) metabolism, which includes all of the reactions and pathways necessary for the plant's survival, and secondary (or specialised) metabolism, which plays a crucial role in the plant's development and growth and also allows it to communicate with its environment (Fig. 1.2). Many of the hundreds of secondary metabolites that have been characterised so far are synthesised from products of primary metabolism such as glycolysis, the TCA cycle, and the shikimate pathway (Kroymann, 2011).

1.2.1 Lipids

Lipids are relatively small hydrophobic or amphiphilic molecules that comprise sterols, waxes, fatty acids, and phospholipids that are insoluble in water but soluble in organic solvents. Plants store chemical energy in the form of lipids in seeds. They also play a role in signal transduction, which allows them to control the metabolic activity of cells (Ohlrogge & Browse, 1995; Li-Beisson *et. al*., 2013). The most prevalent kind of lipid in plants is glycerolipid, whereby the carboxyl group of the fatty acid is ester-linked to the hydroxyl group of glycerol. Polar lipids are amphipathic in the sense that half of the lipid molecule is hydrophobic (aliphatic or aromatic groups), while the rest is hydrophilic (ionic or hydroxyl residues). In neutral or non-polar lipids, the later groups are usually missing. Polar lipids are nearly exclusively found in cell membranes (with the exception of some seeds and fruits) (Quinn & Williams, 1978). Glycosylglycerides and phospholipids are the two primary groups of lipids found in plant tissues.

Recent research has focused on the finding of transcription factors that govern lipid synthesis (Lee *et al*., 2018), the involvement of galactolipids in photosynthesis (Li & Yu, 2018), the production and transport of cuticle lipids (Fich *et al*., 2016; Lee & Suh, 2015) and lipid remodelling during development and stress (Yang & Benning, 2018). Studies on the alteration of fatty acid composition and the augmentation of oil content are now ongoing to improve the qualitative and quantitative characteristics of plant oil in terms of application (Lee *et al*., 2015; Vanhercke *et al*., 2019).

1.2.2 Carbohydrates

Carbohydrates are the most ubiquitous kind of organic component in living things. They make up three-quarters of the dry weight of plants and are found in a broad range of different living forms (Whistler & Corbett, 1966). Plant carbohydrates contain a tremendous amount of energy, which may be used as food, animal feed, or fuel. Some carbohydrates and their derivatives have been investigated as chemotherapeutic treatments for a variety of diseases, including cancer (Whistler, 1966). Carbohydrates provide energy (sugar, starch), taste (sweetness), and bulk (cellulose and hemicellulose). Simple carbohydrate biosynthesis mostly occurs through the gluconeogenesis and pentose phosphate shunt pathways (Sharkey, 2021). Carbohydrates, polyalcohols, and certain amino acids (notably proline) are not only serve nutritional purposes in cells, but they also play an intriguing role as osmoregulatory substances, particularly under

stress (Sami *et al*., 2016; Versluys *et al*., 2017). Emil Fischer was the first to synthesise carbohydrate material in the laboratory in 1889 (Fischer, 1890). Cereals, sweets, fruits, root crops, and tubers are examples of dietary carbohydrates that are eaten by people all over the globe. The class of carbohydrates derived from plants includes a variety of simple sugars, including mono- and disaccharides, sugar alcohols, and polymers, such as starch and cellulose (Fig. 1.3) (Bryant *et al*., 1999; Ernst *et al*., 2000). The most prevalent organic compound on Earth is cellulose, a polymer of glucose that serves as the primary structural component of plants.

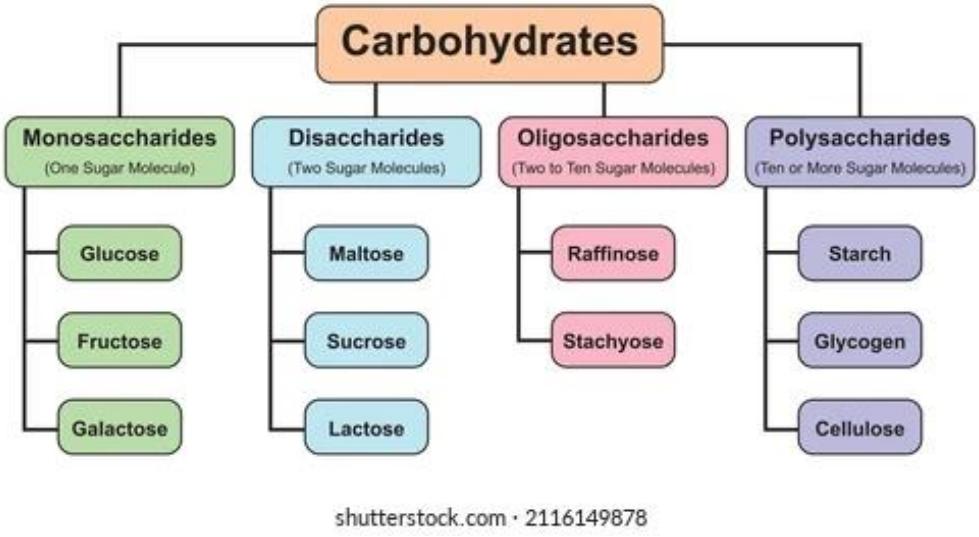


Figure 1.3: Classification of carbohydrates

Despite the fact that carbohydrates are main metabolites, they are integrated into several secondary metabolites through glycosidation bonds. In addition to their vital biological and structural roles in plants, certain carbohydrates, such as mucilage, have therapeutic properties. Mucilage, a viscous, sticky material produced by most plants and microorganisms, thickens plant membranes for protection and stores water, food for seeds. Chemically, it's polar glycoprotein and exopolysaccharide. In medical, mucilage is used as a demulcent. Cacti and *Linum usitatissimum* are major mucilage suppliers.

Additionally, the carbohydrate has a critical role as hormone-like signalling molecules has been discovered and is now being extensively researched (Koch, 1996; Koch, 2004; Rolland *et al*., 2006; Sheen *et al*., 1999; Smeekens *et al*., 2010). This is why sugars are seen as part of an intricate communication network that is important for coordinating metabolism with growth and development as well as coping with a variety of environmental changes and stressors (Rolland *et al*., 2002, 2006). Sugars, in particular the disaccharides sucrose and trehalose,

raffinose family oligosaccharides, and fructans, have a role in the production of reactive oxygen species (ROS) by plants in response to abiotic stressors. Enzymatic scavengers (superoxide dismutase, ascorbate peroxidase, glutathione peroxidase) and non-enzymatic metabolites (ascorbate, glutathione, -tocopherol) are the known antioxidants in plants. Furthermore, there is mounting evidence that carbohydrates play a function as antioxidants due to their ROS-scavenging capabilities. Therefore, sugars might be regarded as essential components of an integrated cellular redox network.

Plants generate a wide variety of secondary or specialised metabolites, which play essential roles in plant adaptation to varying environmental situations. In addition to their extensive usage in human nutrition and medicine, phytochemicals are also frequently used in human nutrition and health. Secondary metabolism pathways show far higher variation between species, organs, tissues, cells, and developmental stages than primary metabolism responses, which are well conserved (Wink, 2010).

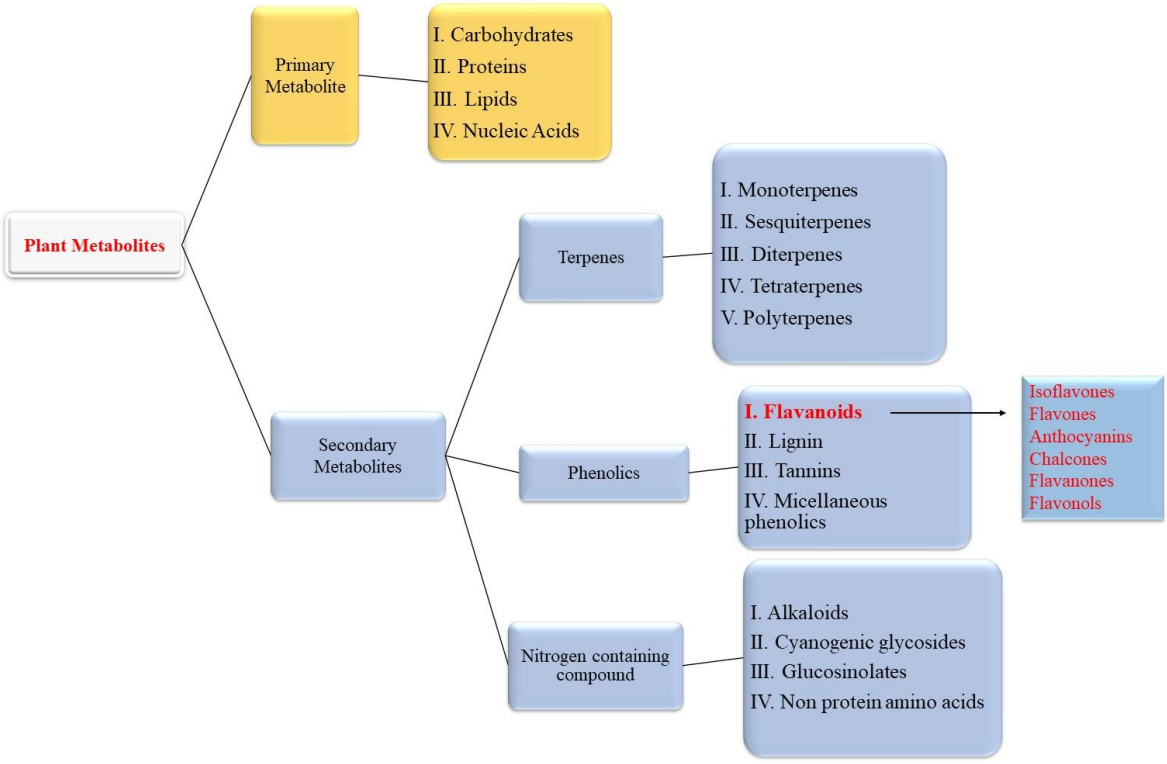


Figure 1.4: Classification of Phytochemicals

Around the years, the successful use of plant products to prevent and treat infectious illnesses has caught the interest of scientists all over the world (Falodun *et al*., 2006). Plants' therapeutic abilities come from their unique biochemical capabilities, which include the production and storage of a vast array of secondary compounds. Recent years have seen a phenomenal growth in interest in secondary metabolites due to their undeniable relevance in plant stress physiology and their enormous practical consequence for pharmacological, nutritional, and aesthetic uses (Ngo *et. al*., 2013). The therapeutic efficacy of these plants is due to the presence of certain chemical compounds that have a specific physiological effect on the human body. There are over a thousand identified phytochemicals, each of which operates in a unique way.

1.3 Importance of plant secondary metabolites

Albrecht Kossel, Nobel Prize laureate in physiology or medicine in 1910, was the first to establish the notion of secondary metabolite (Jones, 1953). Secondary metabolites are a diverse group of chemicals from several metabolite families that are significantly inducible in stress responses. These chemicals are not required for cell architecture or life maintenance, but they are frequently implicated in plant defence against biotic or abiotic stressors. In the plant kingdom, nearly 50,000 secondary metabolites have been found. Secondary plant metabolites are responsible for the activities of medicinal plants and many contemporary drugs. Secondary plant metabolites are categorised into many groups based on their chemical structures. Secondary plant metabolites are classified into the following categories:

1.3.1 Phenolics

Phenolics are one of the most widespread class of secondary metabolites in the plant world (Boudet, 2007). They include a highly expansive and varied range of aromatic compounds that share the benzene ring (C6) and one or more hydroxyl groups as their distinguishing features (Bhattacharya *et al*., 2010). They are abundant in plants and contribute greatly to the colour, taste, and flavour of a broad variety of herbs, foods, and beverages. Plant phenolics, which include simple phenols, phenolic acids, flavonoids, coumarins, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins, are the most abundant secondary metabolites, produced primarily via the shikimate pathway from L-phenylalanine and L-tyrosine and containing one or more hydroxyl groups attached directly to the aromatic ring (Chirinos *et al*., 2009; Kumar & Pruthi, 2014). Phenolics are further categorised based on their structural characteristics or biosynthetic origin. Phenolics are categorised into three groups based on their chemical structures:

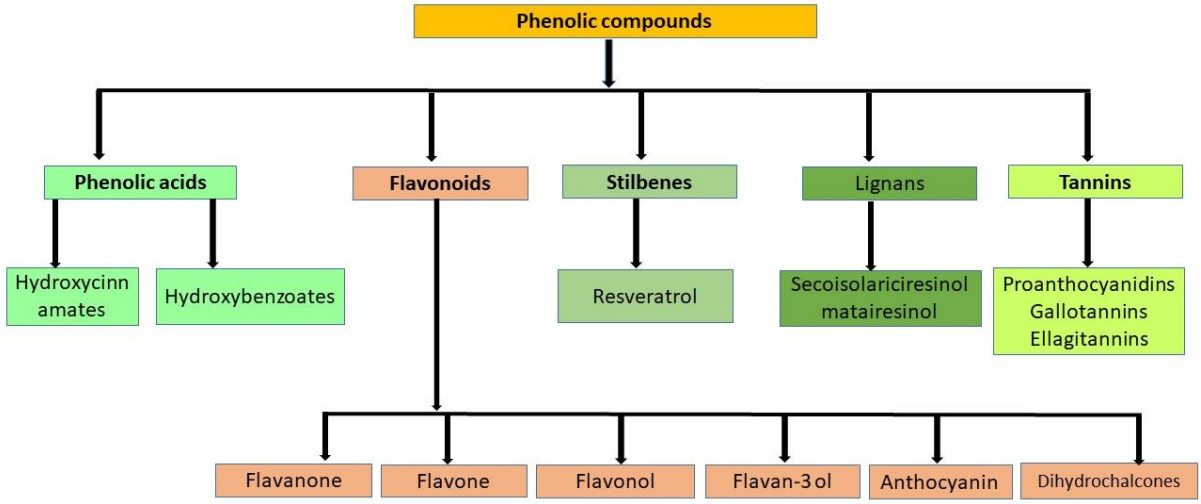


Figure 1.5: Classification of phenolic compounds

1.3.2 Phenolic Acids

The phenolic compounds with a single carboxylic acid group are referred to as "phenolic acids." phenolic or phenolcarboxylic acids (a form of phytochemical known as a polyphenol) are one of the principal types of phenolic compounds found in plants. Many plant-based products contain these compounds, with the largest amounts being found in seeds, fruit skins, and vegetable leaves. They are usually found in bound forms such as amides, esters, or glycosides, and very rarely in free form (Pereira *et al*., 2009). The two main subgroups of phenolic acids are hydroxybenzoic acid and hydroxycinnamic acid (Clifford, 1999). *P*-hydroxybenzoic, protocatechuic, vanillic, and syringic acids are the four most prevalent hydroxybenzoic acids, while ferulic, caffeic, *p*-coumaric, and sinapic acids are the most common hydroxycinnamic acids that are found in plants.

1.3.3 Flavonoids

Flavonoids are a class of hydroxylated phenolic compounds that plants produce in response to microbial infection (Dixon *et al*., 1983). Structure has an important role in their actions. Flavonoids' chemical nature relies on their structural class, degree of hydroxylation, various substitutions and conjugations, and degree of polymerization (Heim & Bobilya, 2002). Flavonoids are now considered as an indispensable component in a variety of nutraceutical, pharmaceutical, medicinal and cosmetic applications. The possible health advantages resulting from the antioxidant properties of these polyphenolic chemicals have sparked a recent interest in these substances. Flavonoids exert their antioxidant actions by scavenging free radicals

and/or chelating metal ions (Kumar *et al*., 2013; Kumar & Pandey, 2007). Flavonoids are claimed to have health-promoting properties as a dietary component owing to their strong antioxidant activity in both in vivo and in vitro systems (Cook & Samman, 1996). Flavonoids have the capacity to stimulate protective enzyme systems in humans. Numerous studies have demonstrated that flavonoids provide protection against a variety of infectious (bacterial and viral illnesses) and degenerative (cardiovascular diseases, malignancies, and other age-related disorders) diseases (Pandey, 2007; Cook & Samman, 1996). They also influence plant growth factors like auxin (Agati *et al*., 2012).

Based on the carbon of the C ring to which the B ring is attached, as well as the degree of unsaturation and oxidation of the C ring, flavonoids are divided into distinct subgroups. Isoflavones are flavonoids in which position 3 of the C ring is linked to the B ring. Those in which the B ring is connected in position 4 are known as neo-flavonoids, while those in which the B ring is linked in position 2 may be further classified depending on the structural properties of the C ring. These substances consist of flavones, flavonols, flavanones, flavanonols, flavanols or catechins, anthocyanins, and chalcones (Fig. 1.5 & 1.6).

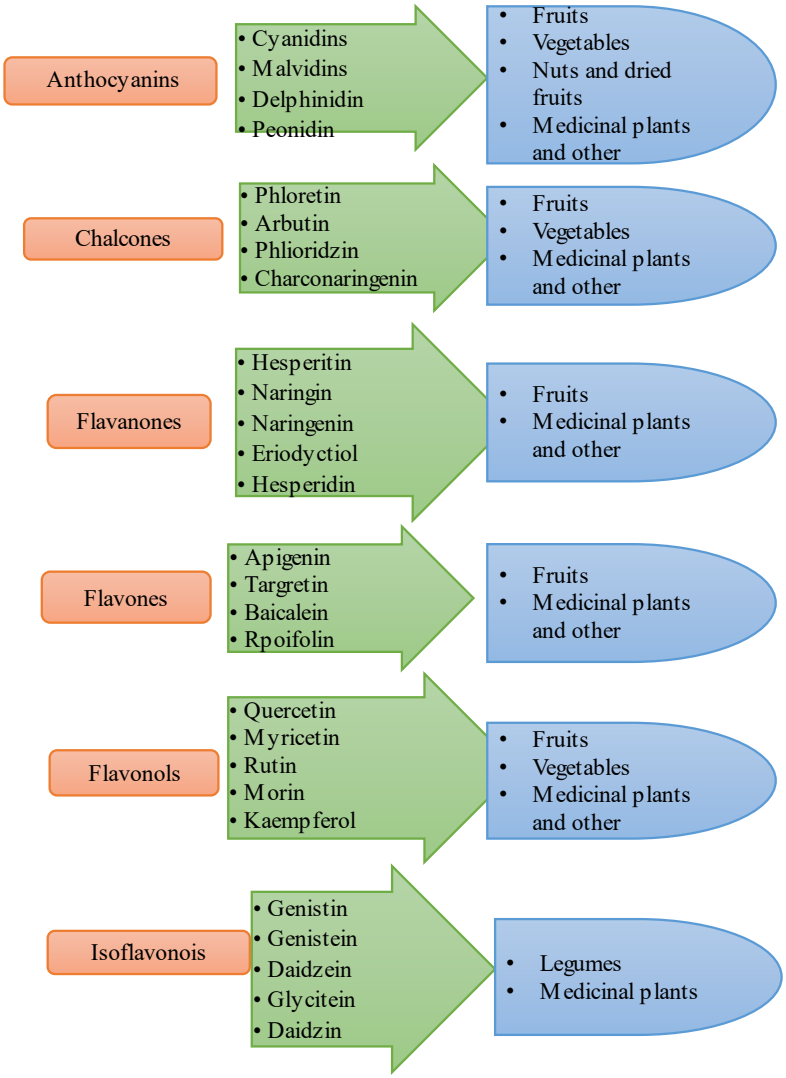


Figure 1.6: Flavonoid Classes, Subclasses and natural Sources

1.3.4 Stilbenes

Stilbenes are a small but significant family of non-flavonoid polyphenols that share a 14-carbon skeleton consisting of two benzene rings connected by an ethylene bridge. Currently, over 400 distinct stilbene compounds are known (El Khawand *et al.* 2018), mostly trans-resveratrol (3,5,40-trihydroxy-trans-stilbene), with some variation in structure across various plant species (Chong *et al.*, 2009). At least 72 plant species have been found to contain stilbenes, with evidence coming from 31 genera and 12 distantly related families. These include the Pinaceae (e.g., *Picea abies* (L.) Karst. and *Pinus nigra* J.F. Arnold), Gnetaceae (e.g., *Gnetum parvifolium* (Warb.) W.C. Cheng and G. africanum Welw.), Fabaceae (*Arachis hypogaea* L. and *Robinia pseudoacacia* L.), Vitaceae (e.g., *Vitis vinifera* L. and *V. amurensis* Rupr.), Moraceae (e.g., *Morus alba* L. and *M. macroura* Miq.), and Polygonaceae (e.g., *Polygonum cuspidatum* Sieb.

et Zucc. and *P. multiflorum* Thunb.) (Riviere *et. al*., 2012; Dubrovina & Kiselev, 2017). Mainly, stilbenes have a role in both constitutive and inducible plant defence mechanisms against biotic (such as phytopathogenic bacteria and herbivores) and abiotic (such as UV radiation and tropospheric ozone) stress (Chong *et al*., 2009; Jeandet *et al*., 2010). Stilbenes posses activities like antibacterial, antifungal (Albert *et al*., 2011; Chalal *et al*., 2014) nematocidal (Suga *et al*., 1993), and insecticidal (Torres *et al*., 2003; Liu *et al*., 2013).

1.3.5 Lignans

Lignans are 1,4-diarylbutan compounds that are formed from the shikimic acid metabolic pathway (Lewis and Davin, 1999; Imai et al., 2006). Traditionally, lignans are classified as a type of secondary metabolites derived by the oxidative dimerization of two or more phenylpropanoid units. Lignans possess a basic structure of two or more phenylpropanoid units (Ayres & Loike, 1991) with cinnamic acid, cinnamyl alcohol, propenyl benzene, and allyl benzene serving as their monomers. Lignans have been identified in over 70 families and so far, more than 200 classical lignans and 100 neolignans have been identified (Pan *et al*., 2009). The majority of lignans in plants are free but some may react with glycon to produce glycosides and other derivatives. Lignans possess several appealing pharmacological activities including anticancer (Capilla *et al*., 2001), antioxidant (Lu & Liu, 1992), antibacterial (Kawazoe *et al*., 2001), immunosuppressive (Hirano *et al*., 1991) and anti-asthmatic effects (Iwasaki *et al*., 1996). Many lignans have been shown to have antiviral properties as well (Charlton, 1998). Lignans are extensively dispersed throughout the plant world and are found in plant roots, rhizomes, stems, leaves, flowers, fruits, seeds, xylem, and resins. In addition, plants belonging to the family Annonaceae, Orchidaceae, Berberidaceae and Schisandraceae are rich in lignans and neolignans (Lu & Liu, 1992; Wu *et al*., 2019).

1.3.6 Tannins

According to Barbehenn and Constabel (2011), tannins are the primary polyphenolic secondary metabolites that are extensively dispersed and account for 5 to 10% of the dry vascular plant materials. Tannins are mostly found in the bark, stems, seeds, roots, buds, and leaves of the plants (Barbehenn and Constabel, 2011; Giovando *et al*., 2019; Tomak and Gonultas, 2018). Foods including grapes, blackberries, strawberries, walnuts, cashew nuts, hazelnuts, mangoes, and tea also contain tannins (Clifford and Scalbert, 2000). Tannins serve as a plant's first line of defence against fungal infections, insect pests, and herbivores (De Bruyne et al., 1999; Hagerman et al., 1998; Khanbabaee and van Ree, 2001; Sharma, 2019). Tannins are divided into three categories: condensed tannins, hydrolyzable tannins, and complex tannins

(Khanbabaee & van Ree, 2001) (Fig. 1.7). Hydrolyzable tannins have fewer natural sources compared to condensed tannins (Haslam, 1982; Hillis & Rozsa, 1985). As a result, more than 90% of all commercial tannins in the world are condensed tannins (Filgueira et al., 2017; Pizzi, 2008). Tannins have been primarily used by the leather industry for millennia for both hide treatment and leather colouring (Kemppainen *et al*., 2014; Pizzi, 2008). Tannins' phenolic structure provides the potential for usage in various applications, such as adhesives for the wood industry (Pizzi, 2006), insulating foams (Tondi *et al*., 2009), mineral industry, wine production industry, animal nutrition, oil industry (Pizzi, 2006), water treatment plant (Combs, 2016) and protecting metal from corrosion (Luo *et al*., 2019; Ostovari *et al*., 2009).

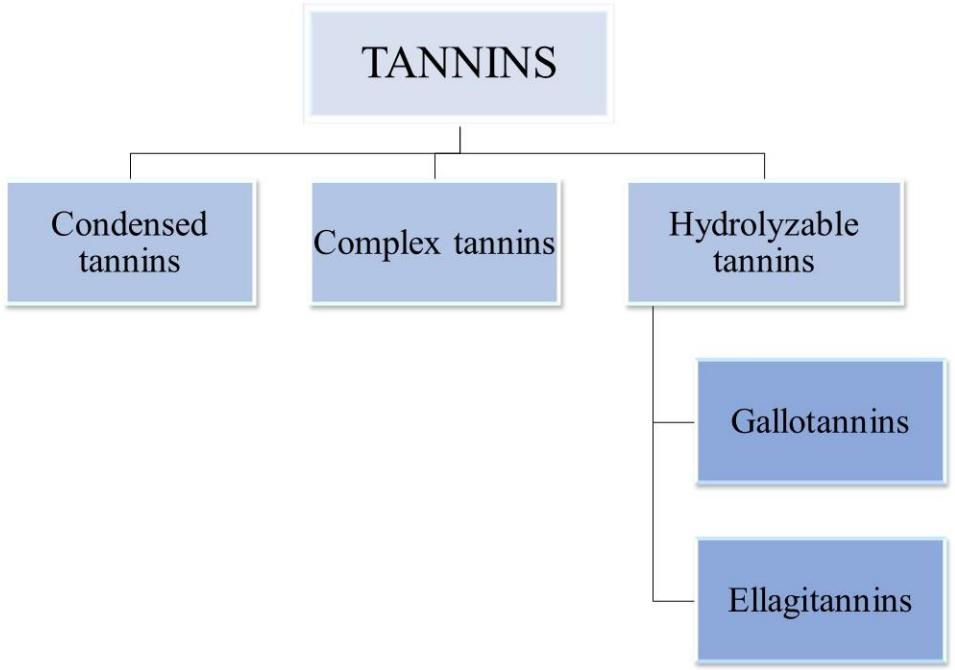


Figure 1.7: Classification of Tannins (Vermerris & Nicholson, 2007)

Many tannins may be hydrolytically fractionated into their constituents, and these tannins are referred to as 'hydrolysable tannins.' Gallotannins and ellagitannins are two types of hydrolyzable tannins (Kashiwada *et al*., 1992; Weinges & Plieninger, 1999). Condensed tannins are non-hydrolyzable oligomeric and polymeric proanthocyanidins (Würdig & Woller, 1989). Plant extracts containing tannin are used in medicine, particularly in Asian (Japanese and Chinese) natural healing, as astringents, against diarrhoea (Yoshida *et al*., 1992), as diuretics (Hatano & Yazaki, 1991; Okuda *et al*.,1983), against stomach and duodenal tumours (Saijo *et al*.,1989) and as anti-inflammatory, antibacterial, and haemostatic medicines (Haslam, 1989).

1.3.7 Alkaloids

Morphine, the first alkaloid to be isolated in crude form, was described by Sertürner in 1805; it is still a significant pharmaceutical compound derived from opium (Suzuki & El-Haddad, 2017). A few years thereafter, the important antimalarial agent quinine was obtained Kaufman & Rúveda, 2005). Alkaloids are present in around 20% of plant species (Srivastava & Srivastava, 2013), but their synthesis (including biotechnology), extraction, and processing are key areas of study (Yu *et al*., 2002; Djilan *et al*., 2006). Alkaloids are formed from l-lysine, l-ornithine, l-tyrosine, l-tryptophan, l-histidine, l-phenylalanine, nicotinic acid, anthranilic acid or acetate (Aniszewski, 2007). Additionally, this category contains several related chemicals that exhibit neutral (McNaught, 1997) and even mildly acidic (Manske, 1965) characteristics. The presence of nitrogen atom(s) in a negative oxidation state at their structures (Kurek, 2019), gives alkaloids their characteristic alkaline characteristics and associated therapeutic advantages (Rosales *et al.*, 2020; Kurek, 2019; Babbar, 2015). Alkaloids have been classified in a variety of ways by various scholars. One of the most often used classifications, which divides a complex class into three categories i.e., True alkaloids, proto-alkaloids and pseudo-alkaloids (Eagleson, 1994).

The human diet includes a wide variety of alkaloids, including those found in coffee seeds (caffeine), cacao seeds (theobromine and caffeine), tea leaves (theophylline and caffeine), tomatoes (tomatine), and potatoes (solanine) (Cushnie *et al*., 2014). Alkaloids have been utilised for thousands of years in human medicine to treat anything from neurological problems (Hussain *et al*., 2018), cancer (Dey, 2019), metabolic dysfunction (Feng, 2019), and infectious infections (Kishore, 2009). They are among the most varied, effective, and medicinally important plant compounds.

1.3.8 Saponins

Saponins are a wide and diversified collection of secondary metabolites that are typically characterised as amphipathic glycosides with glycosyl residues connected to a triterpenoid (triterpene or steroidal) aglycon (Hostettmann & Marston 1995; Osbourn 2003; Gu¨c¸lu¨-U¨stu¨ndag˘ and Mazza 2007; Vincken *et al*. 2007). Their structural diversity is mirrored in their physicochemical and biological characteristics, which are used in a variety of conventional (as soaps, fish poison, and molluscicides) and industrial applications (Price *et al*., 1987; Oakenfull, 1981; Hostettmann & Marston, 1995; Oakenfull & Sidhu, 1989). Saponins are most often found in angiosperms (Wink 2003; Henry 2005), although they are also present

in certain ferns (Hanus *et al*. 2003). Saponins are a broad set of chemicals found across the plant kingdom that have a structure that includes a triterpene or steroid aglycone as well as one or more sugar chains.

Saponins are amphiphilic glycosides with polar glycone structure moieties (sugars) separated from nonpolar aglycone structure moieties (also known as sapogenins) (Sparg *et al.*, 2004). According to their aglycone counterparts, saponins are classed as steroidal saponins and triterpenoid saponins (Moghimipour & Handali, 2015; Sharma *et al*., 2012) (Fig. 1.8).

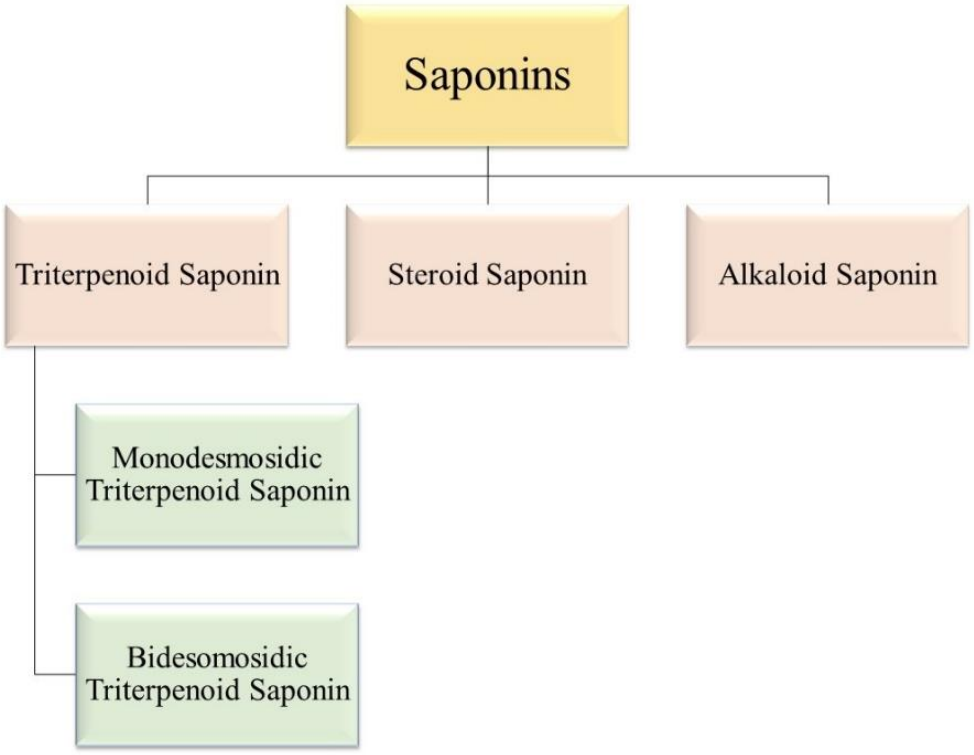


Figure 1.8: Classification of saponins

1.3.9 Terpenoids

Terpenes are the biggest and most diversified collection of naturally occurring chemical that are found in both plants and animals and has a variety of therapeutic benefits (Gershenzon & Dudareva, 2007). Till date, about 50,000 terpenoids have been discovered in nature (SUN *et. al.*, 2017), and the majority of which have been extracted from plants only. Gibberellin is just one example of a terpenoid that functions as a plant hormone to regulate growth and development; others include the carotenoid pigments involved in photosynthesis, the phenylpropanoid pigments responsible for plant defence, and the phenylpropanoid pigments involved in interspecific competition as interspecific sensing molecules (Arimura *et al*., 2000).

Menthol and perillyl alcohol are two examples of the many volatile terpenoids that find application as ingredients in the spice, flavouring, and cosmetic industries (Martin, 2003). Terpenoids are formed either the active cytosolic mevalonate (MVA) route or the plasticidal 2-C-methyl-D-erythriol 4-phosphate (MEP) pathway. The isoprene rule is an empirical characteristic used to classify terpenoids based on the quantity and structural organisation of carbons created by the linear arrangement of isoprene units, which is followed by cyclization and rearrangements of the carbon skeleton (Zwenger Chhandak Basu *et al*., 2008). Terpenoids are classified into eight categories depending on the amount of isoprene units present as shown in the Fig. 1.9. As per the recent study, terpenes improve skin permeability and facilitate medication absorption by disrupting the lipid arrangement in the intercellular sections of the stratum corneum (Cal *et al*., 2006). Thymol, a monoterpenoid, and menthol derivatives shown to have insecticidal properties (Robledo *et al*., 2005; Kiuchi *et al*., 2002). Terpenoids are common in medicinal plants and are in high demand due to their numerous possible uses and potential.

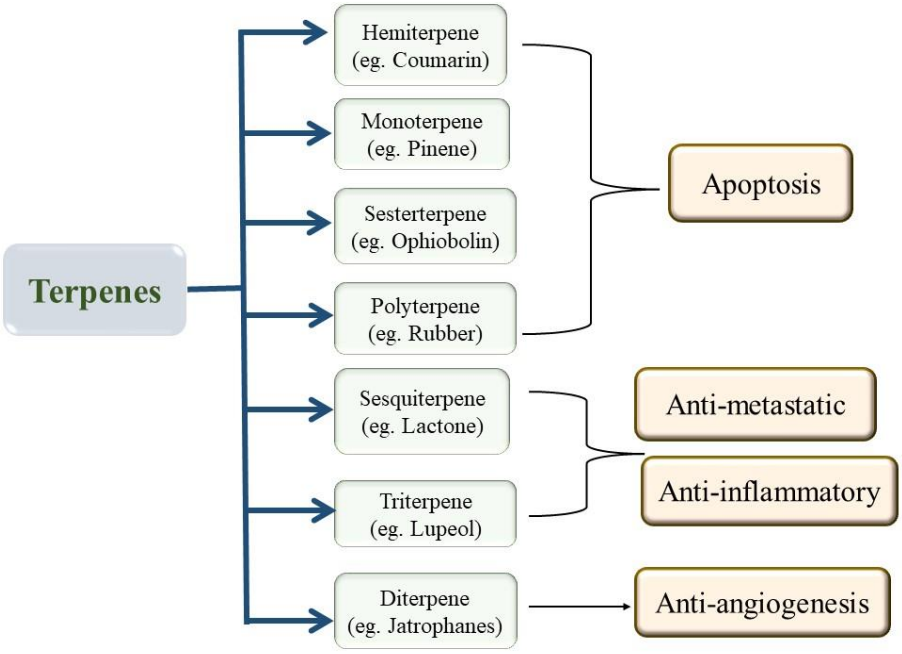


Figure 1.9: Classification of Terpenoids

1.4 Characterisation of phytocompounds

Pure chemicals or standardised extracts from medicinal plants provide an infinite supply of novel therapeutic leads due to their unparalleled chemical diversity (Cosa *et al*., 2006). Natural product screening programmes are becoming more diverse, with a growing focus on edible

plants as a potential source of therapeutic medicines. As many as 20% of all known plants have been employed in pharmaceutical investigations, which have had a good influence on the healthcare system, such as curing cancer and other hazardous disorders (Naczk & Shahidi, 2006). Natural remedies satisfy the primary health-care needs of most people in under developed nations and are gaining favour in wealthier countries due to high health-care expenses and little side effects. Plant produces various bioactive substances and almost all of the medicinal, botanical and herbal medications include these bioactive chemicals. The phytochemical characterisation relies on the refinement of methods for the efficient and selective separation of these bioactive compounds (Fig. 1.10).

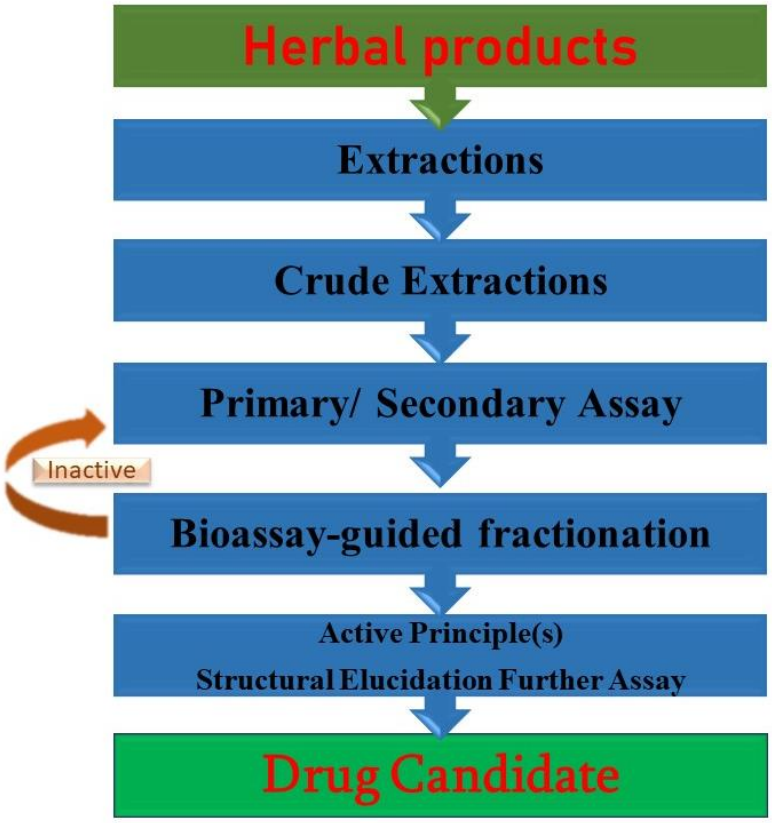


Figure 1.10: Schematic diagram for characterization of a bioactive compound from natural sources

It is the utmost duty of regulatory agencies to ensure that customers get medications that guarantee purity, safety, potency, and effectiveness. The quality monitoring of crude pharmaceuticals and herbal formulations is of utmost relevance to their acceptance in the current medical system (Dhiman *et al*., 2016). However, the lack of a strict quality control profile for herbal ingredients and their formulations is one of the most significant challenges facing the herbal medication sector (Archana *et al*., 2011).

Figure 1.11: A brief summary in Extraction, Isolation and characterisation of Natural products from medicinal plant.

There is a large scope in determining an appropriate extraction technique that extracts the phyto-components from the plants. Selection of solvent is an important aspect in any extraction; in general, solvents, such as methanol, ethanol, acetone, propanol and ethyl acetate, have been commonly used for the extraction of phytocomponents from the plants (Durling *et al*., 2007) (Fig. 1.11). Appropriate extraction technique coupled with the optimization of the parameters involved in extraction and using a right optimization technique proves essential in order to capture medicinal components for further processing in pharmaceutical industries.

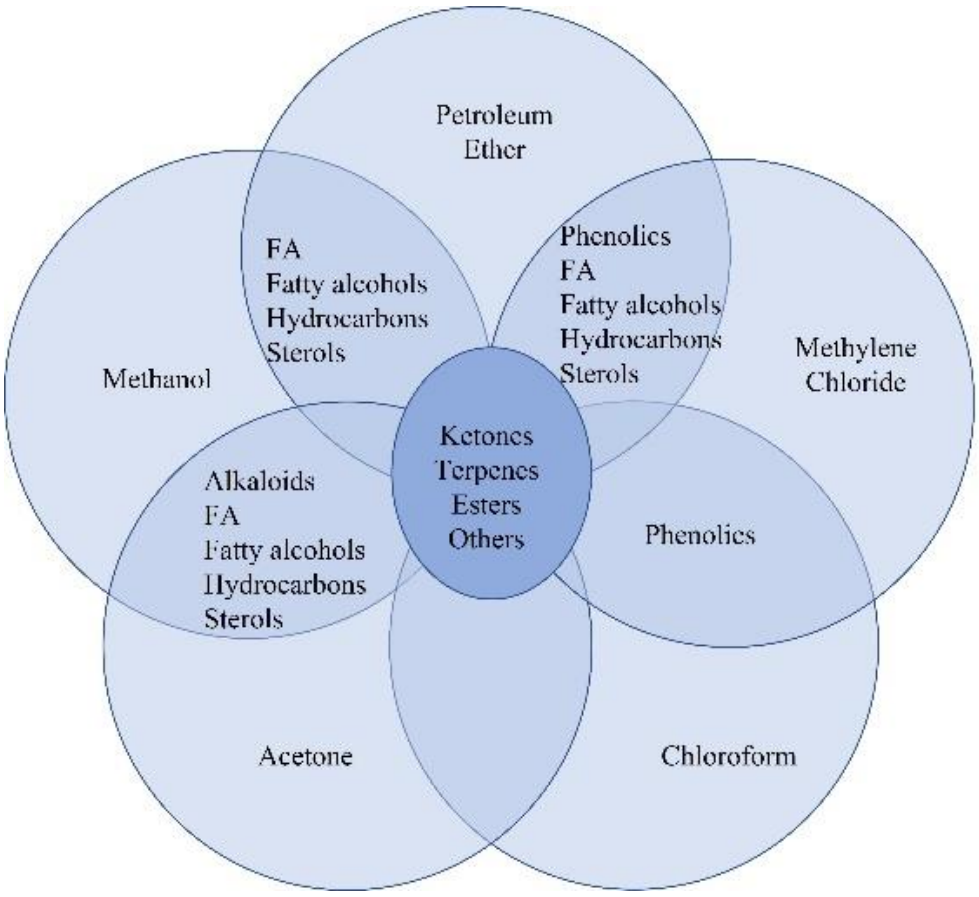


Figure 1.12: Venn diagram elucidate the overlapping of compounds belonging to different identified chemical groups between different extraction solvents.

Analysis of medicinal plants begins with the extraction of the required chemical components from the plant materials because it is important to extract the desired chemical components from plant materials for subsequent separation and characterisation. The fundamental steps for the extraction of any phyto-components included are pre-washing, drying of plant materials by either air drying or freeze drying, grinding to obtain a homogenous sample, and frequently improving the kinetics of analytical extraction and also increasing the contact of sample surface with the solvent system. During the process of preparing an extract from plant materials, the appropriate precautions need to be followed to avoid compromising the stability of any possible active ingredients, which might result in the extract's ineffectiveness. The choice of solvent solution is heavily influenced by the type of the bioactive chemical being targeted. To extract the bioactive component from natural sources, a variety of solvent systems are available. To extract hydrophilic compounds, polar solvents such as methanol, ethanol, and ethyl acetate are used. For the extraction of more lipophilic substances, dichloromethane or dichloromethane/methanol in a 1:1 ratio is used. In certain cases, hexane extraction is employed to remove chlorophyll (Cosa *et al*., 2006).

Plant samples are extracted using a variety of processes, including sonification, heating under reflux, soxhlet, and others ("Pharmacopoeia of the People's Republic of China.," 2000; "The Society of Japanese Pharmacopeia.," 2001; USP, 2002). Other methods are also employed for the extraction such as macerating or percolating fresh green plants or powdered plant material in water or organic solvent systems. The following are some of the most commonly used extraction techniques (both traditional and modern) for medicinal plants:

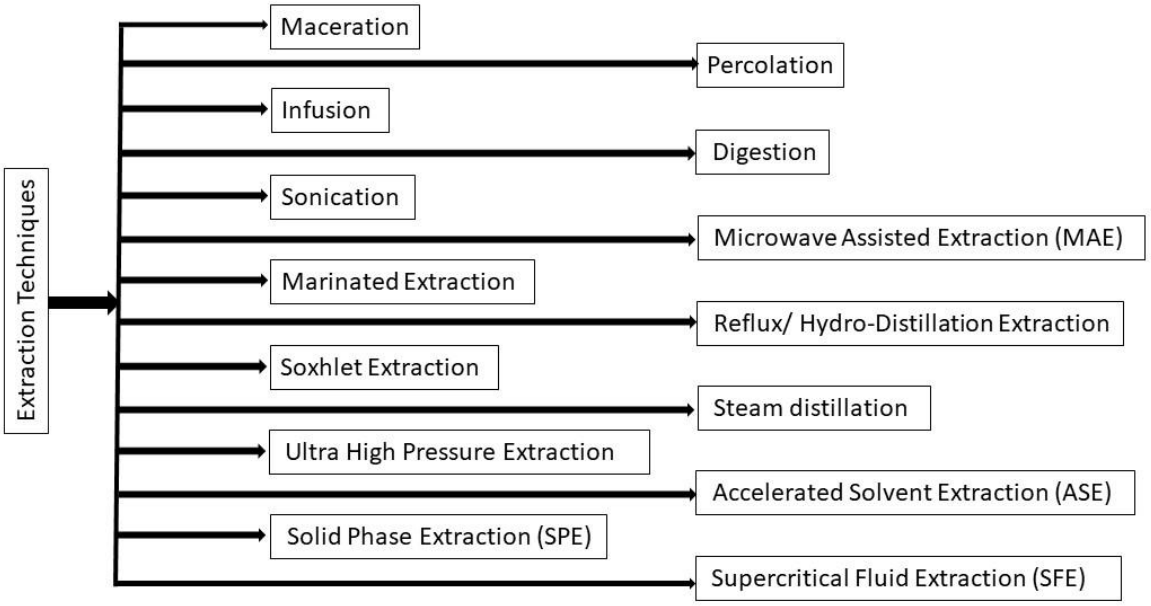


Figure 1.13: Types of Extraction methodology used

1.4.1 Maceration

In this method, solid plant pieces are put in a stoppered container with the whole solvent and left to stand for 3-7 days with regular agitation, until soluble component is dissolved. The combination is then strained, the marc pressed, and the mixed liquids purified by filtering or decantation. When the solvent is water and maceration is extended, alcohol can be added to limit microbial development.

1.4.2 Percolation

This is the most common method for extracting tincture and fluid extract components. Plant material is placed in a cotton-plugged percolation tube with a filter and stopcock (Pan *et al*., 2014). Solvent is poured to plant material and left for 4 hours in a well-closed container before packing and closing the percolator. The complete system is stored for 24 hours at room temperature, and the solvent along with the extracted substance is collected by opening the stopper below.

1.4.3 Infusion

In this extraction method, the plant material is macerated with either cold or hot water for a brief amount of time (Vidyadhar *et al*., 2010). It is a diluted solution of the crude drugs which is easily soluble constituents.

1.4.4 Digestion

This is a kind of maceration in which the extraction process is carried out with the use of moderate heat (40-60°C). The method may be altered by utilising a magnetic stirrer, mechanical stirrer, or manual shaking to mix the substance with the solvent. The extract is filtered after 8 to 12 hours, and new solvent is added, and the procedure is repeated until all the required components have been extracted.

1.4.5 Sonication/ Ultrasonic Extraction

A high-frequency sound wave damages the cell wall, allowing natural chemicals to escape. Immiscible solvent mixes, such as hexane and methanol/water, may be employed with ultrasound aided extraction. Compounds that are heat sensitive may be decomposed by the method. As a result, the extraction container is submerged in an ice bath.

1.4.6 Microwave Assisted Extraction (MAE)

Using a microwave with a standard solvent extraction method, this process is referred to as "microwave extraction". As a result of microwave aided organic syntheses, simple molecules may be assembled into larger polymers in a fraction of the time previously required (Jacob, 2012). In order to enhance analyte partitioning from the sample matrix into the solvent, microwave heating of solvents and plant tissue accelerates extraction kinetics (Trusheva *et al*., 2007). Polar and polarizable materials are heated at their surfaces by microwave radiation, which is then transported by conduction. Hydrogen bonding is disrupted, allowing dissolved ions to migrate and solvents to penetrate the matrix (Altemimi *et al*., 2015); this process is facilitated by microwave electromagnetic dipole rotation of molecules. Since energy is only moved by dielectric absorption in non-polar solvents, heating isn't very good (Popova *et al*., 2009).

1.4.7 Marinated Extraction

The marinated extraction process is carried out in a room-temperature. For this approach, consider the kind of solvent, the herb-to-solvent ratio, and the extraction period. This process takes longer since it uses neither heat nor force. The extraction products are often filtered. This procedure needs no special equipment or a lab setting. Long extraction time is a drawback of this approach (Wu *et al*., 2014). This approach was used by Laghari et al., in 2011 to extract

Cassia angustifolia flowers and Zhu *et al.*, in 2010 to extract Portulaca oleracea within 48 hours at room temperature.

1.4.8 Reflux/ Hydro-Distillation Extraction

The extraction process that uses hydro-distillation is very similar to the extraction process that uses the Soxhlet method; the only difference is that the solvent that is used for extraction in this case is water. In the process of isolating volatile and non-volatile polar components from plants (Grosso *et al*., 2007), both methods are often employed with only minor differences in the kind of solvent used and the configuration of the apparatus. It is preferable to use flasks with a spherical bottom since the condenser on top of the container can better recycle the solvent vapour. A hydro-distillation extractor must adhere to a precise temperature restriction in order to avoid the deterioration of thermal compounds. Hydro-distillation extraction has the benefit of not using a costly organic solvent.

1.4.9 Soxhlet Extraction

Soxhlet extraction is used not only for phytochemical extraction, but also as a reference to compare contemporary extraction procedures (Tatke & Jaiswal, 2011). The sample is introduced into a thimble holder, which is progressively filled with new solvent from a distillation flask. When the liquid reaches the overflow level, a syphon aspirates the solute from the thimble holder, returning the aliquot to the distillation flask and transporting the extracted analytes into the bulk liquid. This technique is repeated until the extraction is complete. As the solvent is recirculated through the sample, the system runs continuously. The extracts are extracted by filtering, and the solvent is evaporated in a rotary evaporator at predetermined temperatures and decreased pressure (Lope Pihie *et al*., 2012). In Soxhlet extraction, the most appropriate solvent for extracting the desired chemicals is also important since various solvent polarities will dissolve different compounds (Zarnowski & Suzuki, 2004).

1.4.10 Steam distillation

The steam distillation-extraction technique is often used to extract essential oils. To deliver steam to the solvent and plant raw material combination, steam distillation equipment is required. Steam is given at a pressure and saturation temperature that are both higher than the boiling point of the mixture. This lets evaporation happen at temperatures below the boiling point of the mixture. The evaporated combination of water and chemicals runs into a condenser where it is condensed into a liquid and then collected in a separator. Using a rotary evaporator, the solvent is eventually evaporated and separated from the chemical. As the temperature of steam distillation can be precisely regulated, it could avoid the degradation of organic

compounds caused by high temperatures. So that it may be operated below the temperatures at which the chemicals decompose.

1.4.11 Ultra-High Pressure Extraction (UHPE)

In this technique of extraction, high pressure refers to a cold, isostatic, ultra-high hydraulic pressure. Under high pressure, natural chemicals are more soluble in any solvent (Von Rohr & Trepp, 1996). A fluid was employed in conjunction with an ultrahigh pressure booster pump to provide pressure to the vessel. Extraction was conducted at ambient temperature and high pressure (100 MPa to 1,000 MPa) for a definite period of time (5 to 15 minutes). The ultra-high pressure used in UHPE increases the solubility of the chemical and shortens the extraction time. Typically, organic molecules in plants are heat sensitive (Shouqin *et al*., 2004). Heat may cause organic substances to denature, lose biological function, or break down into new chemicals. Using UHPE that functions at room temperature, compound breakdown may be prevented (Shouqin *et al*., 2004). However, the extract impurity is a major difficulty and downside of the UHPE technique.

1.4.12 Accelerated Solvent Extraction (ASE)

Accelerated solvent extraction (ASE) is carried out at increased temperatures (50 to 200 °C) and pressures (between 10 and 15 MPa) while keeping the solvent in liquid state. As a result, the extraction process is likely to proceed more quickly under these circumstances. This system maintains a consistent temperature and pressure. The sample and solvent are introduced in the sealed container inside the pressurized vessel. The ASE approach is often used for environmental matrices containing thermally stable organic contaminants. The high temperature required by the ASE approach may cause heat-sensitive compounds to degrade, making it inappropriate for thermally labile compounds. Kaufmann and Christen (Kaufmann & Christen, 2002) examined the performance of ASE and soxhlet extraction for extracting steroids from the leaves of Lochroma gessoides. In terms of recovery, repeatability, and selectivity, both techniques were shown to provide identical outcomes. The use of the ASE may minimize extraction time and solvent use (Kaufmann & Christen, 2002; Wang & Weller, 2006).

1.4.13 Solid Phase Extraction (SPE)

This quick, affordable, and accurate method employs several cartridges and disks types with a range of sorbents, where the solute molecules are selectively bonded over the stationary phase. Solid phase extraction devices are offered in normal phase, reverse phase, and ion exchange

configurations. For instance, using 'Sep-Pak C18' cartridges (reverse phase), it is feasible to remove polar components while the residual low-polar components may be eluted later.

1.4.14 Supercritical Fluid Extraction (SFE)

SFE is a popular extraction technique in the herbal processing industry because of its high yield and excellent quality when it comes to extracting useful constituents from plants (Bernardo-Gil *et al*., 2011). SFE is also being widely researched as an alternative to traditional extraction techniques (Bernardo-Gil *et al*., 2011). One of the most commonly used solvents for SFE is carbon dioxide (CO_2) (Eller, 2002). Furthermore, since CO_2 is non-toxic, less costly, non-flammable, and non-reactive with extraction components and equipment, it makes it an excellent choice for extraction procedures. One of the most significant advantages of using CO_2 instead of organic solvents in SFE processes is the decrease in environmental and health risks (Geng *et al*., 2007; Martinelli *et al*., 1991). In SFE, a thick liquid is created by compressing gases, such as CO_2, into a supercritical state. In order to extract the substance, this liquid is first circulated through the cylinder. It is then pushed into an extraction chamber, where the extract is separated from the gas, and the gas is reclaimed for further extraction. It is possible to control CO_2 solvent characteristics by changing the pressure and temperature. Because CO_2 evaporates entirely, no solvent residues are left behind, well behind SFE (Patil & Shettigar, 2010).

Using SFE for extraction and separation of essential oils and their derivatives in the culinary, cosmetics, pharma, and other sectors is a well-established process. The essential oils produced by SFE techniques are also of higher quality and have more economically viable compositions than those produced by traditional steam distillation methods (Ehlers *et al*., 2001; Özer *et al*., 1996). By adjusting pressure or temperature without crossing phase boundaries in the SFE technique, extraction fluid physicochemical parameters such density, diffusivity, dielectric constant, and viscosity can be effectively adjusted (Bravi *et al*., 2007). Whereas, despite using the same quantity of solvent, sonication extraction is simpler than MAE. MAE requires less extraction time than sonication, but the equipment is costly and difficult to operate (Wang & Weller, 2006; Zhang *et al*., 2009, 2011). (Chen *et al*., 2007) compared steam distillation, Soxhlet, and MAE and found that MAE reduced extraction time, enhanced yields, improved selectivity, and produced higher-quality targeted extracts. MAE was cheaper than Soxhlet extraction (Kaufmann & Christen, 2002; Sticher, 2008). Soxhlet extraction has been the most used extraction technique worldwide for decades, surpassing the performance of other extraction alternatives and being used as an efficiency reference for the comparison of its

conventional and new counterparts (Ahmad *et al*., 2010). Being a continuous–discrete technique, it shows some important advantages. Specifically, in Soxhlet extraction, the sample is repeatedly brought into contact with fresh portions of solvent facilitating the displacement of the transfer (Luthria, 2008). There are number of other variables such as temperature, extraction time, material and solvent play a big role in the extraction yield of extract (Fig. 1.14).

According to the most cutting-edge forthcoming equipment, the aforementioned conventional extraction techniques have a few limitations, such as the fact that they might be laborious and risky due to the large amount of solvents required. Additionally, the conventional extraction raises severe problems due to the use of organic solvent and the potential presence of harmful solvent residues in the final extract. Furthermore, heat sensitive active chemicals in the extract may be damaged due to the high temperature operation during traditional extraction (Da Porto & Decorti, 2009; Khan *et al*., 2010; Kimbaris *et al.*, 2006; Adjé *et al*., 2010).

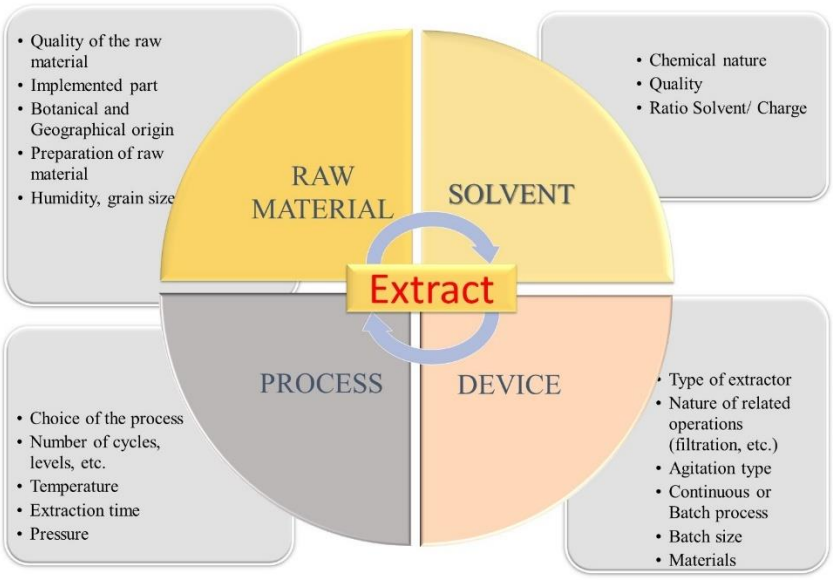


Figure 1.14: parameters influencing extract quality

The process of identifying and characterising bioactive compounds is made more difficult by the fact that plant extracts typically occur as a combination of many different types of bioactive compounds or phytochemicals, each of which has a different degree of polarity (Sasidharan *et al.*, 2011). This makes the separation of these components a particularly difficult task. When isolating these bioactive compounds, it is standard practise to use a variety of different separation methods to obtain pure compounds. Some examples of these methods include thin-layer chromatography, column chromatography, flash chromatography, Sephadex

chromatography, and high-performance liquid chromatography (HPLC). After that, the pure chemicals are put to use for the purpose of figuring out the structure and the biological activity. In addition to this, non-chromatographic methods, such as immunoassays, which make use of monoclonal antibodies (MAbs), phytochemical screening assays, and Fourier-transform infrared spectroscopy (FTIR), may also be used to acquire and make the identification of bioactive substances easier.

Analytical chemistry (Sakodynskii & Chmutov, 1972) is a subfield of chemistry concerned with the separation, identification, and quantification of sample constituents. It is the science of quantitative measurement, which involves a foundational understanding of chemical and physical ideas. Analytical chemistry has traditionally been divided into two primary kinds, qualitative and quantitative, which aim to determine the presence and quantity of a specific element or chemical in a sample, respectively. Modern analytical methods play a crucial role in evaluating the quality requirements of pharmaceuticals.

The pure substance isolated from the mixture and identified using chromatographic and non-chromatographic methods in order to identify the required secondary metabolites. The active components of a mixture are separated and/or identified using chromatographic procedures. Industrial and/or academic chromatographic methods are used to separate and purify the results of various syntheses. There are several chromatography methods, ranging from thin-layer chromatography (TLC) through gas chromatography and liquid chromatography with mass spectrometry (Harborne,1998; Sasidharan *et al*., 2011). Among all analytical procedures, 'CHROMATOGRAPHY' is the technique most commonly used to test the quality of drugs.

1.5 Chromatography

Chromatography is the process of separating a mixture of chemicals into its subcomponents using a mobile phase and a stationary phase (Coskun, 2016; Patil *et al*., 2020). It was initially developed in 1903 by Mikhail Semyorivich Tswett, an Italian-born Russian botanist who was subsequently called the "Father of Chromatography" (Ettre, 1979; Ebere *et al*., 2019). Before the publication of the well-known work by Martin and Synge in the early 1940s, this approach of Tswett remained completely undetected in the literature. They also laid the theoretical groundwork for the fundamental chromatographic process owing to which they were awarded the 1952 Nobel Prize in Chemistry. Next, James and Martin developed gas-liquid chromatography, which was a big stride forward in this discipline. Giddings' 1965 book,

Dynamics of Chromatography, was a major factor in the development of contemporary chromatography because of its detailed study of chromatographic theory. Later, a long list of notable scientists contributed to the creation of what is now known as high-pressure or high-performance liquid chromatography.

The technology is divided into several types based on the separation principle, method geometry, and chromatography mode (Fig. 1.15). The first phase of drug development requires partitioning when a large number of structurally similar molecules are generated. Monitoring and confirming purity is crucial. It is difficult to determine the purity and/or other impurities of synthesised substances without chromatography. Chromatography is crucial in a variety of preclinical and clinical research.

TLC is widely employed for the phytochemical assessment of medicinal plants because it offers quick overview with precise quantification and requires just a minimal amount of solvent. A chromatographic fingerprint of a herbal medicine (HM) is the extract of common chemical components with pharmacologically active or chemical properties. This chromatographic profile should demonstrate "integrity" and "fuzziness" or "sameness" and "differences" to chemically reflect the herbal medicine tested (Shettigar, 2010). It is suggested that chromatographic fingerprints can be used to authenticate and identify herbal medicines accurately (integrity) even if the amount and/or concentration of the chemically characteristic constituents are not exactly the same for different samples of this herbal medicine (hence, "fuzziness") or that they can show both the "evenness" and "differences" between various samples. This necessitates a comprehensive evaluation of the elements of Herbal Medicine extracts, as opposed to a focus on only one or two markers for evaluating product quality. Any herbal medicine and its extract include hundreds of unknown, low-quantity components. Thus, accurate chromatographic fingerprints of pharmacologically active and chemically distinctive Herbal Medicine components are crucial. In TLC fingerprinting, the data that can be recorded using a high-performance TLC (HPTLC) scanner includes the chromatogram, retardation factor (Rf) values, the color of the separated bands, their absorption spectra, λ max and shoulder inflection/s of all the resolved bands. All of these, together with the profiles on derivatization with different reagents, represent the TLC fingerprint profile of the sample.

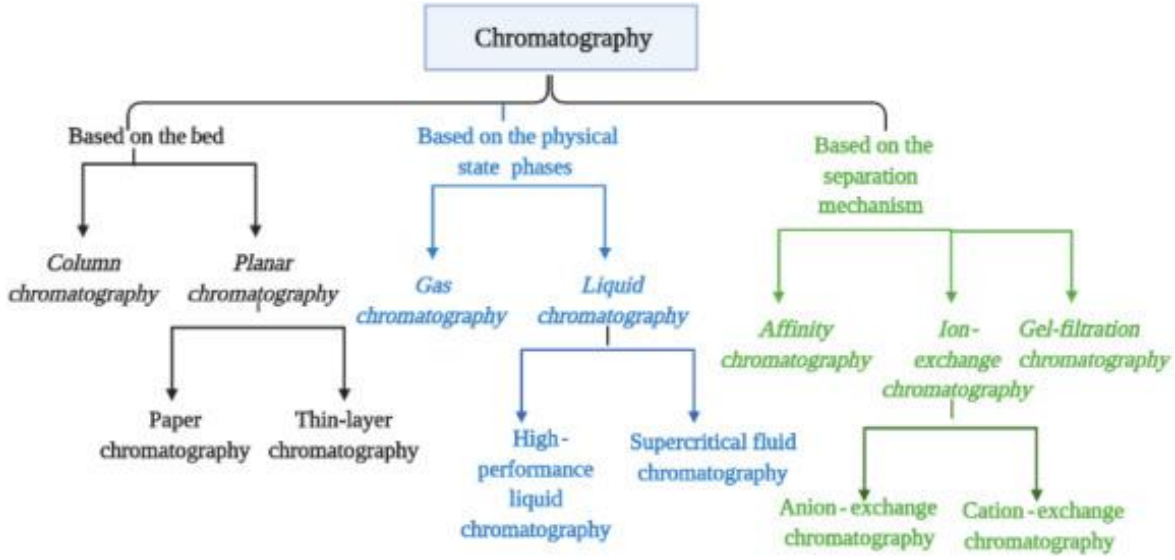


Figure 1.15: Types of Chromatography (Kumari et al., 2022)

1.5.1 High Performance Thin Layer Chromatography (HPTLC)

HPTLC is an advanced instrument approach based on the comprehensive capabilities of thin-layer chromatography. The advantages of automation, scanning complete optimization, selective detection principle, little sample preparation, hyphenation, etc. make it a potent analytical instrument for chromatographic information of complex mixtures of inorganic, organic, and biomolecular compounds.

The improvement in separation and resolution, as well as in resolution speed, is attributable to use of premade HPTLC plates as the stationary phase with optimised adsorbent layers having tiny particle with consistent particle size distribution.

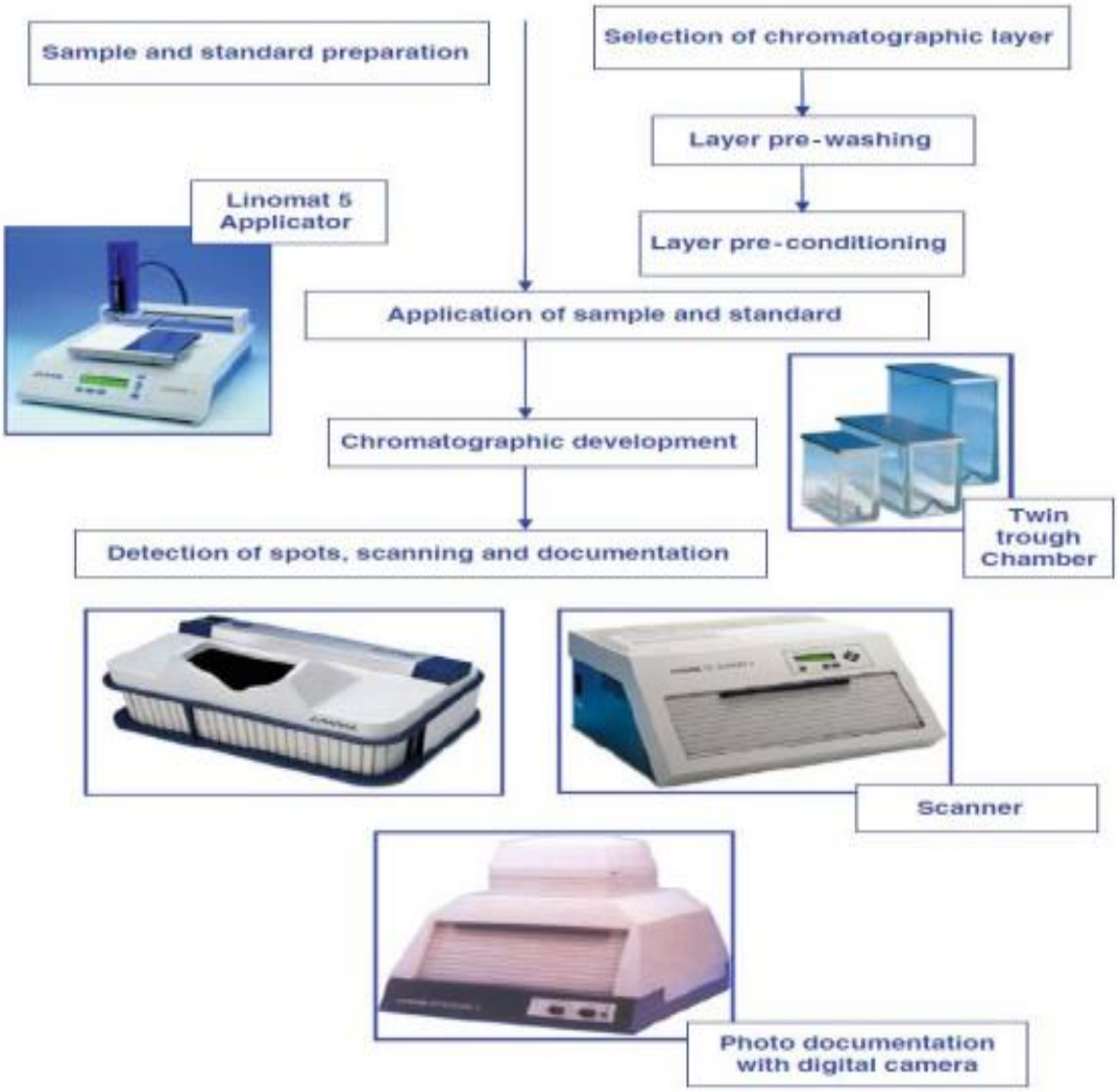


Figure 1.16: Schematic procedure for HPTLC method development

It uses HPTLC plates with tiny, uniform particles. HPTLC plates improve resolution, detection sensitivity, and in situ quantification for pharmaceutical densitometric analysis. NP- HPTLC uses a less polar mobile phase, such as chloroform–methanol. RP-HPTLC uses lipophilic C-18, C-8, C-2, phenyl-modified silica gel phases, and hydrocarbon-impregnated silica gel plates produced with a polar aqueous mobile phase, such as methanol–water or dioxane–water. Plates are not pretreated unless contamination causes chromatographic impurity fronts. Prewashing solvent is usually methanol.

1.5.2 High-Performance Liquid Chromatography (HPLC)

Liquid chromatography refers to the use of a liquid as the mobile phase in this procedure. In liquid chromatography (LC), two non-miscible phases are separated by the difference in

species distribution between them. The mobile phase is a liquid that flows through the stationary phase in a column. Long, wide-diameter glass columns were the standard for early liquid chromatography. Modern technology has helped lower particle sizes to below 10 nm by replacing glass columns with stainless steel ones. Pumps were used to increase the flow rate of the mobile phase, which resulted in an increase in performance. As a result, high-performance liquid chromatography or high-pressure liquid chromatography came to be referred to as "high-performance liquid chromatography" (HPLC).

Macromolecules of biological interest, natural products that may be used, and other less stable and/or high molecular weight substances are excellent for HPLC separation. It is possible to achieve a broad range of HPLC selectivity by using a wide range of distinct column packings (stationary phase). In addition, HPLC provides a broad range of detection techniques since a number of distinct detectors are on the market. With post-column derivatization procedures, HPLC may be extended to trace determinations of chemicals that don't generally yield enough detector response. Gradient flow of mobile phase is often used during method development because it allows for challenging separation to be achieved in a shorter run time. As a result of its automated instrumentation and computation, HPLC provides a significant reduction in human work. Most scientists use HPLC as a primary separation method because of its extensive range of applications in the area of scientific investigation.

There are several parts to a liquid chromatograph: a reservoir for the mobile phase, an injector for introducing samples into the mobile phase, a column for retaining analytes (column temperature control may also be used), a detector for detecting analyte response, and a data collection device like a computer, integrator, or recorder for storing results. Even with the current HPLC system shown in (Fig. 1.17), the degasser with vacuum pump and pre-column facility may be used.

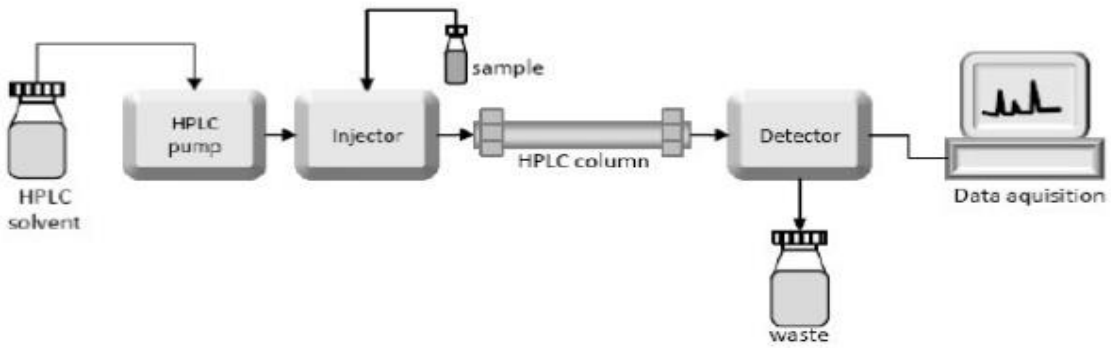
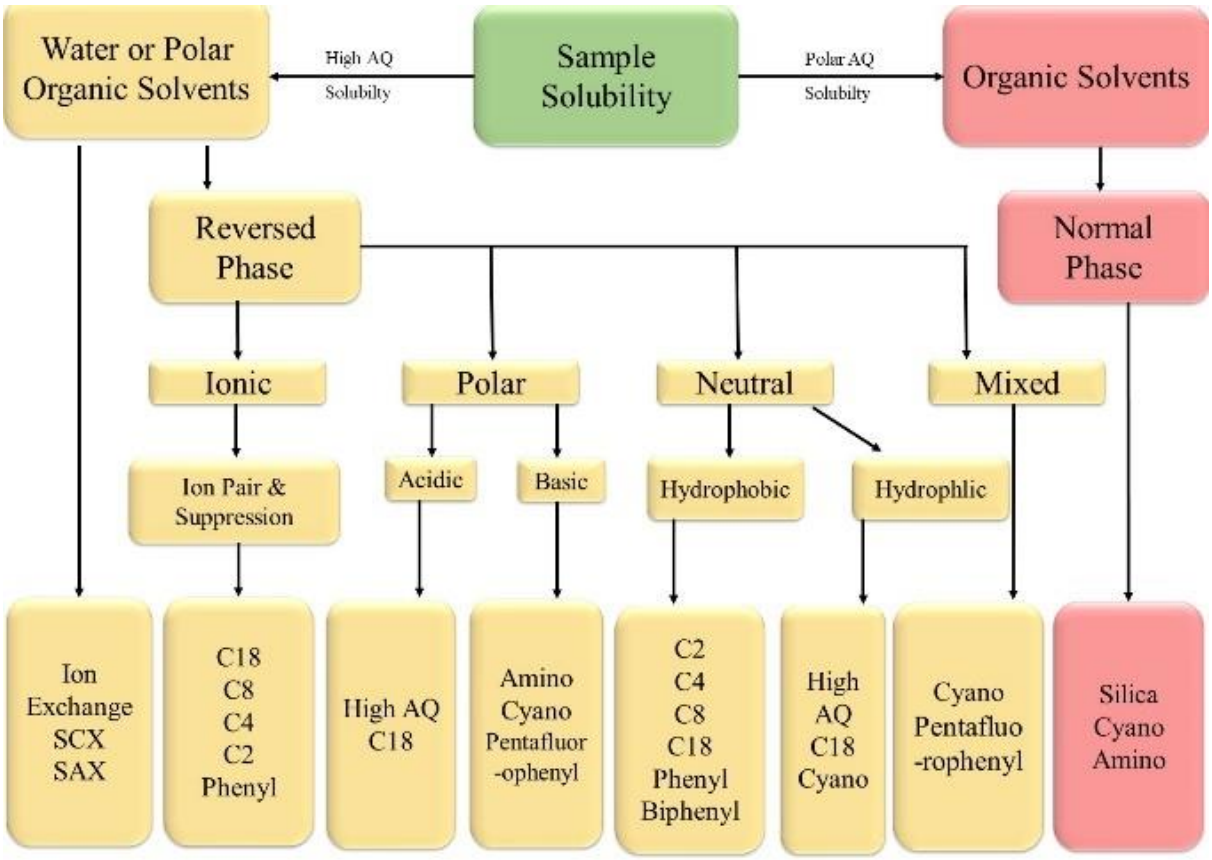


Figure 1.17: Schematic diagram of a typical HPLC instrument setup.

The column or stationary phase is at the heart of every chromatographic device. The Columns are available in a variety of lengths, bore diameters, and packing materials. There are several column dimensions available, including prepared columns, normal-bore columns, micro- and mini-bore columns, and capillary columns. Different column diameters, packing materials, and flow rates may be used for various kinds of separations. For HPLC separations, silica-based packing materials are the most typically used. The most prevalent material is octadecyl-silica (ODS-silica), although it also contains C18 coating aterials with C1, C2, C4, C6, C8, and C22 coatings. The selection of the column is totally depends on the nature of the sample and its polarity (Fig. 1.17).



*SCX/SAX: strong cation/anion exchange

Figure 1.18: Selection of column chemistry depending on sample solubility.

The detector is located at the end of the system. It is responsible for analysing the solution eluting from the column. The concentration of each component of the analyte is proportional to the electrical signal emanating from that component (Bhardwaj *et al.*, 2015; Sharma, 2006). The most often used detectors are UV/Visible spectrophotometers, including diode array

detectors. Depending on the application, several detectors including as UV, PDA, RI, ELSD, FL., ECD, and CAD, are available commercially.

1.5.3 Liquid Chromatography and Mass Spectroscopy (LCMS)

The analytical method Liquid Chromatography-Mass Spectrometry (LC-MS) is a combination of Liquid Chromatography (LC) and Mass Spectrometry (MS) (MS). HPLC (LC) separates mixture components by passing them through a chromatographic column. In most cases, the segregated components cannot be definitely identified by LC alone. Mass Spectrometry is also used for the identification of new and identified substances, as well as the elucidation of their structures. Mass spectrometry alone is inadequate for detecting mixes due to the fact that a mixture's mass spectrum is a complicated overlap of spectra from distinct individual components. It is challenging to link liquid chromatography (LC) with mass spectrometry (MS). Utilizing an interface, liquid eluents are transferred from LC to MS. LC-MS is increasingly used in research of invitro dissolution, bioavailability, bioequivalence, and pharmacodynamics (Lim & Lord, 2002). Rapid mass-directed purification of individual compounds from significant mixtures in fundametal research, pharmaceutical, agrochemical, food, and other sectors is possible using preparative LC-MS systems (Hanai & Hanai, 1999). The LC-MS schematic block diagram is seen in the image below (Fig. 1.19).

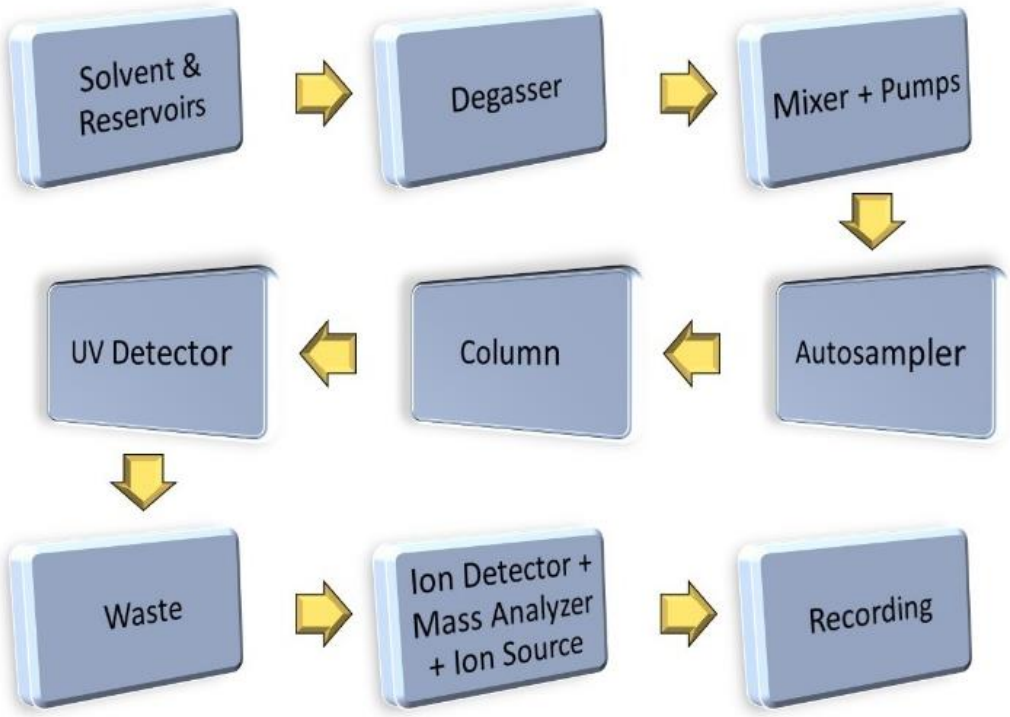


Figure 1.19: Schematic block diagram of LC-MS instrument

Due to the sensitivity and specificity of MS in comparison to other chromatographic detectors, coupling MS with chromatographic methods has long been desired. LC-MS combines the mass analysis skills of MS (identification of chemicals with high molecular specificity and detection sensitivity) with the separation capacity of LC (generation of pure or nearly pure fractions in a chemical mixture). The most potent analytical tool for non-volatile chemicals exists today as a consequence of this orthogonal hyphenation, which offers distinctive answers to a broad variety of structural characterisation difficulties (Loos, 2016). Applications in drug development (discovery, preclinical, and clinical phases), metabolism studies (in vitro and in vivo), and the identification of impurities and degradation products have increased as a result of the continuous improvement of LC-MS interface technologies and the availability of powerful tools for structural analysis (Rao, 2011).

The design and type of the ionisation source have a significant effect on the performance of an LC-MS technique. Electrospray ionisation (ESI) is still the popular ionisation technique in pharmaceutical analysis (Gadepalli *et al*., 2014; Vickers, 2017; De Meulder *et al*., 2016; Ortega *et al*., 2017). For the analysis of thermally labile, non-volatile, and polar compounds, followed by atmospheric pressure chemical ionisation (APCI) and atmospheric pressure photo-ionization (APPI) for nonpolar or less polar compounds with lower molecular weights (Farhan *et al*., 2016; Zheng & Zheng, 2015; Kim *et al*., 2016). Since the majority of small molecule medications are weak bases that are readily protonated, positive ionisation mode is often used in pharmaceutical analysis. Negative ionisation mode may be used for acidic functional groups that readily lose a proton. Several modern equipment can also alter polarity within tens of milliseconds, allowing for the simultaneous investigation of chemicals ionised in multiple polarities (Yusop *et al*., 2016; Beccaria *et al*., 2017; Yamada *et al*., 2015). Despite the fact that APPI is an interesting option to ESI and APCI owing to its application to a wide variety of chemicals, it is not extensively employed because to its low sensitivity (Fig. 1.20). A dopant is often added to the mobile phase to increase the sensitivity of the analysis and boost the ionisation efficiency of the analytes. However, this makes APPI more difficult to use and less flexible. Unlike ESI and APCI, APPI may be utilised in conjunction with normal-phase conditions containing combustible mobile-phase solvents, since there is no explosion risk, as is the case with ESI and APCI. In this regard, it has been shown that some mobile phase solvents (e.g., hexane-based mobile phases) are self-doping, rendering the insertion of dopants unnecessary18. As an alternative to ESI, APCI, and APPI, Waters Corporation recently introduced a new atmospheric pressure ionisation source called UniSpray. Another ionisation

addition was Electrospray Ionization Inlet (ESII) and it performs better at low flow rates, whereas Unispray is primarily advantageous at higher flow rates (Fenner *et al.*, 2017).

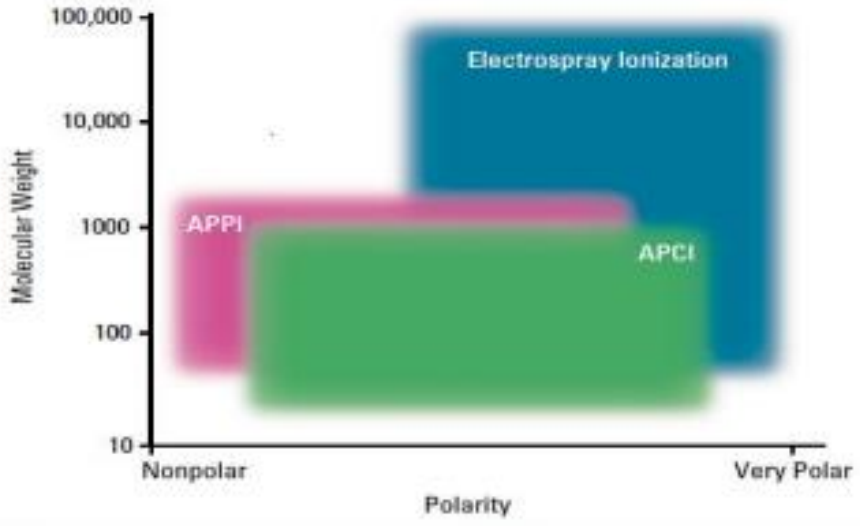


Figure 1.20: Applications of different LC/MS ionisation methods

In pharmaceutical analysis, the most common kinds of mass analyzers include ion trap (IT), quadrupole (Q), Orbitrap, and time-of-flight (TOF) instruments, as well as a variety of hybrid instruments with a high resolution, higher sensitivity, and/or increased mass accuracy across a broad dynamic range. Among them, the triple quadrupole (QQQ), quadrupole TOF (Q-TOF), ion trap Orbitrap (ITOrbitrap) and quadrupole-Orbitrap (Q-Orbitrap)35 are widespread (Lin *et al.*, 2015).

A mass spectrometer is more sensitive and selective than all other LC detectors for the majority of substances. It is capable of analysing chemicals without a sufficient chromophore. It may also detect components in unresolved chromatographic peaks, hence eliminating the necessity for flawless chromatography. Mass spectrometers function by ionising molecules and classifying and identifying ions based on their mass-to-charge (m/z) ratios. The ion source, which produces the ions, and the mass analyser, which classifies the ions, are two essential components of this process. Several distinct kinds of ion sources are often used in LC/MS.

1.6 Analytical Method Validation

Quality of pharmaceutical products can be assessed by using a newly developed analytical procedure. It is necessary to ensure that the developed analytical procedure's performance characteristics meet the requirements for the analytical application intended for use. Method validation refers to the process of using laboratory tests to verify that the results are accurate.

In order to demonstrate that analytic processes are appropriate for their intended application and assist the identification, strength, and quality of pharmaceutical substances and products, method validation is carried out. The literature, industry groups, and government bodies have all given method validation a lot of attention. Analytical Procedures and Methods Validation Industry Guidance was also proposed by the FDA in the United States.

A section of ISO/IEC 17025 deals with the validation of techniques using a list of validating criteria. Consensus statement on validation of analytical processes has been developed by the ICH In addition, ICH produced recommendations that included specific methods. A plan for testing and verifying procedures.

An iterative approach to method development and validation is necessary. The validation step provides an opportunity to examine how operational parameters affect the method's performance, something that was not possible during the method's development or optimization stages. The most important issue presented in the validation process is that only laboratory research can verify a method's validity. Rather of relying only on previous research, it is necessary to undertake new laboratory tests aimed at validating the approach under consideration. These new investigations should be well-planned and documented. All of this information should be laid out in a way that clearly explains the method's intended usage and functioning, as well as its validation criteria and the reason for selecting them. Acceptance criteria and an explanation of the analytical technique must also be included.

1.6.1 Parameters for Method Validation

ICH Q2(R1) presents a formal summary of the requirements necessary for the complete validation of an analytical technique (ICH, 2005). Method validation is a statutory requirement of the three ICH regional regulatory agencies (FDA, EMA (European Medicines Agency), PMDA (Pharmaceutical and Medical Device Agency)) and other international and transnational organisations (e.g., BRIC (Brazil, Russia, India, China), ASEAN (Association of South East Asian Nations), and WHO). The method validation language and accompanying definitions used in ICH Q2(R1) were necessary to "bridge the gaps" between the several national pharmacopoeias, compendia, and these three regulatory organisations (ICH, 2005). While ICH Q2(R1) describes the validation requirements applicable to a restricted number of techniques used for identification, assay, impurities, and limit tests for impurities, it was always the intention of the writers of this guideline that it would also apply to additional analytical processes (Borman & Elder, 2017). The following is a summary of the ICH and other regulatory organisations' established validation parameters.

1. Specificity study
2. Linearity and range study
3. Limit of detection and Limit of quantitation study
4. Precision study
5. Accuracy study
6. Robustness study
7. Solution stability study
8. System suitability

1.6.2 Specificity study

Specificity is defined by ICH as "the capacity to evaluate unambiguously the analyte in the presence of components that may be anticipated to be present." This may typically consist of contaminants, degradants, matrix, etc." USP1225> refers to the same definition but also notes that IUPAC and AOAC use the word "selectivity" with the same meaning. This restricts the usage of the term "specific" to techniques that give a response for a single analyte. Because it needs a technique to be "selected" rather than "specific," ISO/IEC probably shares the same concept. Our objective is to differentiate and measure the reaction of the target chemicals relative to all other molecules. Selectivity is the capacity to precisely and specifically measure the analyte in the presence of other components that may be present in the sample matrix. Specificity for an assay guarantees that the signal evaluated originates from the substance of interest, with no interference from excipient, degradation products, or contaminants. This may be determined by analysing the identity and purity of the peak.

1.6.3 Linearity and Range Study

The capacity of an analytical procedure's test findings to be directly proportional to the concentration (amount) of analyte in a sample is known as its linearity. This refers to the method's capacity to provide findings that are directly or mathematically proportionate to the concentration of the analyte in a certain range. Analyte concentration is used to calculate the regression line, which is then compared to that line to evaluate if it is linear or not. Peak area vs analyte concentrations were used to create a calibration curve.

It has been shown that an analytical technique is accurate, precise, and linear up to and including a range of analyte concentrations in the sample. This range is called the analytical procedure's range. As with the test findings, range is often given in the same units. In an assay test, the ICH specifies a minimum concentration range of 80 to 120 percent.

1.6.4 Limit Of Detection (LOD) and Limit Of Quantitation (LOQ) Study

Detection limit is the lowest quantity of analyte that can be detected but not necessarily quantified as an exact value by an individual analytical method. The formula used to compute LOD (limit of detection) was:

LOD = 3.3 x σ/S Where,

σ = Standard deviation, S = slope of the regression equation

The lowest quantity of analyte in a sample that can be quantitatively measured with acceptable precision and accuracy is the quantitation limit of a particular analytical method. Low-level chemical quantitation limit is a key quantitative test parameter used to identify contaminants and/or degradation products in sample matrices.

LOQ = 10 x σ/S Where,

σ = Standard deviation of response, S = Slope of regression equation.

1.6.5 Precision Study

The precision of an analytical technique is the degree of agreement (dispersion) between a series of measurements obtained from successive samplings of a homogenous sample under specified circumstances. Replicate standard preparations and sample preparations are used to determine the accuracy of an analytical procedure. LOQ findings are often reported as variance, standard deviation, or level of confidence for a set of measurements. Repeatability, reproducibility, and intermediate precision serve to achieve precision (ruggedness). Repeatability describes the accuracy under same operating circumstances over a short period of time. Repetition is sometimes referred to as intra-assay precision. Reproducibility demonstrates the consistency across labs. By testing aliquots from the same homogenous batches, the repeatability of an analytical procedure is assessed. Intermediate precision reflects differences within a laboratory; various days, different analysts, different equipment, etc. The purpose of intermediate precision validation is to confirm that the same findings will be obtained in the same laboratory after the development phase is complete. In addition, the purpose is to confirm that the approach will provide same findings in various labs (ruggedness).

1.6.6 Accuracy Study

Accuracy in an analytical technique may be expressed as the degree to which the value acknowledged as a conventional true value or a conventionally accepted reference value and the value discovered are in agreement. True value and test results provided by the procedure are sometimes referred to as accuracies. To ensure accuracy, the ICH publication on validation

methodology advises a minimum of nine measurements at a minimum of three concentration levels, each with three replicates, to cover the necessary range. Percentage recovery by assay of a known quantity of standard in the sample or difference between mean and recognised actual value, together with confidence intervals, should be used to describe accuracy.

By conducting recovery tests utilising standard addition at three distinct levels (80%, 100%, and 120%), the method's accuracy was evaluated. Known quantities of analyte-containing Standard solutions were added to pre-quantified sample solutions in order to achieve 80,100, and 120 percent concentrations, respectively. Percent recovery was estimated for these samples by injecting a sample solution into them. The following formula was used to figure out the percent recovery in this investigation.

$$\frac{\text{Area of Spiked Sample} - \text{Area of Unspiked Sample}}{\text{Area of Standard}} \times \frac{\text{Conc. Of Standard}}{\text{Conc. of Added Drug}} \times 100$$

Acceptance limit for % recovery is 98-100%

1.6.7 Robustness Study

The robustness of an analytical technique is a measure of its ability to stay unaffected by tiny, but intentional, modifications in method parameters and is indicative of its dependability under regular conditions of use. Tests of robustness assess the influence that operational factors have on the outcomes of an analysis. For the purpose of determining a technique's robustness, method parameters such as pH, flow rate, column temperature, column lot, and mobile phase composition are adjusted within a realistic range, and the quantitative effect of the variables is determined. If the effect of the parameter falls under a previously determined tolerance, the parameter falls inside the robustness range of the procedure.

1.6.8 Solution Stability Study

Prior to chromatographic studies, many solutes easily break down, for instance, during the preparation of the sample solutions, extraction, cleaning, phase transfer, or storage of prepared vials (in refrigerators or in an automatic sampler). In these cases, the stability of the analytes and standards in solution form should be examined during method validation (in analytical preparations). The standard and test preparations are kept for the designated duration at the designated temperature, and their stability is assessed by contrasting solution preparations made at various intervals with those made initially.

1.6.9 System Suitability Study

In addition, prior to beginning laboratory investigations to verify method validity, some sort of system appropriateness must be performed to show that the analytical system is operating correctly. The system's appropriateness should be evaluated by analysing the standard or reference solution in duplicate. When the RSD, theoretical plates, tailing factor, and resolution parameters computed on the data acquired at various time intervals do not exceed the prescribed limit for the associated system precision value, the system is deemed suitable.

Table 1.1:System Suitability Test Parameters and Recommendations

Parameters	**Recommendation**
Theoretical Plates (N)	should be > 2000
Relative retention(k")	The peak should be well-resolved from other peaks and the void Volume, generally k">2.0
Relative retention	Not essential as long as the resolution is stated.
Resolution (Rs)	Rs of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard) etc.
Tailing Factor (T)	T of </=2

1.6.10 Stability Indicating Assay Method

Patients using a pharmaceutical treatment for a certain ailment anticipate that the medicine will be both safe and effective. Pharmaceutical regulatory bodies across the globe require that the product maintains its identity, quality, purity, and efficacy during its commercial availability. Therefore, the agencies anticipate to see stability evidence supporting the suggested expiry date in the marketing proposal. Therefore, stability studies are required to forecast, assess, and assure drug product safety. In accordance with ICH criteria, drug substance stability tests including acid hydrolysis, base hydrolysis, oxidation, and thermal and photolytic stress testing are a component of the development approach. These investigations give information on the intrinsic stability of a medicine and assist confirm the analytical procedures used to evaluate its stability. Currently, stability tests are being developed using the stress testing methodology of the ICH guidelines, Q1A[R2]. The method has been expanded to include medication combination stress testing. These techniques provide precise and reliable measurement of medicines, their degradation products, and their interaction products.

Table 1.2: Important validation parameters suggested by regulatory agencies

Parameters	ICH	USP	ISO 17025
Specificity	X	X	-
Selectivity	-	-	X
Precision	-	X	-
Repeatability	X	-	X
Intermediate precision	X	-	-
Reproducibility	X	X(Ruggedness)	X
Accuracy	X	X	X
Linearity	X	X	X
Range	X	X	-
Limit of detection	X	X	X
Limit of quantification	X	X	X
Robustness	X	X	X
Ruggedness	X	X	-

1.7 National and International Scenario of Herbal Market

The future of medicine is anchored in the past, before scientists attempted to create synthetic silver bullets for all diseases and before pharmaceutical firms tethered our collective health to what has become a multibillion-dollar cash cow for them. In the past, almost all medications were derived from plants; plants were man's sole chemist for generations. Herbal 'renaissance' is occurring all over the world, and more and more people are becoming aware of herbal remedies for treating a variety of conditions. There is a rising concern about the dependency and safety of pharmaceuticals and surgery, which is the key reason for the popularity of herbal remedies. Furthermore, many of the most prevalent health issues are not being successfully treated by contemporary medicine. Finally, several natural methods are proving to be as effective as, if not more so than, pharmaceuticals and surgical procedures, without the negative consequences associated with their use.

The term ‘Nutraceutical’ refers to foods or extracts claimed to have a medicinal effect on human health. Nutraceutical products as substitute for synthetic vitamins, minerals and other nutrients were further accentuated by the emergence of ‘antioxidants’ as a class of health products (Devi *et al*., 2010).

The structure of this industry varies according to the particular medicines being produced, but typically there are several stages: collection from the wild or cultivation, followed by the purchase of materials by exporters, importers, wholesalers, brokers, or traders. Materials may

then be tested for contamination, powdered, or extracted by processing companies or by manufacturers of the finished products. These may then be handled by specialized distributors before retailing to consumers. Revenues from these products can be very large. For example, annual sales of medicinal Ginkgo, Garlic, Evening Primrose, and Echinacea in Europe average $350 million (Kate & Laird, 1999). The global sales of raw botanical materials by leading U.S. suppliers amount to approximately $1.4 billion (Kate & Laird, 1999).

Herbal medicines, health products, pharmaceuticals, nutraceuticals, food supplements, cosmetics, etc., are becoming increasingly popular around the world because of their low cost, little or no toxicity, and compatibility with the body's natural ecosystem (Dubey *et al*., 2004; Sharma *et al.,* 2008). Similarly, the rise of new commodities such as health meals, natural cosmetics, and hygiene products, among others, enhanced the demand for herbal products. In the year 2000, the worldwide market in medicinal plants and their products was valued at US$ 60 billion, with an average annual growth rate of 7%, and it was anticipated to reach US$ 5 trillion by 2050 (Govt. of India, 2000). China and India are the major exporting nations, whereas Hong Kong, Japan, the United States, and Germany are the biggest importing nations (Jadhav *et al.,* 2020). Following terpenoids, glycosides were the second most popular class. In terms of income, flavonoids, saponines, anthraquinones, and digitalis chemicals were among the most valuable classes (Jadhav *et al.,* 2020). Germany, France, Italy, and Switzerland are home to the vast majority of enterprises specialising in herbal medications. Some are over a century old, and the majority are still privately owned. In Europe, major pharmaceutical corporations have acquired smaller herbal enterprises. Examples include Boehringer Ingelheim (Sweden), Lederle (Australia) and Schering (Belgium and Hungary).

India's ancient traditional medicine systems include the Rigveda, Atherveda, Charak Samhita, and Sushruta Samhita texts. Traditional (tribal) remedies are also essential to the indigenous healthcare system. Historically, India has been recognised as a trove of medicinal herbs from ancient civilizations. India's woods are the primary source of an abundance of medicinal and fragrant plants (De *et al*., 2010; Kamboj, 2000; Mukherjee, 2008). The study of medicinal plants, the separation of bioactive ingredients, and pharmacological screening may assist in the discovery of novel therapeutically useful medications. It is believed that there are around 6000 higher plant species included in codified systems such as Ayurveda, Siddha, and Unani traditions as well as in folk medicine of India, comprising approximately 40% of the country's total higher plant variety (Ved and Goraya, 2008). India is on the cusp of a herbal revolution and is equipped to produce medicinal plant resources to fulfil the rising global demand due to

its abundant supply of herbs. According to Volza's India Export statistics (volza, 2022), 948 shipments of medicinal plants were shipped from India by 58 India Exporters to 140 Buyers, including Premier Herbal Inc., Phytomed Medicinal Herbs Ltd., and Allied Express International. India sells the majority of its medicinal plants to Canada, New Zealand, and the Philippines, and is the world's second-largest exporter of medicinal plants. The top three exporters of medicinal plants are the United States (1,183 shipments), India (1,072 shipments), and China (734 shipments). The top three categories of medicinal plant exports from India are classified under the Harmonized System (HS) codes are 12119029 (Oil Seeds and Oleaginous Fruits; Miscellaneous Grains, Seeds and Fruit; Industrial or Medicinal Plants; Straw and Fodder), 12119099 (Plants And Parts Of Plants used primarily in perfumery, pharmacy or insecticidal, fungicidal or similar purpose) and 12119022 (Plants And Parts Of Plants used primarily in perfumery, pharmacy or insecticidal (leaves powder flowers and pods: senna leaves and pods) (Volza, 2022).

1.7.1 National and International Status of Licorice

Licorice is a traditional medicinal plant used worldwide for both food and medicine. In addition, owing to the properties of its natural products, licorice is extensively utilised in food, cosmetics, and the light industry, and has grown popular globally (Hayashi and Sudo, 2009). Licorice (Glycyrrhiza Linn.) is a perennial plant widespread across the globe, particularly in northwest China, Central Asia, and West Asia (Brinckmann, 2020). Licorice is primarily used in the domains of medicine, food, and cosmetics on a global scale. As the different pharmacological benefits of licorice are steadily validated, it is increasingly employed to treat a variety of disorders. Previous research has demonstrated that flavonoids in licorice extracts have anticancer properties (Zhang *et al*., 2021); 6″-O-acetylliquiritin may be a shortlisted compound for antithrombosis (Yi *et al*., 2017); liquiritigenin has the effect of lowering blood glucose levels (Carnovali *et al.*, 2019); licochalcone A has been shown to improve obesity and non-alcoholic (Sultan *et al*., 2014). In the food sector, the use of natural powerful substances boosts the perceived safety of goods relative to synthetic additions (Aziz & Karboune, 2018). Glycyrrhizinic acid is used as a sugar substitute, antioxidant, antibacterial agent, and flavour booster, among other applications (Iida *et al*., 2007). In addition, licorice extracts are frequently utilised in skin care products due to their anti-bacterial, anti-inflammatory, brightening, and anti-allergic activities (Kolbe *et al*., 2006; Lv *et al*., 2020; Sulzberger *et al*., 2016). However, as more applications for licorice are found, the world's demand for licorice is also expanding, and a supply crisis has formed for licorice resources

(Bailly and Vergoten, 2020; Li *et al*., 2019; Wang *et al*., 2019). There are around 20 species in the genus Glycyrrhiza (Committee and others, 2018), however the three most often utilised species are as follows: *Glycyrrhiza uralensis* Fisch., *G. inflata* Bat., *G. glabra* L. (N. P. Committee, 2020). The majority of licorice is found in Eurasia, with the highest concentration in central Asia. In addition to China, the Middle East, Central Asia, and Eastern Europe also possess rich licorice resources (Han *et al*., 2022).

As seen in figure 1.21, the supply and demand chain for licorice extracts has been generally consistent for a long time. In 2019, Afghanistan to India, Uzbekistan to China, the United Arab Emirates to China, and Iran to Germany are the primary trade flows for licorice extracts. Afghanistan, France, Uzbekistan, United Arab Emirates, and Germany are the top five exporting nations of licorice extracts in 2019, accounting for 49.95 percent of the total global export value. It comprises fifty percent of the world's licorice extracts. Afghanistan led the list of exporting nations, accounting for 27.65% of world commerce. When reviewing the leading importers of licorice extracts, it was determined that Germany, China, the United States, the United Arab Emirates, and France accounted for 62.04% of the global import value (Han *et al*., 2022) (Fig. 1.21; 1.22 (A &B).

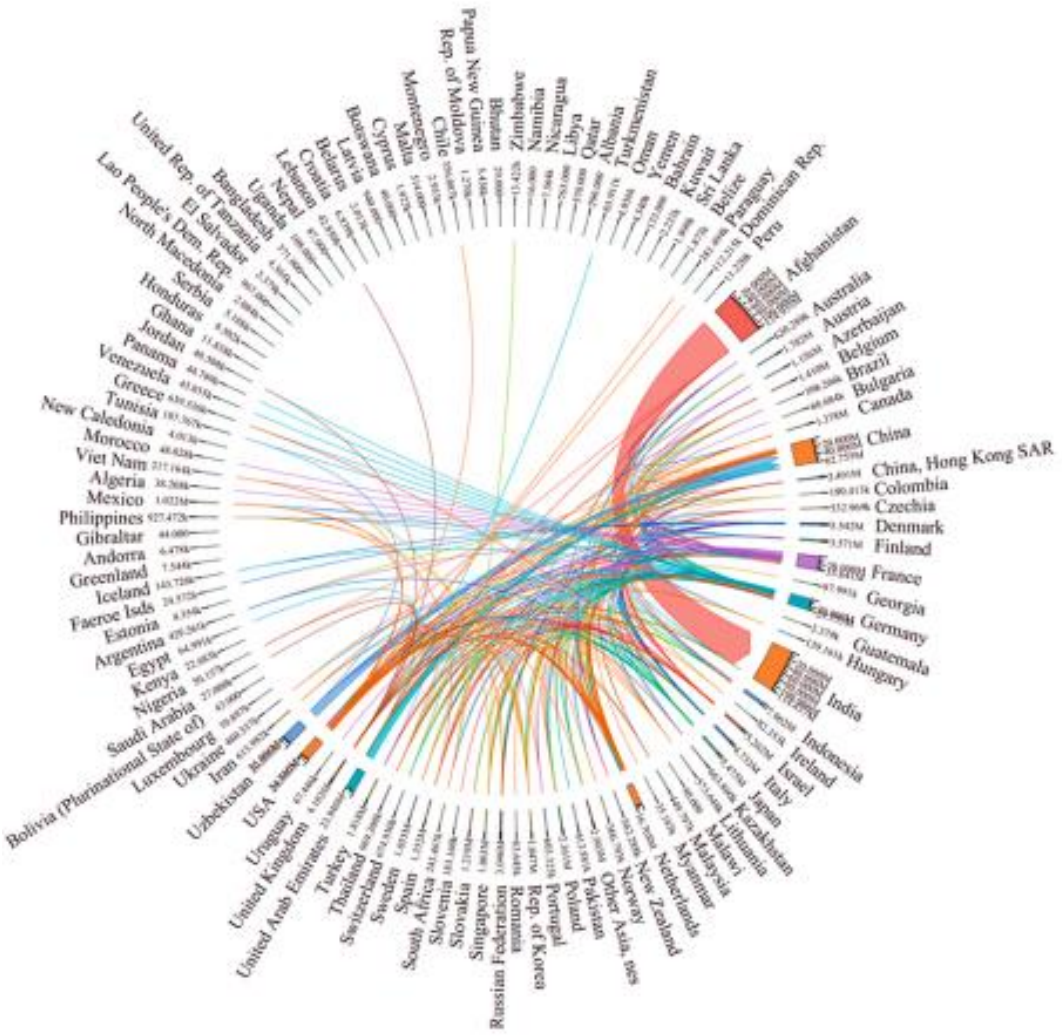
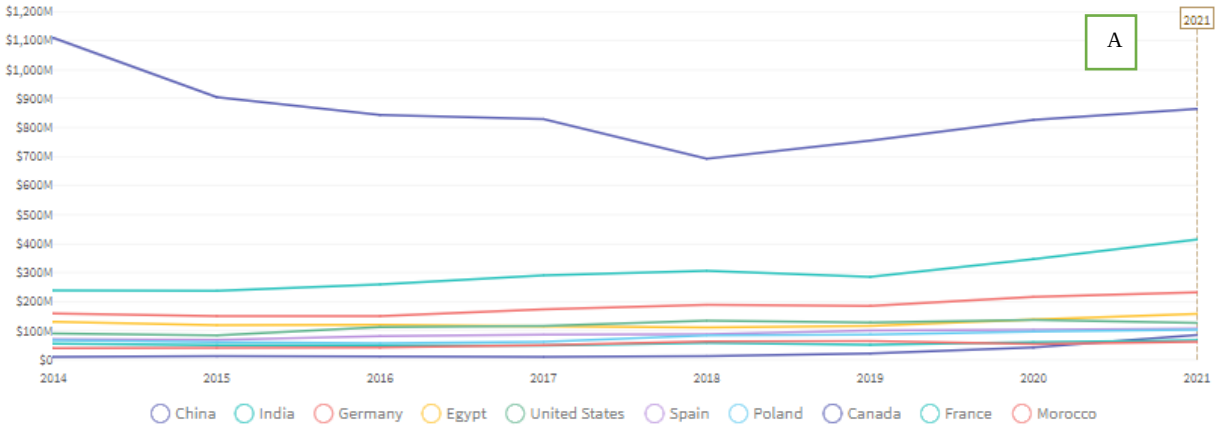


Figure 1.21: Global licorice extract trade flow chart in 2019



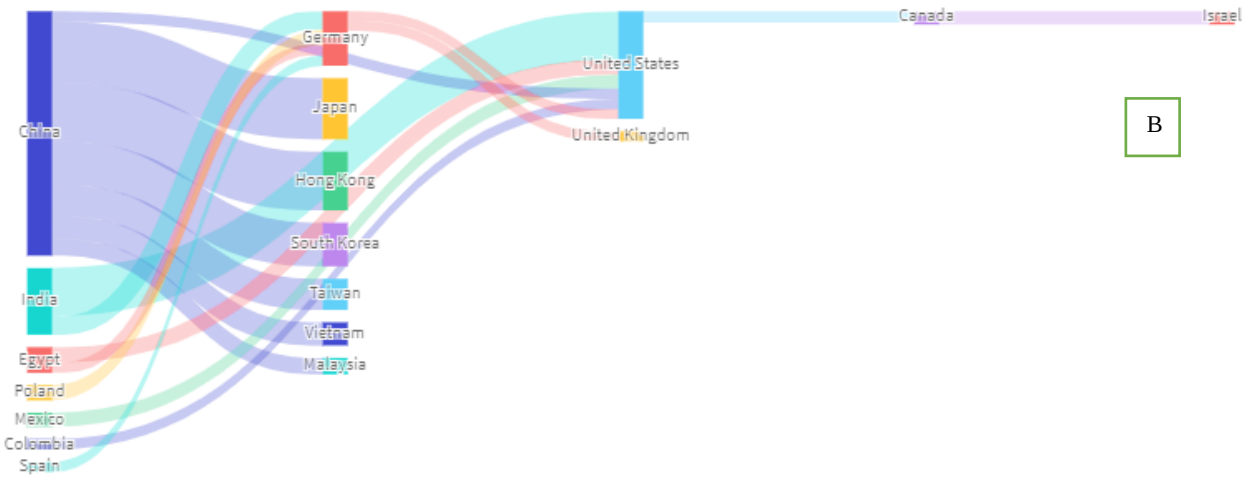


Figure 1.22 A & B: National Scenario Figure: top export flows of Licorice Root in 2021

The top export flow in 2021 was from China to Japan, with an export value of USD 177.89M

Table 1.3: Last 5 years major licorice export data

Trade Flow	Export Value 2021, USD	1-Year Growth in Export Value 2020-2021	3-Year Growth in Export Value 2018-2021	5-Year Growth in Export value 2016-2021
China to Japan	$177.89M	3.79%	1.25%	8.68%
China to Hong Kong	$170.69M	3.52%	55.41%	-41.30%
India to United States	$140.15M	25.36%	43.46%	55.99%
China to South Korea	$126.67M	7.20%	27.59%	5.95%
China to Taiwan	$90.56M	10.85%	30.50%	104.28%
China to Vietnam	$67.24M	29.89%	214.86%	239.90%
India to Germany	$53.76M	19.02%	49.84%	77.08%
China to Malaysia	$47.70M	-7.88%	20.61%	24.48%
Egypt to United States	$43.95M	6.18%	36.90%	41.00%
Poland to Germany	$43.74M	6.16%	5.68%	67.03%

The most recent information from tridge.com reveals that China is the largest exporter of licorice, with an export value of 3.39 billion US dollars, while the United States is the largest importer, with an import value of 3.52 billion US dollars (Table 1.3 & 1.4).

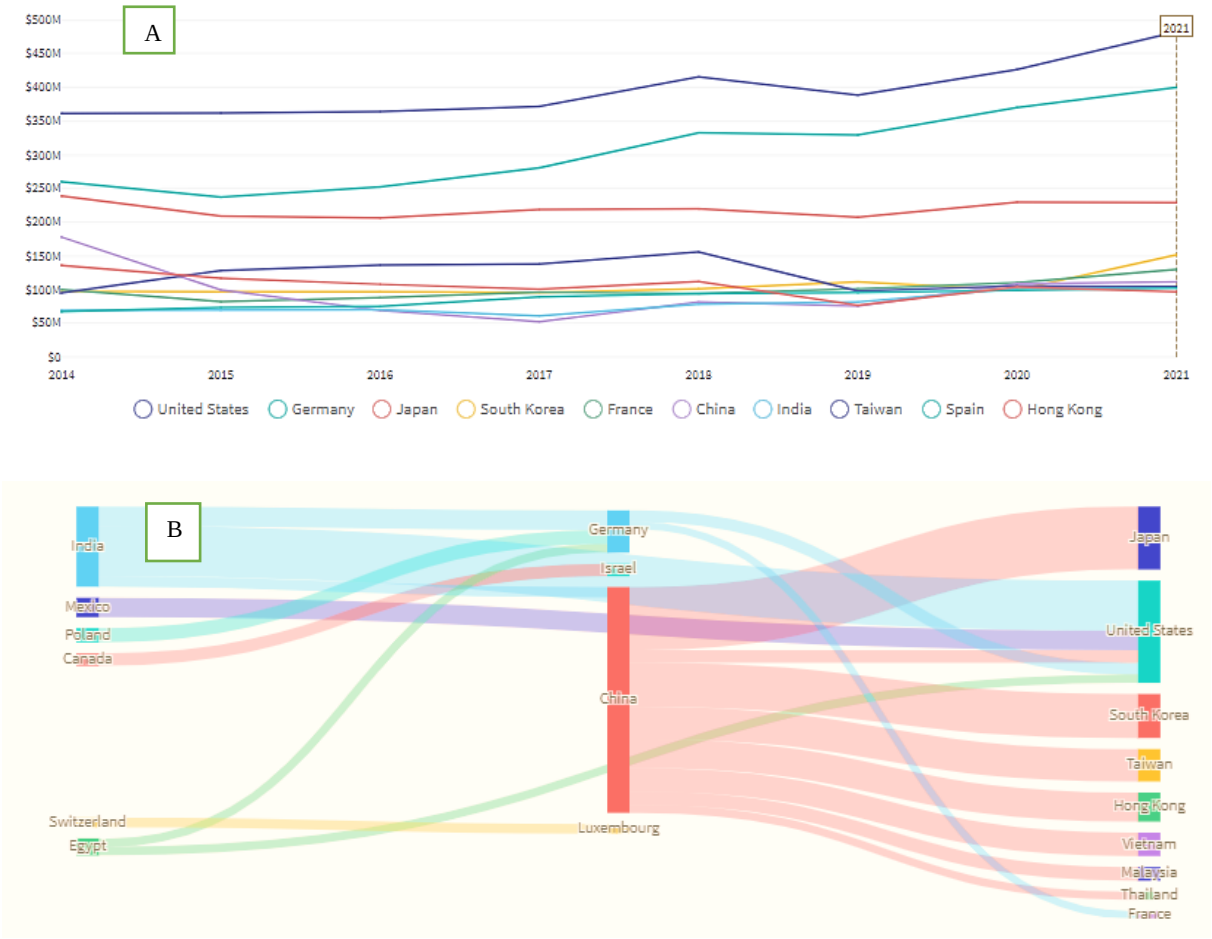


Figure 1.23A & B: top import flows of Licorice Root in 2021. The top import flow in 2021 was from Japan to China, with an import value of USD 181.07M

Table 1.4: Last 5 years major licorice import data

Trade Flow	Import Value 2021, USD	1-Year Growth in Import Value 2020-2021	3-Year Growth in Import Value 2018-2021	5-Year Growth in import value 2016-2021
China to Japan	$181.07M	5.31%	6.95%	14.29%
India to United States	$144.41M	30.48%	47.36%	65.09%
China to South Korea	$126.67M	71.30%	78.82%	73.48%
China to Taiwan	$93.05M	-3.80%	-17.45%	-4.65%
China to Hong Kong	$83.52M	-11.43%	11.03%	28%
China to Vietnam	$67.24	33.97%	175.12%	328.80%
India to Germany	$56.42M	34.62%	47.97%	98.41%
Mexico to United States	55.61M	1.38%	30.34%	44.58%
Poland to Germany	$40.73M	-5.08%	25%	54.44%
China to Malaysia	$38.77M	1.01%	-15.90%	-21.16%

However, wild licorice resources have been taken indiscriminately in recent years due to commercial interests and rising demand. This has led to a progressive deterioration in the resource's overall amount and quality. As wild licorice is regularly picked, the population stability of wild licorice is endangered (Zhou *et al.*, 2020). In such a circumstances, *Taverniera cuneifolia*, a native plant, is showing promise as a prospective alternative for *G. glabra*. *Taverniera cuneifolia* (Roth) Ali is an ethnobotanically important traditional medicinal plant of semi-arid region of India belonging to the family of Fabaceae. It is known for its sweet component from the roots which is similar to that of G. glabra (Zore, 2008). Taverniera DC. genus consists of 17 species distributed in African, Middle east and Asian countries, out of which two species occur in India (Mangalorkar, 2013). Native people of these region use this plant for various purposes such as traditional as well as folk medicinal usages because of its potential medicinal properties. The study of literature on Taverniera genus has revealed the presence of some leading phyto-molecules such as Saponins, Phenols, Terpenes and Glycosides. So far, total 117 compounds were identified and isolated, which includes terpenoids, flavonoids, triterpenoid saponins, phenolic acids, saponins, organic acids, fatty

acids, amino acids, sugars, vitamins, saikosaponins and sterols. Out of these, flavonoids and terpenoids were the most prominent. The pharmacological activities shown by various Taverniera species are Anti-carcinogenic, Anti-HIV, Anti-inflammatory, Antimicrobial, Analgesic and Antipyretic, Antioxidant, Antitussive, Gastroprotective, Memory enhancer, Nematicidal, Anti-dementia, Spasmolytic, Wound healing properties (Khalighi *et al*., 2014; Alexiades and Laird, 2002; Zore *et al*., 2008; Keymanesh *et al*., 2009; Dagne *et al*., 1990; Jamdhade *et al*., 2015; 2013; Heba 2016; Salah *et al*., 2016; Prajapati & Patel, 2015; Noamesi *et al*., 1990; Mangalorkar, 2013).

In the present thesis, an effort has been made to standardized and validate the active phytoconstituents present in the roots of Taverniera cuneifolia. The qualitative and quantitative analysis was done for various phytoconstituents along with the complete validation of the sugars. The seasonal and geographical variations of the sugars have been done by HPTLC analysis. The LCMS/MS analysis of the sweet component i.e. Glycyrrhizin has also been covered in this thesis. Various medicinally important phytoconstituents analysis such as liquirtigenin, Naringenin, Glabridin, Kaempferol, Quercetin etc. is done by using LCMS instrument.

1.8 Hypothesis

T. cuneifolia has been stated as a substitute of G. glabra. However, when it comes to validation of active compound in *T. cuneifolia* with that of *G. glabra*, the presence of Glycyrrhizin, Glabridin, Naringenin, Apigenin, 18 alpha Glycyrrhetinic acid, 18 beta Glycyrrhetinic acid, Kaempferol, Liquiritigenin, Stigmasterol, Beta Sitosterol is always a dogma whether *T. cuneifolia* could be used as substitute of *G. glabra* or not.

Objectives of the present research work: on the basis of literature analysis, the objectives of research work are as follows:

1.9 Objectives

- Selection and segregation of appropriate germplasm from different biogeographical zones of Gujarat.
- Standardization and validation of sugars (sweeteners) and amino acids.
- Standardization and validation of active phyto-constituents of *T. cuneifolia.*
- Purification of the active fractions by Column chromatography.