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Review Article

Biocompounds from Medicinal Plants

Review On Isolation Methods of Bio-Active Compounds from Traditional Medicinal Plants

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Abstract: Herbal plants are very important in traditional use and enhance our plant biodiversity. According to the World health Organization (WHO), more than 80% of world's population depends on traditional medicine for their primary health care needs. The sequence of Extraction and analytical technique for isolating and characterizing the bioactive compounds from plants, act as potential Lead compounds in the drug exploration process. A Broad range of Bioactive chemical compounds have been acquired from the medicinal plants as pure form (or) as Homogeneous Extract. Some plants having the medicinal value, which contain in form of chemical substances that produce a specific biological action on the Human body are called Phytochemicals. Phytochemicals in the herbal extracts for traditional use include a wide variety of bioactive metabolites of pharmaceutical and pharmacotherapeutic nature and many phytomedicines for various therapeutic areas. So, High Through put Screening programs seeking new leads in phytochemical research are gaining popularity. Today, it is very crucial to develop effective and selective methods for the isolation of those phytochemicals. The focus of the this review article includes, the brief elucidation of various Isolation techniques such as Preparative TLC, Preparative HPTLC, Preparative HPLC, Column chromatography, counter current chromatography, gas chromatography, preparative gas chromatography etc are employed in the plant extract in which the Phytochemicals compound are present and some structural examples of compounds separated using this techniques were also provided. In this article, some non chromatographic separation methods such as acid basic solvent method, dialysis method, crystallization method etc also were provided. In this article various types of Solvent System used in chromatography techniques also were given. This review covers on the various types of isolation methods of compounds from plant extracts and the various type of solvent systems used in chromatography techniques that where missed by some early articles. This review paper gives an idea about the research of those isolate compounds from the medicinal plant extracts and also gives idea about the various solvent systems used in the chromatography techniques.

KEYWORDS: Chromatography, isolation, spectroscopy.

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1. INTRODUCTION

For the wellbeing of individuals and populations, medicinal plants are of great importance. Some bioactive compounds that produce a definite physiological effect on the human body are of medicinal value to these plants. Plants have formed on the Basis of advanced conventional therapeutic systems that have existed for thousands of years and continue to give new remedies to humanity. The increasing presence of natural medicines in modern medicine demonstrates the importance of plants in human and animal health. Indeed, in the world today, natural products and their derivatives account for more than 50 percent of all medications in clinical use.[1] Natural products, such as Plant Extracts, either as pure compounds or as standardized extracts, offers unlimited opportunities for New Drug Discoveries because of the unmatched availability of chemical diversity. According to the World Health Organization (WHO), more than 80% of the world's population relies on traditional medicine for their primary healthcare needs. The use of herbal medicines in Asia reflects a long history of Human Interactions with the Environment.[2] The traditional knowledge of medicinal plants if harnessed, can give in-sights into the vital role that medicinal plants play in drug development. Often, a single medicinal plant can have multiple uses and sometimes different parts of the same plant may be used for the treatment of more than one disease condition.[3] Plants used for traditional medicine contain a wide variety of substances that can be used to treat chronic as well as infectious diseases. Men turned to ethnopharmacognosy due to the development of adverse effects and Microbial resistance to the chemically synthesized Drugs. They found literally thousands of phytochemicals from plants as safe and broadly effective alternatives with minimum side effects. Many beneficial biological activity such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity were reported. Certain natural or herbal products claim the good benefit for people in many cases.[2] The crude extracts and phytochemical constituents isolated from Neorautanenia mitis have shown antidiarrheal, acaricidal, insecticidal, antinocicetive, anti-inflammatory, larvicidal, mosquitocidal , cytotoxicity, and antimicrobial, activities.[3] Calicotome villosa leaf methanol extract have led to the isolation and identification of many phenolic compounds, alkaloids and anthraquinones . A biological study has shown antimicrobial activity of the Leaf methanolic extract of this species against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus lentus*, *Pseudomonas aeruginosa*, *Providenica rettgeri*, and *Morganella morganii*.[4] Current advances in research have made it possible to extract medically essential compounds from plants used in conventional medicinal practices. The bio-active compounds in plants are known as phytochemicals. These phytochemicals are obtained from various plant components such as leaves, barks, and seeds, coats of seeds, flowers, roots and pulps and are thus used as direct medicinal agents.[1] The phytochemicals rich in plants have shown to be beneficial for prevention of diseases as well as long- term Health. Plants are generally consumed as sources of essential compounds such as saccharides, coumarins, lignans, flavonoids, terpenoids, and steroids. The health benefits and the composition from plant have been described more and more in the literature. Because of complexity of phytochemicals, they must be obtained in pure form via extraction and isolation before structure identification, bioactivity screening and so on. The first step for phytochemistry research and also necessary work before the isolation of effective constituents is Extraction. The purpose of extraction is to get the objective chemical constituents to the utmost extent and avoid or reduce the separation of unwanted constituents. The Separation of Phytochemicals is a process of isolating the constituents of plant extracts or effective parts one by one and purifying them into monomer compounds by physical and chemical methods. Classical Isolation methods, includes Solvent Extraction, Precipitation, Crystallization, Fractional Distillation, Salting-Out and Dialysis which are also still used commonly .On the other hand, modern separation technologies such as column chromatography, high performance liquid chromatography, ultrafiltration and high performance liquid drop counter current chromatography also play an important role in the separation of Phytochemicals.[5]

2. ISOLATION

The components in the extracts are complex mixture and contain various types of natural products with different polarities.[6] Isolation is a Separation technique in which we can obtain a purified compound. Hence we can call it "Purification" as well. In this technique, all the foreign or contaminating substance can be removed in order to isolate the desired compound. To get a highly pure compound, a series of extraction need to be done.[7] To obtain pure bioactive compound, further separation and purification is needed. Their separation remains a big challenge for the process of identification and characterization of pure bioactive natural product. Purification and isolation of natural products has undergone new development in recent years.[6] The example flow chart of series of extraction is given in figure no 1.

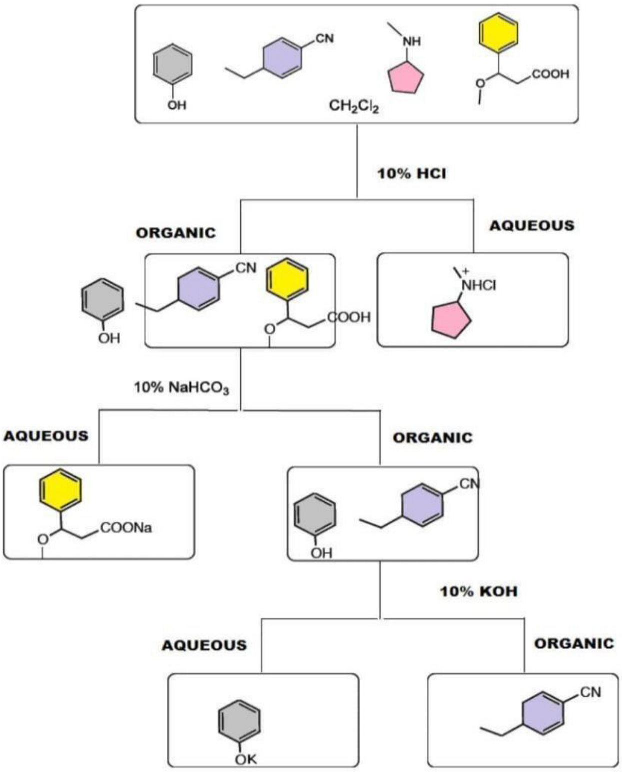


Fig no 01: A Flow chart for a series of extractions.[7]

2.1 VARIOUS ISOLATION TECHNIQUES

Many bioactive natural products have been isolated and purified by using different separation techniques such as,

2.1.1 CHROMATOGRAPHY

Preparative Thin layer chromatography
High performance thin layer chromatography
Preparative High performance liquid chromatography
Column chromatography
Gas chromatography
Preparative Gas chromatography
Fast Protein Liquid Chromatography
Supercritical Fluid Chromatography
Affinity Chromatography
Reversed Phase Chromatography
Two Dimensional Chromatography
Pyrolysis Gas Chromatography
Counter Current Chromatography.
Optimum performance laminar chromatography
Flash chromatography

2.1.2 SOLVENT METHOD

Acid basic solvent method
Polarity gradient extraction method

2.1.3 PRECIPITATION METHOD

Solvent precipitation method
Exclusive reagent precipitation method
Salting out method
Dialysis method
Fractional distillation method
Crystallization method

3. CHROMATOGRAPHY

3.1 SIMPLE PREPARATIVE THIN LAYER CHROMATOGRAPHY

Numerous Attempts have been made to exploit the advantages of Thin Layer Chromatography for Preparative Work. Such methods are in convenient, quite apart from the increase in scale are required. Thus to elute the components of mixtures directly from chromatograms needs special apparatus and procedures and to recover components by extraction involves subsequent removal of fine adsorbent particles. For best results prior concentration “on the layer” by additional chromatographic steps are needed. Consequently most workers still use thin-layer chromatography only for analysis and for preparative work move from classical column chromatography to the newer “dry column” Method. In the two methods described here these difficulties were avoided, thus opening the way to more widespread use of thin layer chromatography as a preparative tool. Method no.1 is extremely simple and method no.2 though slightly more complex but has the advantage of being able to cope with about 10 times the load per run. Method No.1 needs only a chromatography tank with an upper solvent reservoir (as used for descending paper or thin layer chromatography) and pre-coated thin –layer sheets with flexible plastic backing (alumina, cellulose and silica adsorbent layers may all be used) such as the “polygram” and “chromagram” ranges produced by Macherey Nagel and Eastman, The chromatogram sheet is prepared for use as follows. Edge effects during development are minimized by scrapping off 0.5cm strips of adsorbent from the vertical edges of the sheet (the complete chromatogram sheet is show in figure no 2).

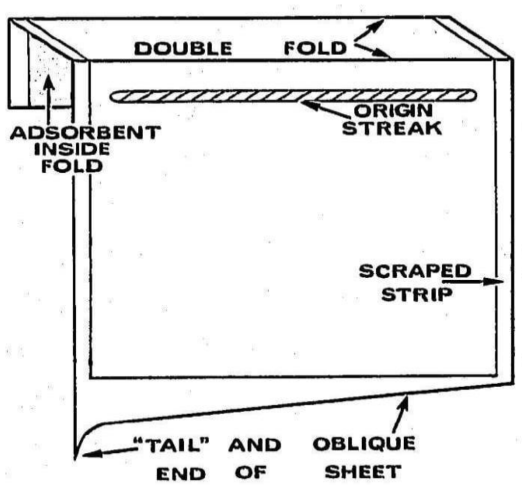
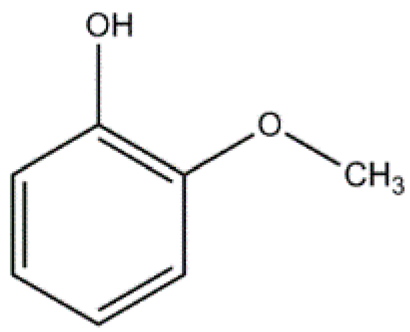


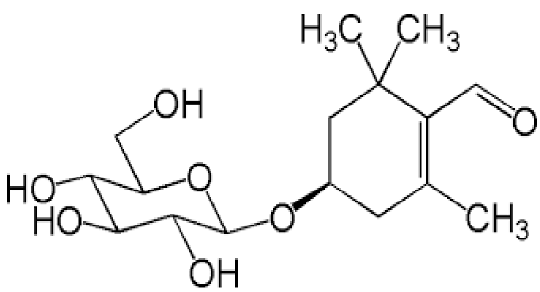
Fig no.2: Completed chromatogram sheet for simple preparative thin layer chromatography.[8]

To ensure the solvent drops readily fall from the sheet during development, its bottom edge is trimmed slightly obliquely leaving at the lowest corner a very sharply pointed 1.0cm long "tail". Resolution of closely spaced components is aided if adsorbent is scrapped off form a straight lower adsorbent edge. A right angled fold is now made parallel to and 3 cm from the upper edge of the sheet. The adsorbent layer should be on the inside of the fold to minimize peeling of the adsorbent. A further right angled fold is made parallel to and some 1.5cm down the sheet from the initial fold. These two folds constitute a "hook" by which the sheet hung over the lip of the solvent through. The developing solvent is chosen with the aid of test strips and direct transfer to the preparative system is possible. Very volatile solvents should be avoided to minimize the risk of the chromatogram drying out. Resolution is often improved by multi-solvent development with the mobile components eluted by non-polar solvents. The chromatogram is run as follows. The sample is applied as a streak terminating some 1.0-2.0 cm from the edges of sheet to limit edge effects. The loaded sheet hooked over the edge of the empty solvent through and a wire clip placed over the 'hook' to prevent it opening out during development. Development is started by filling the solvent trough through a previously stoppered port in the tank lid. Separate fractions are collected by moving the sheet along the trough in such a manner that the "tail" delivers successive fractions into successive collecting tubes standing on the floor of the tank. The development of colourless fractions may be followed by using adsorbents containing fluorescent indicators and an ultra-violet lamp arranged to shine through a port in the tank lid. In this case ultra-violet adsorbing aromatic solvents such as toluene cannot be used. A paper wick on the inside of the "hook" will increase the development rate and through care must be taken for excessive siphoning and thus irregular flow is prevented.[8] The examples of compounds isolated from preparative thin layer chromatography is given in the table no 1.

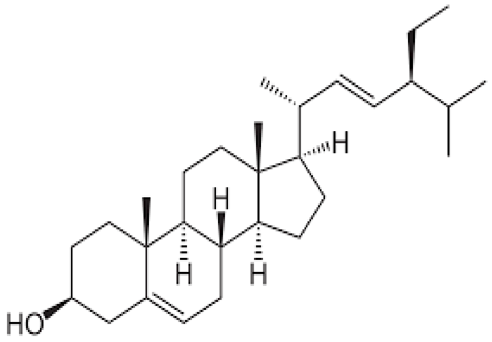
Table No 1 : Compounds Isolated From Preparative Thin Layer Chromatography



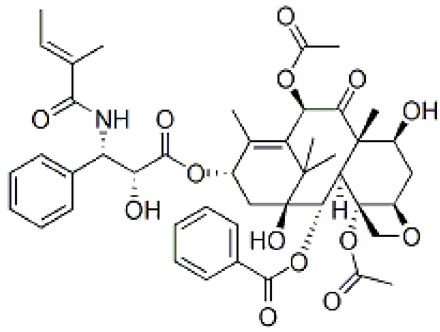
Guaiacol[9]



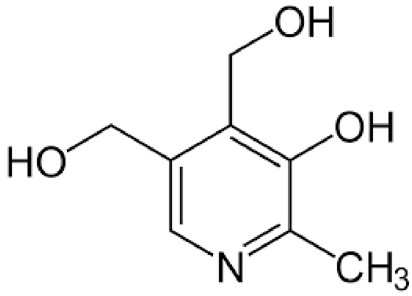
Picrocrocin[10]



Stigmasterol[11]



Cephalomannine[12]

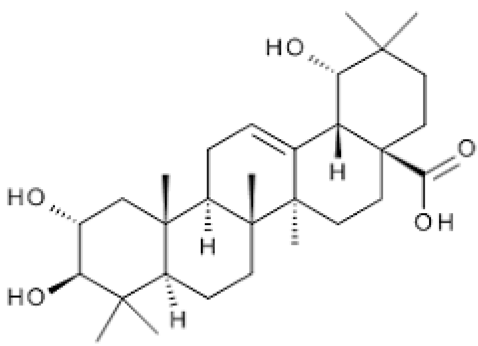


Pyridoxine[13]

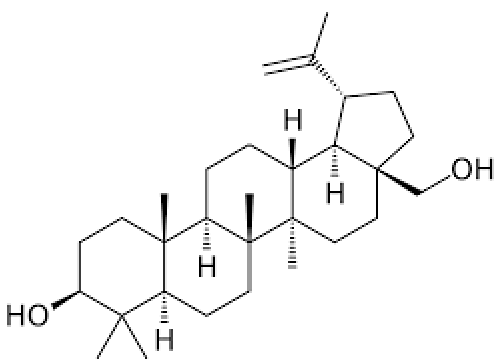
4. HIGH- PERFORMANCE THIN- LAYER CHROMATOGRAPHY (HPTLC)

High-Performance Thin-Layer Chromatography (HPTLC) is an enhanced form of Thin Layer Chromatography (TLC). High-performance thin layer chromatography (HPTLC) is one of the most sophisticated, flexible, robust and cost-effective separation techniques employed in the discovery, development, and analysis of new drugs. HPTLC is an extension of thin layer chromatography (TLC). Thin layer chromatography involves the separation of different components in the sample depending upon the relative affinity of each compound towards the stationary phase or mobile phase, while HPTLC helps in better resolution of compounds with lower limits of detection and quantified separated components with the use of an integrated Software Problem.[14]Separation of natural compounds is achieved on High performance layer with detection and data acquisition. These high performance layers are pre-coated plates coated with a sorbent of particle size 5-7 microns. The reduction in thickness of 150-200 microns. The reduction in thickness of layer and particle size results in increasing the plate efficiency as well as nature of separation. HPTLC plates are substantially more expensive (4 to 6 times more) than normal plates but are an efficient alterative when high sensitivity, accuracy and precision are required in situation demanding high performance.[6] The examples of compounds isolated from High performance thin layer chromatography were given in the table no 2.

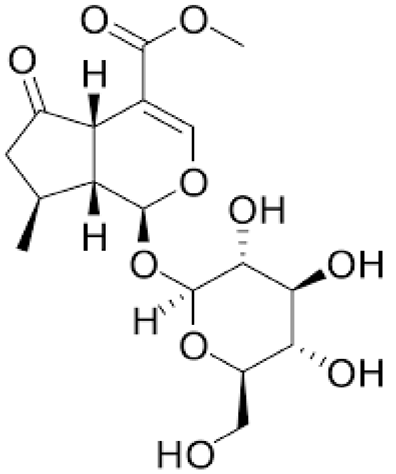
Table No 2 : Compounds Isolated From High Performance Thin Layer Chromatography



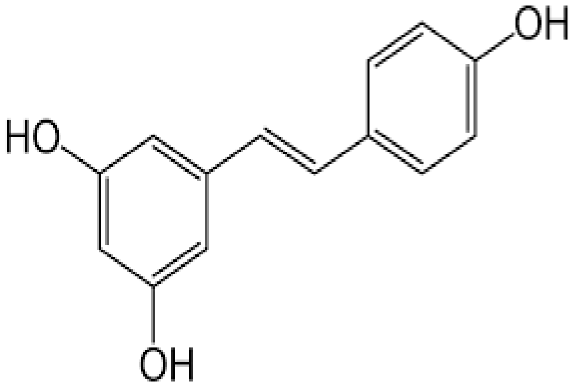
Arjunic acid[15]



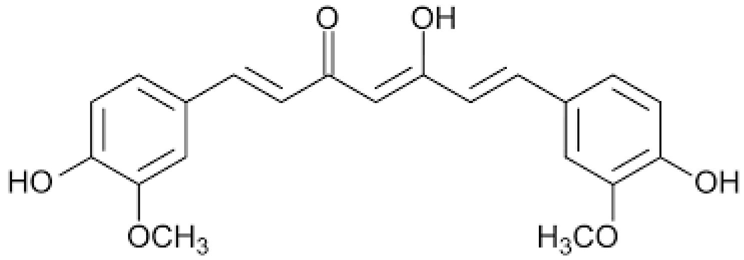
Betulin[16]



Verbenalin[17]



Resveratrol[18]

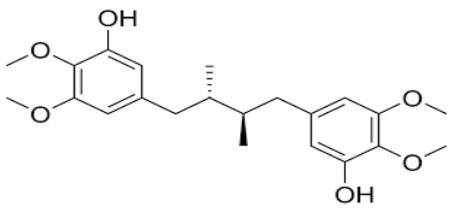


Curcumin[19]

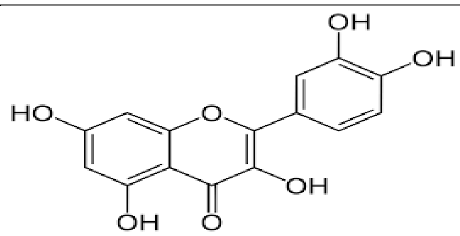
5. *PREPARATIVE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY*

There are Basically two types of Preparative High Performance Liquid Chromatography. One is low pressure (typically under 5 bar) traditional HPLC, based on the use of glass are plastic columns filled with low efficiency packing materials of large particles and large Size Distribution. A more recent form PLC, preparative high performance liquid chromatography has been gaining Popularity in Pharmaceutical Industry. In preparative HPLC (pressure >20 bar), larger stainless steel column and packing materials (particle size 10-30- m of needed. The Examples of Normal Phase Silica columns are Kromasil 10- m, Kromasil 16- m, Chiralcel AS 20- m whereas for Reverse phase are Chromasil C18, Chromasil C8, YMC C18.The aim is to isolate or purify compounds, whereas in analytical work the goal is to get information about the sample. Secondly is traditional PLC, because its higher column efficiencies and faster solvent velocities permit more difficult separation to be conducted more quickly. In analytical HPLC, the important parameters are resolution, sensitivity and fast analysis time whereas in preparative HPLC, both the degree of solute purity as well as the amount of compound can be produced per unit time i.e. through put or recovery are important. This is very important in pharmaceutical industry because new products (natural, synthetic) have to be introduced to the market as quickly as possible. Having available such a powerful purification technique make it possible to spend less time on the synthesis condition.[20] The examples of compounds isolated from High performance liquid chromatography given in the table no 3.

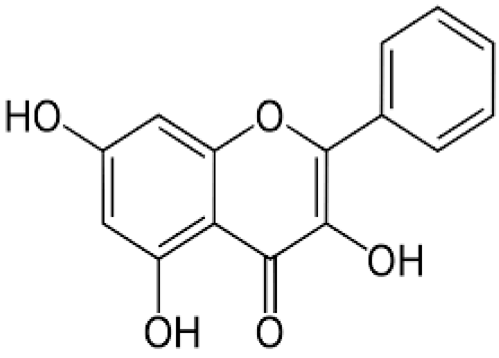
Table No3: Compounds isolated from preparative High Performance Liquid Chromatography



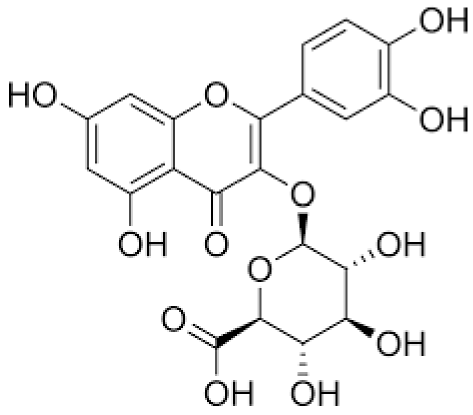
Pregomisin[21]



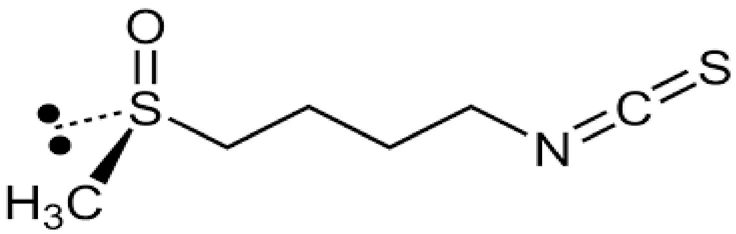
Quercetin[22]



Galangin[23]



Miquelianin[24]



Sulforaphane[25]

6. COLUMN CHROMATOGRAPHY

Column Chromatography is simple and the most popular Separation and Purification Technique. Both solid and liquid samples can be separated and purified by Column Chromatography.[14] In Chemistry, Column chromatography is a technique which is used to Separate a Single Chemical Compound from a mixture dissolved in a Fluid. Column-Chromatography separates substances based on differential adsorption of compounds to the adsorbent as the compounds move through the Column at different Rates which allows them to get separated in fractions. This Technique can be used on a small scale to purify materials that can be used in future experiments. This method is a type of Adsorption Chromatography Technique. When the mobile phase along with the mixture that needs to be separated is introduced from the top of the column, the movement of the individual components of the mixture will be at different Rates. The components with lower adsorption and affinity to the stationary phase travel faster when compared to the greater adsorption and affinity with the stationary phase. The components that move fast are removed first where as the components that move slowly are eluted out last. The adsorption of solute molecules to the column occurs in a reversible manner. The Process of column chromatography is given in the figure no.3. the rate of the movement of the components is expressed as: Rf = The Distance Travelled By Solute / The distance travelled by the solvent . rf is the retardation factor.[26] The examples of compounds isolated from column chromatography given in the table no 4.

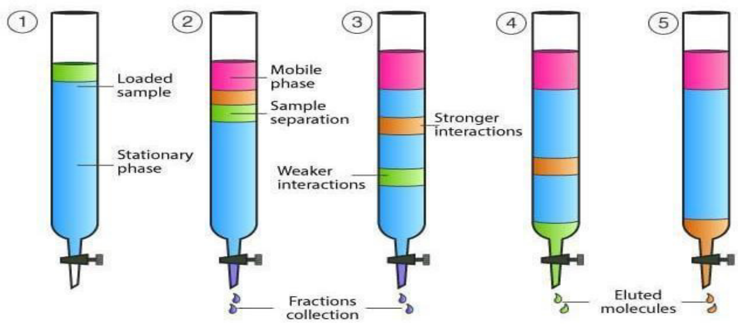
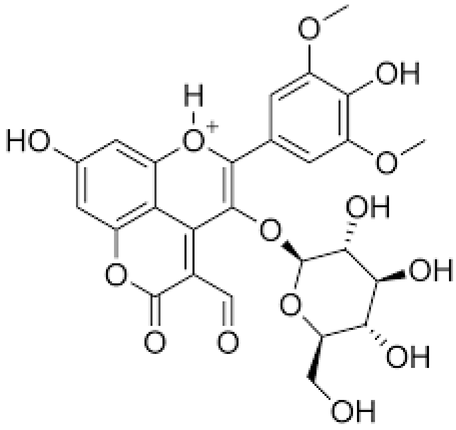
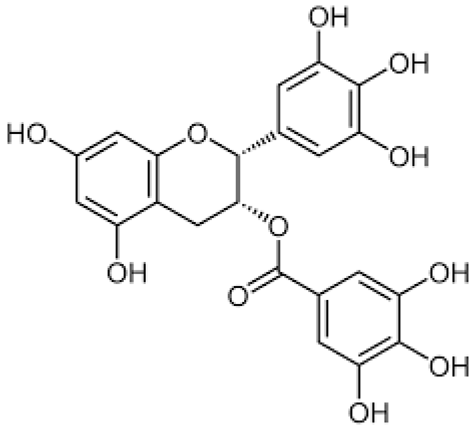


Fig no.3: Process of Column chromatography.[27]

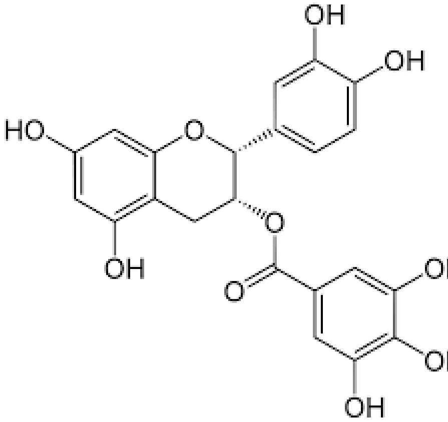
Table No 4 : Compounds Isolated from Column Chromatography



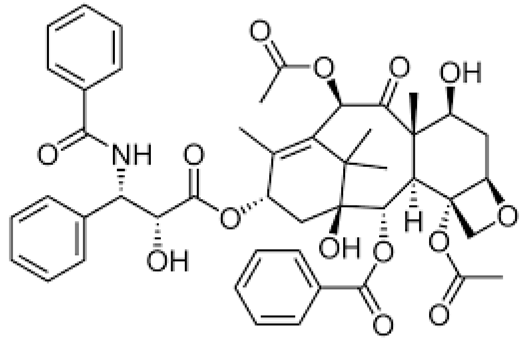
Pyranoanthocyanins[28]



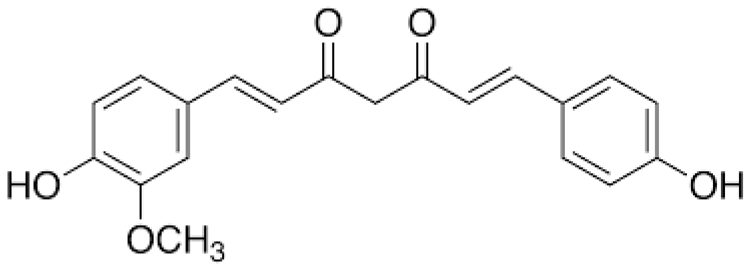
Epigallocatechin gallate[29]



Epicatechin gallate[29]



Paclitaxel[30]



Demethoxycurcumin[31]

7. GAS CHROMATOGRAPHY

It is an analytical technique for separating compounds based primarily on the volatilities. Gas chromatography provides both qualitative and quantitative information for individual compounds present in a sample.[6] Gas chromatography is also sometimes Called as Vapour-Phase Chromatography (VPC) or Gas-liquid Partition Chromatography (GLPC). The Process of Separating Compounds in a mixture by injecting a Gaseous or Liquid sample into a Mobile Phase Typically called the Carrier Gas and Passing the Gas through a Stationary phase Is Gas Chromatography. The Mobile Phase is generally an inert gas or an Unreactive gas such as Helium, Argon, Nitrogen or Hydrogen. The stationary phase is a microscopic layer of viscous liquid on a surface of solid particles an inert solid support inside a piece of glass or metal tubing called a column. in some columns the surface of the solid particles may also act as the stationary phase . In an oven , the glass or metal column through which the gas phase passes is located where the temperature of the gas can be controlled and the constitiuent coming off the column is monitored by a computerized detector.[32] The instrumentation of gas chromatography is shown in figure no.4. The examples of compounds isolated from gas chromatography given in the table no 5.

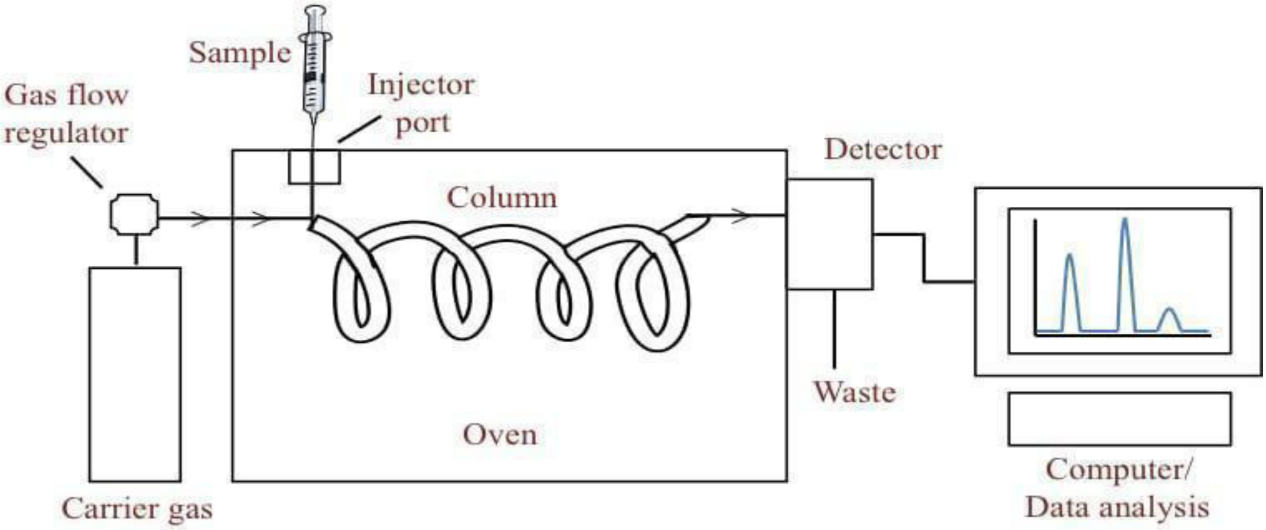
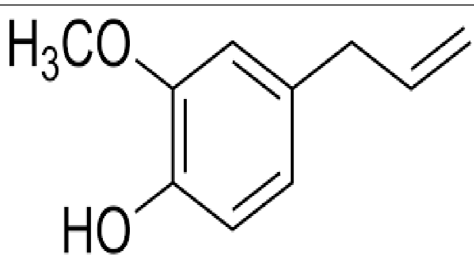
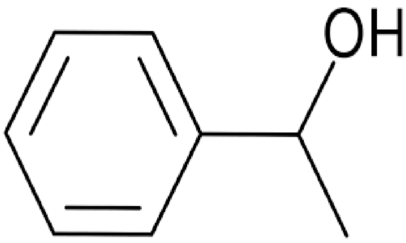
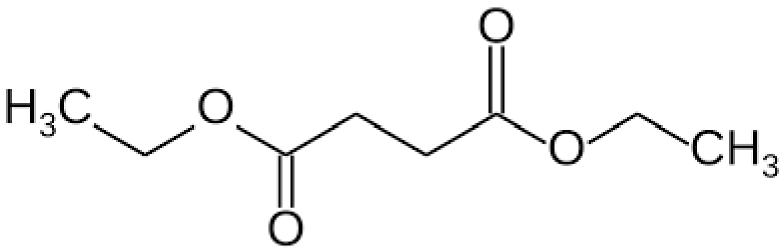
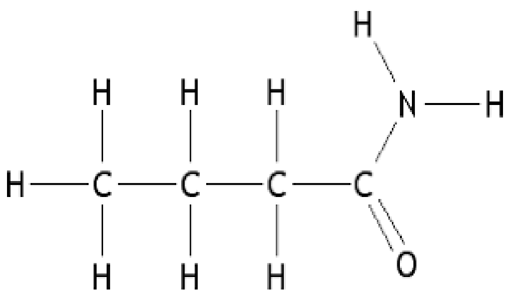
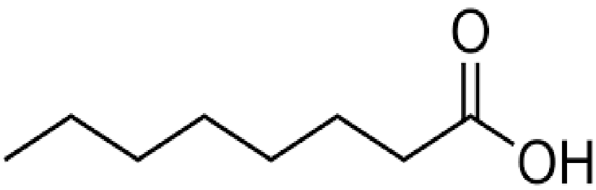


FIG NO.4: Gas chromatography system.[33]

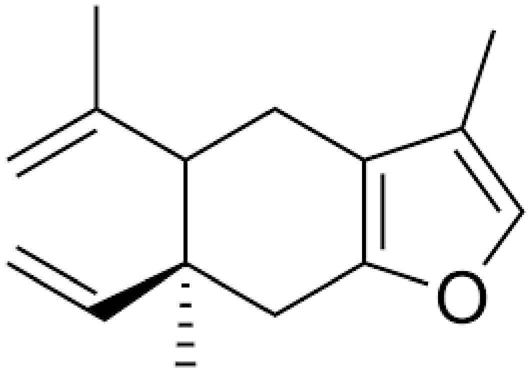
Table No 5 : Compounds Isolated From Gas Chromatography

 **Eugenol**[34]	 **Phenylethanol**[35]
 **Diethyl butanedioate**[35]	
 **Butanamide**[35]	 **Octanoic acid**[36]

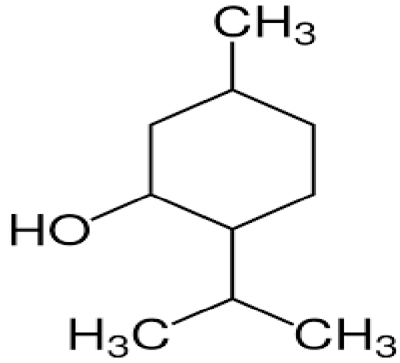
8. PREPARATIVE GAS CHROMATOGRAPHY

Preparative Gas Chromatography Gas chromatography (GC), invented by Martin and Synge in 1941 and was first applied for the separation of a series of fatty acids in 1951. After this, the GC technique developed rapidly and was the first analytical instrument to be controlled by a computer. The term preparative GC usually describes the use of large-diameter columns to purify Preparative gas chromatography (prep-GC) is an important tool for the separation and purification of components of a mixture for further uses such as structure elucidation or for recovery of bulk materials in a pure form for commercial applications.[37] Prep-GC increases the mass of a single compound or zones of compounds isolated from a sample after GC separation. Sample collection includes preparative fraction collection into vials, trapping onto a capillary wall, or using sorbent materials attached to the end of the column or transfer line in order to collect the isolated compound(s).[38] The examples of compounds isolated from preparative gas chromatography given in the table no 6.

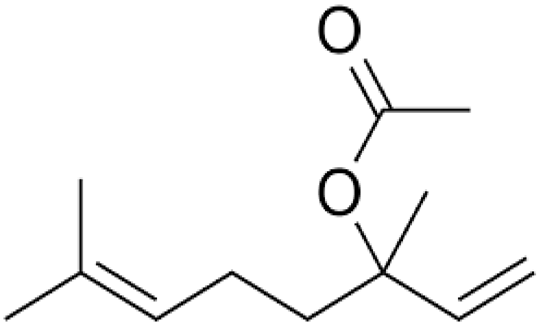
Table No 6: Compounds Isolated From Preparative Gas Chromatography



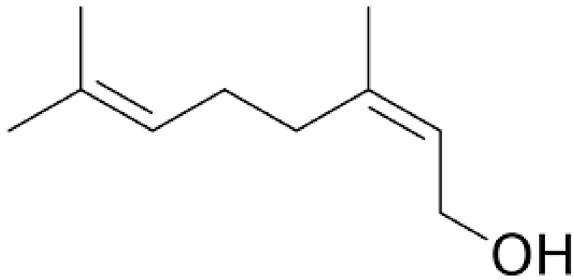
Curzerene[39]



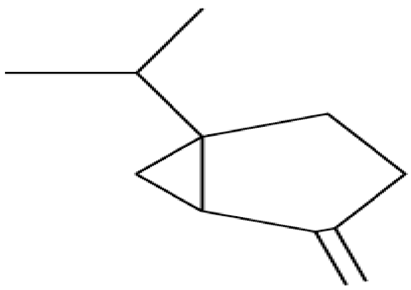
Menthol[40]



Linalyl acetate[41]



Geraniol[42]



Sabinene[43]

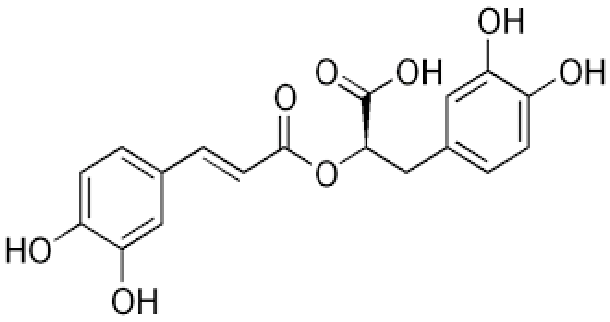
9. *FAST PROTEIN LIQUID CHROMATOGRAPHY*

A Fast Protein Liquid Chromatography (FPLC, formerly named "Fast Performance Liquid Chromatography ") is a form of medium pressure chromatography originally developed for purifying proteins with high resolution and reproducibility.[44] It is very commonly used in biochemistry and enzymology.[45] Its distinguishing feature is that the stationary phase is composed of small diameter beads (generally cross linked agarose) that are packed in glass or plastic columns and have high loading capacity. Resins for Fast Protein Liquid Chromatography are available in a wide range of particle sizes and ligand surfaces, which are selected on the basis of their application. The Fast Protein Liquid Chromatography system allows the use of a wide range of Aqueous Buffers (the mobile phase) and Different Stationary Phases to Perform the Main Chromatography Modes (Ion Exchange, Gel Filtration, Affinity, Chromatofocusing, Hydrophobic Interaction, Reverse Phase). However, Anion exchange and Gel filtration chromatography are the modes most commonly used. In general, the mobile phase is an aqueous buffer solution, whose flow rate through the stationary phase is controlled by a pump (normally kept constant), while the composition of the buffer may vary by mixing two or more solutions contained in external reservoirs. In contrast to high performance liquid chromatography (HPLC), the buffer pressure used in low, typically 5 bar, but the flow rate is high (eg, 1-5ml.min-1). Fast Protein Liquid Chromatography can be scaled up, allowing the analysis of samples containing from milligrams of proteins in 5ml columns to preparative production of kilograms of purified proteins using columns of several liters of volume.[44]

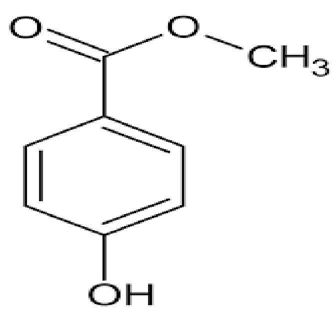
10. SUPERCRITICAL FLUID CHROMATOGRAPHY (SFC)

SFC is the Third Column Chromatography Technique after High Performance Liquid Chromatography and Gas Chromatography. SFC can be more advantageous than High Performance Liquid Chromatography and Gas Chromatography and are suitable for the compounds which are decomposed at high temperature with GC and do not have functional groups to be detected by HPLC detection systems. SFC enables change of some properties during the chromatography process. This turning ability brings an advantage to optimize the analysis. Also, SFC have a broader range of detectors than HPLC.[32] SFC is a form of Normal Phase Chromatography that uses a supercritical fluid such as carbon dioxide as the mobile phase. It is used for the analysis and purification of low to moderate molecular weight, thermally labile molecules and can also be used for the separation of chiral compounds. Principles are similar to those of High Performance Liquid Chromatography (HPLC). In addition, SFC metering pumps require that the pump head be kept cold in order to maintain the carbon dioxide in a supercritical state, where it can be effectively metered at some specified flow rate, composition, and column temperature. In addition, SFC provides, an additional control parameter, pressure, which the chemist similarly sets through the keyboard. From an operational standpoint, SFC is as Simple and Robust as High Performance Liquid Chromatography. Any molecule that dissolve in methanol or less polar solvent is an ideal candidate for SFC.SFC can be even separate polar solutes. Many strong bases that are difficult to separate by other techniques can be separated rapidly and efficiently with good peak shapes.[45] The examples of compounds isolated from Supercritical fluid chromatography given in the table no 7.

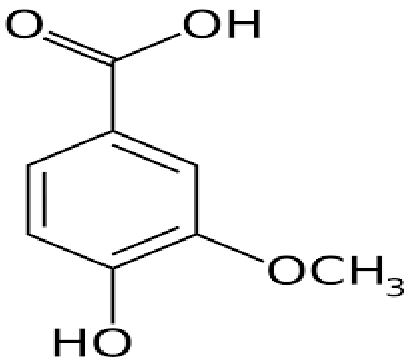
Table No 7: Compounds Isolated From Supercritical Fluid Chromatography



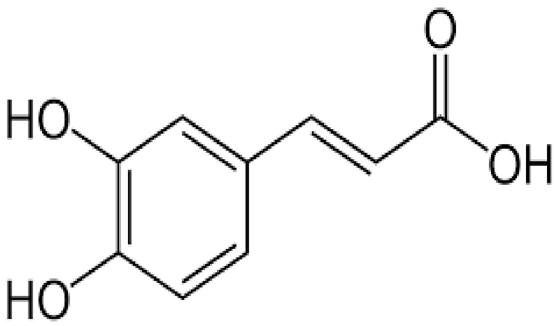
Rosmarinic acid[46]



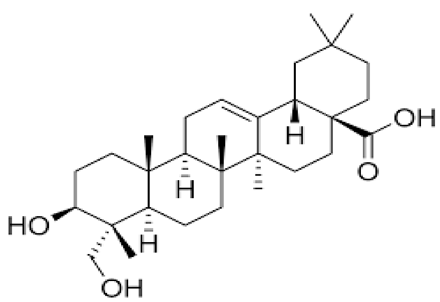
Methyl Paraben[47]



Vanillic acid[48]



Caffeic acid[48]

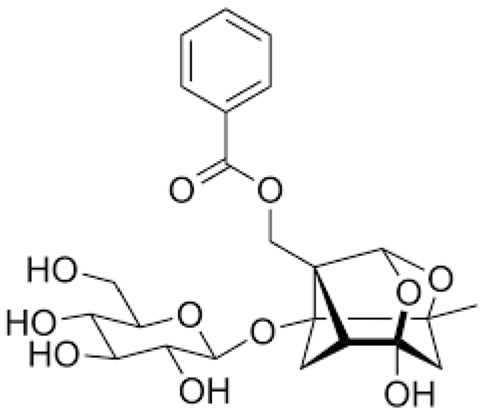


Hederagenin[49]

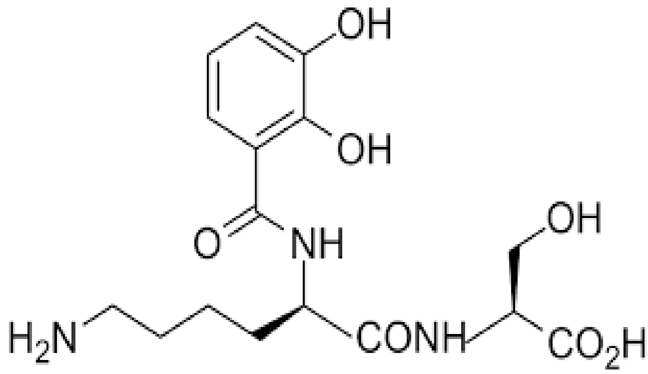
11. AFFINITY CHROMATOGRAPHY

It is a Chromatography Method of separating biochemical mixtures based on a highly specific biologic interaction such as between antigen and antibody, enzyme and substrate or receptor and ligand. Affinity chromatography combines the size fractionation capability of gel permeation chromatography with the ability to design a stationary phase that reversibly binds to a known subset of molecules.[45] The stationary phase consists of a support medium, on which the substrate (ligand) is bound covalently, in such a way the reactive groups that are essential for binding of the target molecules are exposed. As the crude mixture of the substance is passed through the chromatography column, substances with binding site for the immobilized substrate bind to the stationary phase, while all other substance is eluted in the void volume of the Column. Once the other substances are eluted, the bound target molecules can be eluted by methods such as including a competing ligand in the mobile phase or changing the PH , ionic strength or polarity conditions.[50] The examples of compounds isolated from Affinity chromatography given in the table no 8.

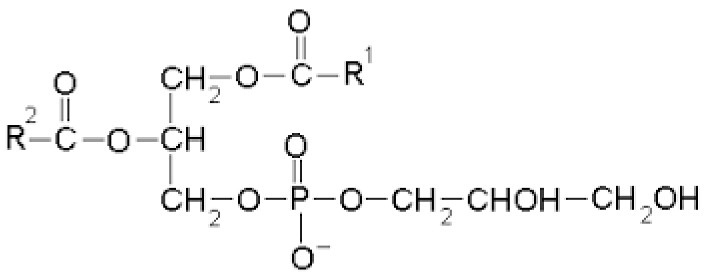
Table No 8: Compounds Isolated From Affinity Chromatography



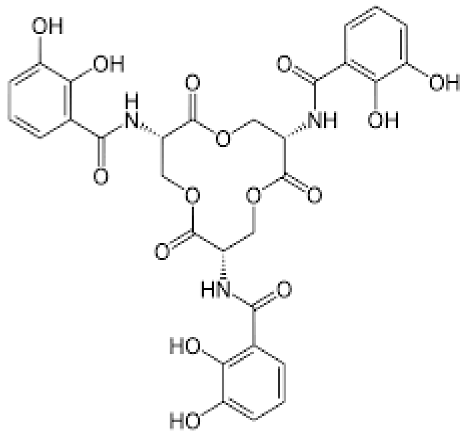
Paeoniflorin[51]



Chrysobactin[52]



Phosphatidylglycerol[53]

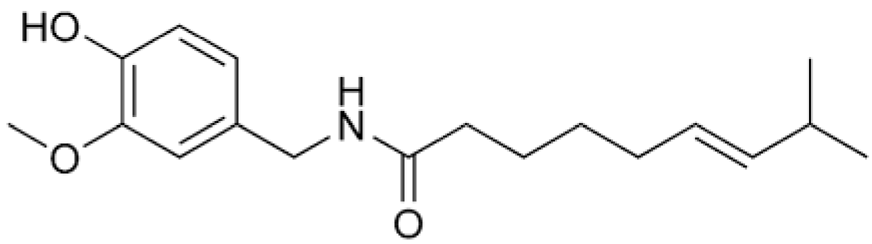


Enterobactin[52]

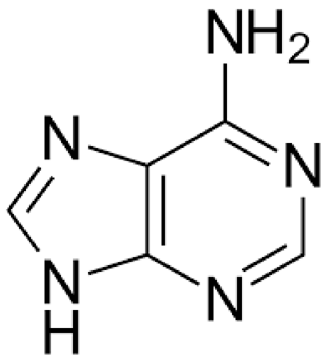
12. REVERSED PHASE CHROMATOGRAPHY

Reversed phase (RP) chromatography is a popular technique for the separation, desalting, and concentration of proteins, in part because the sample is concentrated in a small volume of volatile solvent that can be removed by evaporation.[54] Reverse phase chromatography (RPC) includes any chromatographic method that uses a non-polar stationary phase. All of the mathematical and experimental considerations used in other chromatographic methods apply (i.e., separation resolution proportional to the column length and inversely proportional to the column width). Reversed- phase column chromatography(RPC) is widely used in the pharmaceutical, chemical, and biochemical industry for separating molecules of small molecular weight. In more recent years, RPC has been used to separate larger molecules. Any inert non-polar substance that achieves sufficient packing can be used for reversed-phase chromatography. Common examples include larger hydrocarbons, diphenyl, and divinylbenzene. Typically mobile phases are made of water or a polar organic compound such as acetonitrile or the lighter alcohols. Buffer solutions are used to control the protonation of functional groups. For example, a buffer of high pH will encourage elution of alcohols because they are charged in basic solutions and, therefore, prefer to be in the mobile phase. For the same reason, very acidic solutions encourage the elution of nitrogen containing molecules. Using multiple buffers allows for selective elution of a wide variety of chemicals.[45] The examples of compounds isolated from Reversed phase chromatography given in the table no 9.

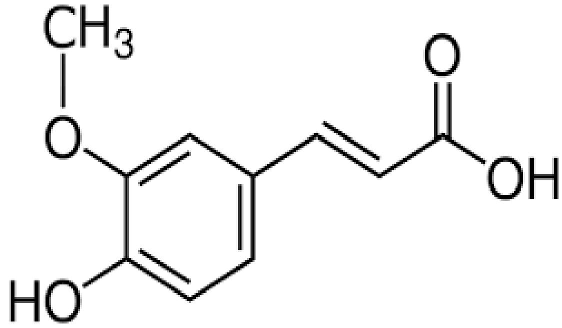
Table No 9 : Compounds Isolated From Reversed Phase Chromatography



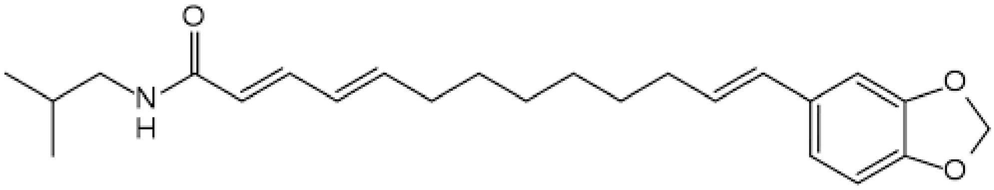
Capsaicin[55]



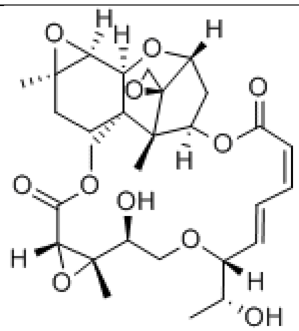
Adenine[56]



Ferulic acid[57]



Guineensine[58]

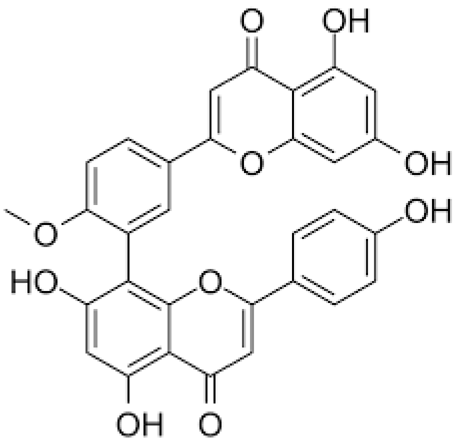


Baccharin[59]

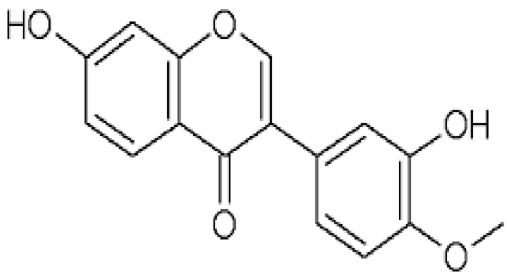
13. TWO-DIMENSIONAL CHROMATOGRAPHY

The Injected Sample is Separated by passing through Two Different Separation Stages this is called as Two- Dimensional Chromatography. Two different Chromatographic columns are Connected in Sequence and the Effluent from the First system is transferred onto the second column.[60] Generally the Second Column has a different Separation Mechanism, so that bands that are Poorly Resolved from the first column may be completely separated in the Second Column (For Example, a c18 reversed-phase chromatography column may be followed by a phenyl column.). Alternatively, the Two Columns might Run at Different . During the second stage of separation, the rate at which the separation occurs must be faster than the first stage, since there is still only a Single Detector. The plane surface is compatible to sequential development in two directions using two different solvents. The examples of compounds isolated from Two-Dimensional chromatography given in the table no 10.

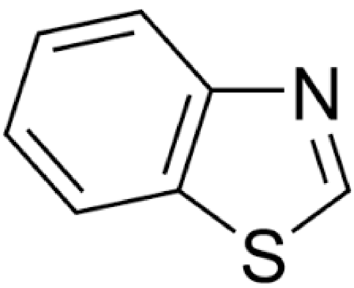
Table No 10 : Compounds Isolated From Two – Dimensional Chromatography



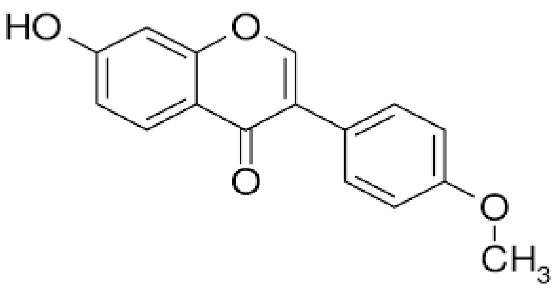
Bilobetin[61]



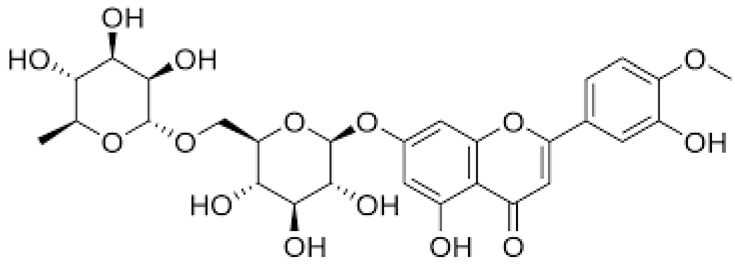
Calycosin[62]



Benzothiazole[63]



Formononetin[62]



Diosmin[64]

14. PYROLYSIS GAS CHROMATOGRAPHY

Pyrolysis Gas Chromatography is a potent Analytical Tool able to Thermally crack (fragment) essentially Non-Volatile Molecules into Fragments suitable for chromatographic analysis. The technique enables a Reproducible and Characteristic "fingerprint" to be generated of a Non-Volatile Sample. The technique can be applied to such varied tasks as bactericidal strain differentiation and forensic characterization of paints, polymers and fibre cross matching.[45]

15. COUNTER CURRENT CHROMATOGRAPHY

This type of chromatography is also called as Liquid-Liquid Chromatography (LLC) because the separation technique does not require any Solid Support. Both Phases are Liquids and no solid matrix is used to retain the stationary phase because Counter current chromatography uses Special Colums. The Liquid Stationary Phase is taken in place by a Centrifugal Force. This support free liquid stationary phase that allows for Preparative Separations and Purifications. The Chemical Compounds of any mixture is separated, Identified and Quantified by the use of our Counter current Chromatography .Counter current chromatography is based on the principle of partioning liquids. Immiscible Liquids are combined and then separated several times. Each individual solute is isolated based on the "Partition Coefficient" in the particular compound present in the solvent phase versus the Diluents Phase. Because, Counter current chromatography is a type of liquid partition chromatography, and has the advantages of operation without the use of a Solid Support. Therefore, all the problems that arise from using a solid support are limited

16. OPTIMUM PERFORMANCE IN LAMINAR CHROMATOGRAPHY

It is a New Concept In Parallel Chromatography. OPLC combines the advantages of both TLC and HPLC.OPLC is both an analytical and preparative tool, suitable for research and quality control laboratories. It is a powerful liquid chromatography separation technique that combine the user-friendly interface and resolution of HPLC with the capacity of flash chromatography and multi dimensionality of TLC. The basis of OPLC is similar to that of other chromatographic techniques in that a pump is used to force a liquid mobile phase through a stationary phase, such as silica. The OPLC column housing structure allows flat planner columns to be used in the someway as cylindrical glass or stainless steel once. Mobile phase is forced through the flat column pressurized up to 50 bars at constant liner velocity via solvent delivery pump.[6]

17. FLASH CHROMATOGRAPHY

Flash chromatography is a rapid, simple and inexpensive approach to separate complex mixtures of compounds. It is a great alternative when other separation techniques cannot be used or difficult. Flash chromatography system allows separations and purifications in an automated way, which saves user time, solvent saving reproducibility. The operating principle of this technique is based on the separation of the compounds between a solid stationary phase and a mobile phase (the eluent). The separation will therefore be performed according to the Greater or lesser affinity that the compounds have for the stationary phase, depending on their structure and the nature of the stationary phase, depending on their structure and the nature of the stationary phase. flash column chromatography uses compressed gas (such as nitrogen or air) or a pump to push solvent through the Column. This technique is particularly advantageous because it allows for faster flow rates of solvent, as opposed to simple gravity flow. Special considerations for flash chromatography include the usage of a finer particle size for the stationary phase (such as silica gel).[65]

18. SOLVENT METHOD

18.1. ACID AND BASIC SOLVENT METHOD

It is carried out according to the different acidity and alkalinity of each component in the mixture. Water-insoluble alkaline organic components, such as alkaloids, could react with inorganic acids and form salt, which can be separated from non-alkaline and water-insoluble components. Acid components with carboxyl or phenolic hydroxyl groups can be salted by bases and dissolved in water. Components with lactone or lactam substructures can be saponified and dissolved in water and then isolated from other water-insoluble components. The total extracts can be dissolved in lipophilic organic solvents (ethyl acetate is commonly used) and extracted respectively with acid water and alkali water, alkaline, and neutral parts. The total extract can also be dissolved in water and extracted with organic solvents after adjusting the pH value. The alkalinity or acidity of the fractions are different and can be separated further by pH gradient extraction. When using the acid and basic solvent method, attention should be paid to the strength of acidity or alkalinity, the contact time with the separated components, heating temperature, and time, so as to avoid the structural changes of some compounds under severe conditions or the chemical structure cannot be restored to the original states.[5]

19. POLARITY GRADIENT EXTRACTION METHOD

This method is to achieve the separation based on the different polarity of each component in plant extracts and the different partition coefficients is two-phase solvents. Generally, different two-phase solvent systems are selected according to the polarity of components in plant extracts. For example, the components with strong polarity can be separated by n-butanol-water system, the components with medium polarity can be separated by ethyl acetate-water system, and the components with weak polarity can be separated by chloroform (or ether)-water system. During the operation, the plant extract should be dissolved by water firstly, and then the solution or suspension is extracted in a separating funnel with different organic solvent which is not miscible with water based on the polarity difference. Usually, the extract was extracted with petroleum ether (or cyclohexane) firstly, then ethyl acetate (or chloroform), and finally with water saturated n-butanol, as shown in figure 5. Petroleum ether layer contains medium polar components with low polarity. Ethyl acetate layer contains medium polar compounds with strong polarity, such as oligosaccharides and other water soluble components. Compounds in water layer possess strongest polarity, such as glycosides with more glycosyl group, carbohydrates, amino acids, proteins and other water soluble components.[5] The flow chart of common polarity gradient extraction method given in figure no.5.

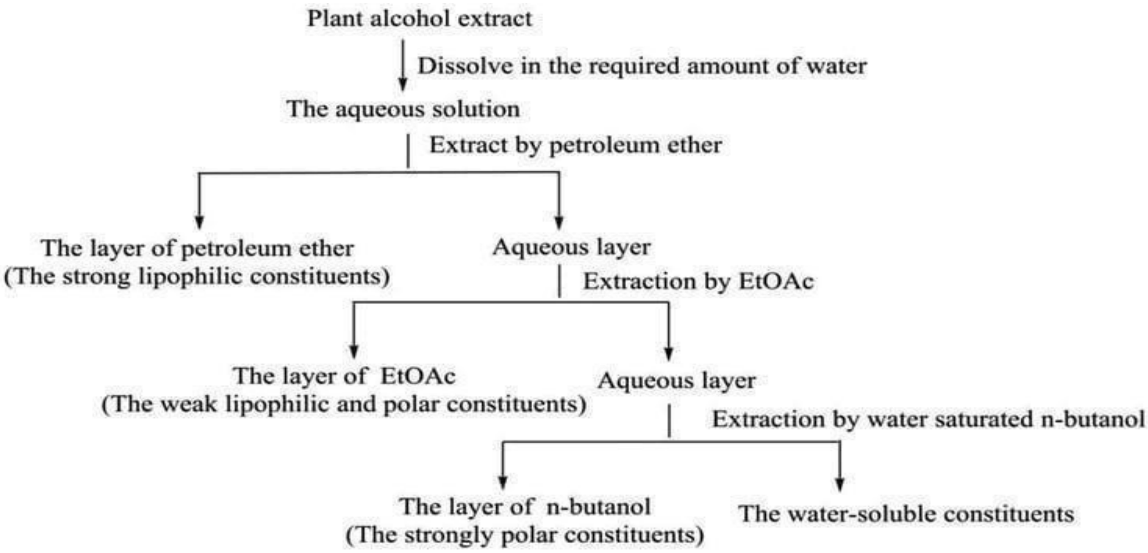


Fig No.5: flow chart of common polarity gradient extraction method.[5]

20. PRECITATION METHOD

It is a method based on the formation of precipitation of some phytochemicals by reaction with specific reagents, or the precipitation of some components from the solution by adding specific reagents, which can reduce the solubility of some components in the solution. The precipitation reaction must be reversible if the target components are required to form precipitation. While if the components are non-target, the precipitation generated will be removed, so the precipitation reaction can be irreversible. According the addition of reagent or solvents, this method could be classified as follows.[5]

21. SOLVENT PRECIPITATION METHOD

The solubility of some components in the mixed component solution can be changed by adding a specific solvent that can be mutually soluble with the solution, so it can be precipitated from the solution. The gradual precipitation by changing the polarity or amount of solvent added is called fractional precipitation. For example, using water as an extracting solvent to extract phytochemicals, ethanol is added to the water extracting concentrate to make its alcohol content more than 80%, and the polysaccharides, proteins, starch, gum and so on will be precipitated and removed after filtration. The preceding procedure is called water extraction and ethanol precipitation. Crude polysaccharides from plants are often separated with this method.[5]

22. EXCLUSIVE REAGENT PRECIPITATION METHOD

Some reagents could react selectively with certain chemical constituents to produce reversible precipitation, and the separation aims are achieved, with is called the exclusive reagent precipitation method. For example, alkaloids precipitation reagent such as Reynolds ammonium salt can precipitate after reacting with alkaloids, which can be used to separate alkaloids and non-alkaloids, or water soluble alkaloids and other alkaloids. As another example, reaction of cholesterol and sterol saponins form precipitates which can be separated from triterpene and saponins additionally, gelatin can precipitate tannins, which can be used to separate or remove tannins. In practical application, appropriate precipitation reagent should be selected according to the properties of target constituents and impurities in plants.[5]

23. SALTING OUT METHOD

Adding inorganic salts to a certain concentration or saturated state in the water extract of plants can reducing the solubility of some components in water, thus they could from water soluble compounds. The inorganic salts commonly used for salting out are sodium chloride sodium sulfate, magnesium sulfate, ferric sulfate, etc. for example, extraction of tetrandrine from daemonorps margaritae and berberine from berberis poiretii could be achieved by salting out with sodium chloride, ammonium sulfate some water soluble substance, such as proto-anemone ephedrine, matrine, are often extracted with organic solvent after adding the certain amount of salt to the water extract.[5]

24. DIALYSIS METHOD

It is a method to let substance selectively penetrate through natural or synthetic semi-permeable membrane (or dialysis bags) under action of concentration difference, pressure difference, or potential difference, so as to achieve the purpose of separation, classification, purification, or concentration. For example, when saponins, protein, polypeptides, polysaccharides and other substance in plant or separated and purified, dialysis can be used to remove inorganic salts, monosaccharides and impurities and other impurities. On the contrary, large molecule impurities left in the semi-permeable membrane from small molecule substance can be separated and purified through the semi-permeable membrane into the solution outside the membrane.[66]

25. FRACTIONAL DISTILLATION METHOD

Fractional distillation is a method of separating components in liquid mixtures based on their different boiling points. It is usually categorize into atmospheric, backup, molecule distillation, and so on. It is mainly used for the separation of volatile oils and some liquid alkaloids in plants. For-example, the boiling points of the two alkaloids in total alkaloids of Cicuta virosa, coniine, and conhydrine are 166-167C for the former and 226C for the latter, which are quite different from each other, and then they can be separated by the fractional distillation method. Generally, if the boiling point difference of compounds in liquid mixtures is above 100C, the separation can be achieved by repeated distillation of the solution . If the boiling point difference of compounds is below 25 ^{0} C, the fractionation column is needed. The smaller the boiling point difference is, the finer the fractionation device is needed.[67]

26. CRYSTALLISATION METHOD

Crystallization is the process of solute precipitation from mother liquor with complex components, and it is an effective method to prepare pure substances. The initial crystallization is often impure and needs to crystallize again, which is called recrystallization. It is a method to separate compounds from the mixture by using the difference of solubility of each component in the solvent. Crystallization is one of the important technologies for plant chemist to prepare pure compounds. When the content of a phytochemical is very high in one plant, crystals can be obtained by cooling or slightly concentrating the extract after extraction with appropriate solvent. [68] Selecting suitable crystallization solvent is the key for the crystallization method. The ideal solvents for crystallization should possess the following characteristics: high solubility for the components to be purified at high temperature, low solubility at low temperature, or soluble for the impurities at high and low temperature, moderate boiling point, no chemical reaction with the components to be crystallized, safe, low price, easy to obtain, and so on. Solvents commonly used for crystallization are methanol, ethanol, acetone, ethyl acetate, acetic acid, pyridine, etc. when crystals cannot be obtained with a single solvent, the crystallization operation can be carried out with a mixture of two or more solvents, one of which has high solubility for the component to be crystallized, and the other has low solubility. Initially, the sample to be crystallized is heated and dissolved in with solvents of high solubility. Then the second solvent with low solubility is added to the hot solution to make it turbid. Then the first solvent is added to dissolve the sample. The solution reaches saturation at this point and crystallize when it is cooled. The purity of crystallization can be preliminary identified by the crystal form, color, melting point, melting range, thin layer chromatography, paper chromatography, etc.[5]

27. SOLVENT SYSTEM

27.1 SEPARATION OF ALKALOIDS

1. Carbon tetrachloride: methylene chloride: methanol: water (2:3:3:2, V/V).[69]
2. Chloroform: ethanol (9:1), methanol: ammonia (99:1), chloroform: methanol: ammonia (60:60:1).[70]
3. Dichloromethane: ethyl acetate: methanol: ammonia (25%), (50:25:5:1, V/V/V/V).[71]

28. SEPARATION OF GLYCOSIDES

28.1 SEPARATION OF CARDIAC GLYCOSIDES

1. Acetonitrile: methanol: water (15:15:19).[72]
2. Chloroform: methanol (80:20), chloroform: methanol (70:30).[73]
3. n-butanol: ethanol: water (4:1:5). Acetonitrile: water (25:75).[74]

29. SEPARATION OF ANTHRAQUINONE GLYCOSIDES

1. Chloroform: methanol: water (5:3:4, V/V/V), methanol: water (52:48, V/V).[75]
2. Chloroform: ethyl acetate: methanol: water (8:1:6:5, V/V).[76]
3. Ethyl acetate: n-butanol: water (4:3:3), benzene: methanol (4:1), methanol: water: acetic acid (80:20:1).[77]

30. SEPARATION OF FLAVONOID GLYCOSIDES

1. Ethyl acetate: n-butanol: water (2:1:3, V/V/V).[78]
2. N-hexane: ethyl acetate: methanol: water (1:5:1:5, V/V/V/V).[79]
3. N-hexane: n-butanol: water (1:1:2, V/V/V).[80]

31. SEPARATION OF FLAVONOIDS

1. Chloroform: ethyl acetate: glacial acetic acid (12:7:1, V/V/).[81]
2. Ethyl acetate: ethanol: acetic acid: water (4:1:0.25:5, V/V).[82]
3. N-hexane: acetonitrile: dichloromethane: water (5:5:1:5).[83]

32. SEPARATION OF SAPONIN

1. n-butanol: water: acetic acid (84:14:7).[84]
2. Chloroform: methanol: isopropanol: water at a volume ratio of (60:60:1:60) and (6:6:1:6).[85]
3. Ethyl acetate: n-butanol: water (1:1:2 or 1:2:1, V:V:V) or ethyl acetate: n-butanol: methanol: water (3:5:1.5:6, V:V:V:V).[86]
4. N-hexane: n-butanol: methanol: 0.1% aqueous formic acid (3:4:1:6, V/V).[87]

33. SEPARATION OF COUMARIN

1. N-butanol: methanol: 0.5% acetic acid (5:1:5:5, V/V).[88]
2. Petroleum: ethyl acetate: methanol: water (5:5:7:4, V/V).[89]

3. N-hexane, ethyl acetate, methanol, and water (6:5:6:5, V/V).[90]
4. Petroleum: ethyl acetate: methanol: water at volume ratios of (5:5:5:5), (5:5:6:4) and (5:5:6.5:3.5).[91]
5. N-hexane: ethyl acetate: ethanol: water (5:5:4:6, V/V) and n-hexane: ethyl acetate: ethanol: water (5:5:6:4, V/V).[92]

34. SEPARATION OF ESSENTIAL OILS

1. Petroleum ether: acetonitrile: acetone (4:3:1, V/V/V).[93]
2. N-hexane: acetonitrile: ethanol (5:4:3, V/V/V).[94]
3. Tetrabutyl ammonium bromide: levulinic acid (1:2).[95]

35. SEPARATION OF TERPENOIDS

1. N-heptane: methanol: ethyl acetate: water (5:2:5:2, V/V/V/V), N-heptane: methanol (1:1, V/V).[96]
2. Chloroform: ethyl acetate: methanol: water (3:1:3:2, V/V/V/V). Petroleum ether: ethyl acetate: methanol: water (1:0.8:1.1:0.6, V/V/V/V).[97]
3. N-hexane: ethyl acetate: methanol: water (1:2:1:2, V/V).[98]
4. N-hexane: ethyl acetate: methanol: water (4:5:4:5, V/V).[99]

36. SEPARATION OF TRITERPENOIDS

1. Hexane: methanol: ethyl acetate (85:10:5).[100]
2. Chloroform: n-butanol: methanol: water (10:0.5:7:4, V/V/V/V).[101]
3. Chloroform: methanol: water (43:37:20, V/V).[102]
4. Hexane: ethyl acetate: methanol: water (1:2:1.5:1, V/V/V).[103]
5. Acetonitrile: trifluoroacetic acid (500:0.3, W/W), water: trifluoroacetic acid (391:0.3, W/W).[104]

37. SEPARATION OF NAPTHOQUINONE

1. Light petroleum: ethyl acetate: methanol: water (5:5:8:2, V/V).[105]
2. Water: acetonitrile (95:5).[106]

38. SEPARATION OF LIGNANS

1. N-hexane: acetone: 1, 4-diaxone (9:1:0.5 by volume).[107]
2. Ethyl acetate: n-butanol: water (2:5:7, V/V/V).[108]
3. N-hexane: ethyl acetate: methanol: water (6:4:5:5, 6:4:6:4, 6:4:8:2, V/V).[109]
4. N-hexane: ethyl acetate: methanol: water (1:1:1:1, V/V), acetonitrile: water (50:50, V/V).[110]
5. Petroleum ether: ethyl acetate: methanol: water (1:0.8:0.6:1.2, 1:0.8:0.8:1, V/V).[111]
6. N-hexane: ethyl acetate: methanol: water at (1:3:1.3:1, V/V) and (2.5:1:2.5:1, V/V).[112]
7. N-hexane: ethyl acetate: methanol: water (1.75:1.5:1:0.75, V/V/V/V).[113]

39. SEPARATION OF PHENOLIC, POLYPHENOLIC, TANNINS COMPOUNDS

1. N-butanol: n-propanol: water (4:1:5,V/V/V), chloroform: methanol: water (7:13:8, V/V/V).[114]
2. N-hexane: ethyl acetate: methanol: water (1:20:1:20, V/V).[115]
3. N-Hexane: ethyl acetate: methanol: acetic acid: water (1:10:0.2:0.2:20), (0.2:10:2:1:5).[116]
4. Chloroform: ethyl acetate: acetic acid (50:50:1), benzene: diaxone: acetic acid (85:15:1).[117]
5. Methanol: water, ethanol:water and acetone: water (80:20, V/V) at 80C.[118]
6. N-hexane: ethyl acetate: methanol: water (1:11:1.2:11, V/V/V/V).[119]

40. SEPARATION OF RESINS

1. N-hexane: ethyl acetate: methanol: water (1:9:1:9, V/V/V/V).[120]
2. Ethyl acetate: n-butanol: water (5:14:12,V/V/V).[121]
3. N-butanol: acetic acid (1%) (5:5, V/V), ethyl acetate: n-butanol: acetic acid (1%) (5:0.8:5, V/V).[122]
4. Ethyl acetate: n-butanol: water (4:1:5, V/V).[123]
5. N-hexane: ethyl acetate: n-butanol: water (1:5:1:5, V/V/V/V).[124]
6. Ethyl acetate: n-butanol: water (13:3:10, V/V/V).[125]

41. SEPARATION OF VOLATILE COMPOUNDS

1. Petroleum ether: acetonitrile: acetone (4:3:1, V/V/V).[93]
2. 0.05% aqueous trifluoroacetic acid: methanol (97.5:2.5, V/V).[126]
3. N-hexane: ethyl acetate: methanol: water (1:1:1:1, V/V/V/V).[127]
4. N-hexane: ethyl acetate: methanol: water (4:5:4:5, V/V): (1:5:1:5, V/V).[128]
5. N-hexane: ethyl acetate: methanol: water (1:6:1:6, V/V).[129]

42. SUMMARY AND CONCLUSION

Over the past few decades, Natural Products have contributed to drug development and continue to do so. In Recent years, Study on Phytochemicals from plants becomes more and more popular due to their Proved health Benefits. The lab intensive and time consuming of extraction and isolation processes, however, have hindered the application of natural products in drug development. As technology continues to develop, more and more new automatic and rapid techniques have been created to extract and separate natural products, which might reach the requirement of high-throughput screening regarding extraction, the most commonly employed technique for preparative separation is reflux extraction. The modern extraction methods, also regarded as green extraction methods, including ultrasound associated extraction, microwave associated extraction , supercritical fluid extraction and pressurized liquid extraction have also been the subject of increased attention in recent years due to their high extraction yields, selectivity, stability of the target extracts and process safety merits. Some of those Green methods have become routine sample preparation methods for analytical purposes regarding isolation, the development of novel packing material could enhance the efficiency of isolation, which although the isolation of pure natural products from complex mixtures remains challenging and we are far from one-step isolation procedures, the application of more selective methods from extraction to fractionation and purification will speed up the time from collecting biological material to isolating the final purified compound. In conclusion, there is a clear and increasing interest in the extraction and isolation of natural products and their advantageous applications. These specific applications are also conditioning the employed extraction methods and Novel Stationary Phases and Mobile phases to be used by these techniques. It is thus expected that these trends will be maintained in the near futures they are mostly motivated by Emerging Consumer Demands and by Safety, Environmental and Regulatory Affairs. Complementary Research is also needed to enhance the potential functionalities of the Phytochemicals in future, where such plants have shown to contain numerous phytochemicals that may be beneficial to human health. The compiled results indicated that many of their bioactive compounds remain to be fully isolated, identified, and characterized (alkaloids, diterpenoids, and so on).therefore, phytochemicals can be considered as the source of natural medicines. The compounds of plants are bioaccessible and bioavailable in humans with some demonstrated health benefits including antioxidant, anti-inflammatory, anti-cancer, anti-microbial, hypoglycemic action, etc. Additional well-designed human intervention studies and clinical trials are needed to validate the health benefits of phytochemicals.

42. AUTHORS CONTRIBUTION STATEMENT

Mr.Gowtham gathered the material with regard to this work. Mr.Gokul and Ms.Nandhini analyzed those data and necessary inputs were given towards the designing of the manuscript. Mr.Gowrisankar K B and Mr. Hari hara sudhan M R provided the valuable inputs towards designing of the manuscript. Dr. N. Asthalakshmi guided the preparation of the manuscript. Dr.M.Surendra kumar provided valuable suggestions for the preparation of the manuscript.Dr. SD Latha identified a correction in the manuscript and contributed to the revision. All authors read and approved the final version of the manuscript.

43. CONFLICT OF INTEREST

Conflict of interest declared none.

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