4.0 MATERIAL AND METHOD

When human life started on earth, the basic needs of humans are fulfilling by plants and them by product in the form of medicine, healthcare, food, clothing, shelter, agriculture, agrochemicals, pharmaceuticals, narcotics, etc. at first the human life were fully depending on the basic essentials of GOD in the form of Prithvi (Earth), Agni (Fire), Jal (Water), Vayu (Air) and Akash (Space). These essentials of nature were visible god and as a result, got security for spiritual, pious cultural and social reasons. There are 72,000 plant species being used in various human cultures approximately the earth for medicinal reason. Out of which, 17,000 flowering plants report from India and 8000 plants are used in a range of systems of Indian medicine. The energetic principles found in medicinal plants are alkaloids, glycosides, tannins, flavonoides or other related compounds of a very complex nature. They are found in root, bark, nuts, stem, leaf, fruit and seed etc. About 70-80% of the rural populations in many steamy budding countries still depend on traditional medicine for their primary physical condition mind, which also means that the people have to depend on medicinal plant life for healing. India is the seventh largest and one of the 12 mega biodiversity countries in the world covering wide diversity in environmental and bio-geographical conditions which lead to the progress of a wide range of plant life types and represents extremely rich plants including a large number of endemic groups.

4.1 Extraction techniques of Medicinal plants

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures. The products so obtained from plants are relatively impure liquids, semisolids or powders intended only for oral or external use.

These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts and powdered extracts. Such preparations popularly have been called galenicals, named after Galen, the second century Greek physician. The purposes of standardized extraction procedures for crude drugs are to

attain the therapeutically desired portion and to eliminate the inert material by treatment with a selective solvent known as menstruum.

The extract thus obtained may be ready for use as a medicinal agent in the form of tinctures and fluid extracts, it may be further processed to be incorporated in any dosage form such as tablets or capsules, or it may be fractionated to isolate individual chemical entities such as ajmalicine, hyoscine and vincristine, which are modern drugs. Thus, standardization of extraction procedures contributes significantly to the final quality of the herbal drug.

Methods of Extraction of Medicinal Plants Maceration In this process, the whole or coarsely powdered crude drug is placed in a stoppered container with the solvent and allowed to stand at room temperature for a period of at least 3 days with frequent agitation until the soluble matter has dissolved. The mixture then is strained, the marc (the damp solid material) is pressed, and the combined liquids are clarified by filtration or decantation after standing.

a. Infusion

Fresh infusions are prepared by macerating the crude drug for a short period of time with cold or boiling water. These are dilute solutions of the readily soluble constituents of crude drugs.

b. Digestion

This is a form of maceration in which gentle heat is used during the process of extraction. It is used when moderately elevated temperature is not objectionable. The solvent efficiency of the menstruum is thereby increased.

c. Decoction

In this process, the crude drug is boiled in a specified volume of water for a defined time; it is then cooled and strained or filtered. This procedure is suitable for extracting water-soluble, heatstable constituents. This process is typically used in preparation of Ayurvedic extracts called “quath” or “kawath”. The starting ratio of crude drug to water is fixed, e.g. 1:4 or 1:16; the volume is then brought down to one-fourth its

original volume by boiling during the extraction procedure. Then, the concentrated extract is filtered and used as such or processed further.

d. Percolation

This is the procedure used most frequently to extract active ingredients in the preparation of tinctures and fluid extracts. A percolator (a narrow, cone-shaped vessel open at both ends) is generally used. The solid ingredients are moistened with an appropriate amount of the specified menstruum and allowed to stand for approximately 4 h in a well closed container, after which the mass is packed and the top of the percolator is closed. Additional menstruum is added to form a shallow layer above the mass, and the mixture is allowed to macerate in the closed percolator for 24 h. The outlet of the percolator then is opened and the liquid contained therein is allowed to drip slowly. Additional menstruum is added as required, until the percolate measures about three-quarters of the required volume of the finished product. The marc is then pressed and the expressed liquid is added to the percolate. Sufficient menstruum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting.

e. Hot Continuous Extraction (Soxhlet)

In this method, the finely ground crude drug is placed in a porous bag or “thimble” made of strong filter paper, which is placed in chamber E of the Soxhlet apparatus. The extracting solvent in flask A is heated, and its vapors condense in condenser D. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber E rises to the top of siphon tube C, the liquid contents of chamber E siphon into fl ask A. This process is continuous and is carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent. This effects tremendous economy in terms of time, energy and consequently financial inputs. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when converted into a continuous extraction procedure on medium or large scale.

f. Aqueous Alcoholic Extraction by Fermentation

Some medicinal preparations of Ayurveda (like asava and arista) adopt the technique of fermentation for extracting the active principles. The extraction procedure involves soaking the crude drug, in the form of either a powder or a decoction (kasaya), for a specified period of time, during which it undergoes fermentation and generates alcohol in situ; this facilitates the extraction of the active constituents contained in the plant material. The alcohol thus generated also serves as a preservative. If the fermentation is to be carried out in an earthen vessel, it should not be new: water should first be boiled in the vessel. In large-scale manufacture, wooden vats, porcelain jars or metal vessels are used in place of earthen vessels. Some examples of such preparations are karpurasava, kanakasava, dasmularista. In Ayurveda, this method is not yet standardized but, with the extraordinarily high degree of advancement in fermentation technology, it should not be difficult to standardize this technique of extraction for the production of herbal drug extracts.

g. Counter-current Extraction

In counter-current extraction (CCE), wet raw material is pulverized using toothed disc disintegrators to produce a fine slurry. In this process, the material to be extracted is moved in one direction (generally in the form of a fine slurry) within a cylindrical extractor where it comes in contact with extraction solvent. The further the starting material moves, the more concentrated the extract becomes. Complete extraction is thus possible when the quantities of solvent andmaterial and their flow rates are optimized. The process is highly efficient, requiring little time and posing no risk from high temperature. Finally, sufficiently concentrated extract comes out at one end of the extractor while the marc (practically free of visible solvent) falls out from the other end.

This extraction process has significant advantages:

i. A unit quantity of the plant material can be extracted with much smaller volume of solvent as compared to other methods like maceration, decoction, percolation.

ii. CCE is commonly done at room temperature, which spares the thermolabile constituents from exposure to heat which is employed in most other techniques.

iii. As the pulverization of the drug is done under wet conditions, the heat generated during comminution is neutralized by water. This again spares the thermolabile constituents from exposure to heat.

iv. The extraction procedure has been rated to be more efficient and effective than continuous hot extraction.

h. Ultrasound Extraction (Sonication)

The procedure involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitation. Although the process is useful in some cases, like extraction of rauwolfia root, its large-scale application is limited due to the higher costs. One disadvantage of the procedure is the occasional but known deleterious effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants through formation of free radicals and consequently undesirable changes in the drug molecules.

i. Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is an alternative sample preparation method with general goals of reduced use of organic solvents and increased sample throughput. The factors to consider include temperature, pressure, sample volume, analytic collection, modifier (co-solvent) addition, flow and pressure control, and restrictors. Generally, cylindrical extraction vessels are used for SFE and their performance is good beyond any doubt.

The collection of the extracted analytic following SFE is another important step: significant analytic loss can occur during this step, leading the analyst to believe that the actual efficiency was poor.

There are many advantages to the use of CO2 as the extracting fluid. In addition to its favorable physical properties, carbon dioxide is inexpensive, safe and abundant. But while carbon dioxide is the preferred fluid for SFE, it possesses several polarity limitations. Solvent polarity is important when extracting polar solutes and when strong analytic-matrix interactions are present. Organic solvents are frequently added to the carbon dioxide extracting fluid to alleviate the polarity limitations. Of late, instead of carbon dioxide, argon is being used because it is inexpensive and more

inert. The component recovery rates generally increase with increasing pressure or temperature: the highest recovery rates in case of argon are obtained at 500 atm and 150° C.

The extraction procedure possesses distinct advantages:

i. The extraction of constituents at low temperature, which strictly avoids damage from heat and some organic solvents.

ii. No solvent residues.

iii. Environmentally friendly extraction procedure.

The largest area of growth in the development of SFE has been the rapid expansion of its applications. SFE finds extensive application in the extraction of pesticides, environmental samples, foods and fragrances, essential oils, polymers and natural products. The major deterrent in the commercial application of the extraction process is its prohibitive capital investment.

j. Phytonics Process

A new solvent based on hydrofluorocarbon-134a and a new technology to optimize its remarkable properties in the extraction of plant materials offer significant environmental advantages and health and safety benefits over traditional processes for the production of high quality natural fragrant oils, flavors and biological extracts. Advanced Phytonics Limited (Manchester, UK) has developed this patented technology termed “phytonics process”. The products mostly extracted by this process are fragrant components of essential oils and biological or phytopharmacological extracts which can be used directly without further physical or chemical treatment.

The properties of the new generation of fluorocarbon solvents have been applied to the extraction of plant materials. The core of the solvent is 1,1,2,2-tetrafluoroethane, better known as hydrofluorocarbon-134a (HFC-134a). This product was developed as a replacement for chlorofluorocarbons. The boiling point of this solvent is -25° C. It is not flammable or toxic. Unlike chlorofluorocarbons, it does not deplete the ozone layer. It has a vapor pressure of 5.6 bar at ambient temperature. By most standards this is a poor solvent. For example, it does not mix with mineral oils or triglycerides and it does not dissolve plant wastes.

The process is advantageous in that the solvents can be customized: by using modified solvents with HFC-134a, the process can be made highly selective in extracting a specific class of phytoconstituents. Similarly, other modified solvents can be used to extract a broader spectrum of components. The biological products made by this process have extremely low residual solvent. The residuals are invariably less than 20 parts per billion and are frequently below levels of detection. These solvents are neither acidic nor alkaline and, therefore, have only minimal potential reaction effects on the botanical materials. The processing plant is totally sealed so that the solvents are continually recycled and fully recovered at the end of each production cycle. The only utility needed to operate these systems is electricity and, even then, they do no consume much energy. There is no scope for the escape of the solvents. Even if some solvents do escape, they contain no chlorine and therefore pose no threat to the ozone layer. The waste biomass from these plants is dry and "ecofriendly" to handle.

Advantages of the Process

Unlike other processes that employ high temperatures, the phytonics process is cool and gentle and its products are never damaged by exposure to temperatures in excess of ambient. • No vacuum stripping is needed which, in other processes, leads to the loss of precious volatiles. • The process is carried out entirely at neutral pH and, in the absence of oxygen, the products never suffer acid hydrolysis damage or oxidation. • The technique is highly selective, offering a choice of operating conditions and hence a choice of end products. • It is less threatening to the environment. • It requires a minimum amount of electrical energy. • It releases no harmful emissions into the atmosphere and the resultant waste products (spent biomass) are innocuous and pose no effluent disposal problems. • The solvents used in the technique are not flammable, toxic or ozone depleting. • The solvents are completely recycled within the system.

Applications

The phytonics process can be used for extraction in biotechnology (e.g for the production of antibiotics), in the herbal drug industry, in the food, essential oil and flavor industries, and in the production of other pharmacologically active products. In particular, it is used in the production of top quality pharmaceutical-grade extracts,

pharmacologically active intermediates, antibiotic extracts and phytopharmaceuticals. However, the fact that it is used in all these areas in no way prevents its use in other areas. The technique is being used in the extraction of high-quality essential oils, oleoresins, natural food colors, flavors and aromatic oils from all manner of plant materials. The technique is also used in refining crude products obtained from other extraction processes. It provides extraction without waxes or other contaminants. It helps remove many biocides from contaminated biomass.

4.2 Parameters for Selecting an Appropriate Extraction Method

i. Authentication of plant material should be done before performing extraction. Any foreign matter should be completely eliminated.

ii. Use the right plant part and, for quality control purposes, record the age of plant and the time, season and place of collection.

iii. Conditions used for drying the plant material largely depend on the nature of its chemical constituents. Hot or cold blowing air flow for drying is generally preferred. If a crude drug with high moisture content is to be used for extraction, suitable weight corrections should be incorporated.

iv. Grinding methods should be specified and techniques that generate heat should be avoided as much as possible.

v. Powdered plant material should be passed through suitable sieves to get the required particles of uniform size.

vi. Nature of constituents:

a) If the therapeutic value lies in non-polar constituents, a non-polar solvent may be used. For example, lupeol is the active constituent of Crataeva nurvala and, for its extraction, hexane is generally used. Likewise, for plants like Bacopa monnieri and Centella asiatica, the active constituents are glycosides and hence a polar solvent like aqueous methanol may be used.

b) If the constituents are thermolabile, extraction methods like cold maceration, percolation and CCE are preferred. For thermostable constituents, Soxhlet extraction (if nonaqueous solvents are used) and decoction (if water is the menstruum) are useful.

c) Suitable precautions should be taken when dealing with constituents that degrade while being kept in organic solvents, e.g. flavonoids and phenyl propanoids.

d) In case of hot extraction, higher than required temperature should be avoided. Some glycosides are likely to break upon continuous exposure to higher temperature.

e) Standardization of time of extraction is important, as:

i. Insufficient time means incomplete extraction.

ii. If the extraction time is longer, unwanted constituents may also be extracted. For example, if tea is boiled for too long, tannins are extracted which impart astringency to the final preparation.

f) The number of extractions required for complete extraction is as important as the duration of each extraction.

vii. The quality of water or menstruum used should be specified and controlled.

viii. Concentration and drying procedures should ensure the safety and stability of the active constituents. Drying under reduced pressure (e.g. using a Rotavapor) is widely used. Lyophilization, although expensive, is increasingly employed.

ix. The design and material of fabrication of the extractor are also to be taken into consideration.

x. Analytical parameters of the final extract, such as TLC and HPLC fingerprints, should be documented to monitor the quality of different batches of the extracts.[113]

MATERIAL

Table. No. 4.1 Equipment List

S. No.	Equipment	Model	Manufacturer
1	Weighing balance	AW 120	Shimadzu Corporation, Japan.
2	Weighing balance	Analytical balance	Sartorius GK Germany
3	Ultra Sonicator	Power Sonic 405	Huwashin Technology, Korea.
4	UV Spectrophotometer	Systronics 2202	Shimadzu Analytical India Pvt. Ltd.
5	Magnetic Stirrer with Hot Plate	LAB.MAG.101555094	Suresh Electricals and scientific works
6	Ostwald Viscometer	RG-93301	Borosilicate Glass wares
7	Specific Gravity Bottles	QLBG 074	Borosilicate Glass wares
8	Refrigerator	RD EDGE 215B TAF TH ST	Godrej and Boyce mfg.co.ltd
9	pH meter	pH Tutor	Eutech Instruments, India
10	FTIR	IRTracer-100	Shimadzu, Japan.
11	Hot Air Oven	DIGITAL MODEL	LABTECHPLUS
12	Muffle Furnace	Digital P.I.D. controller	LABTECHPLUS

All chemicals/reagents used in this study were purchased from SD Fine-Chem Ltd., Mumbai, India. All reagents and solvents used were of analytical grade. Water used in all analyses was ultra-pure distilled water.

METHOD

4.3 PREPARATION OF WALNUT KERNEL SEPTUM MEMBRANES:

Maceration is one of the oldest and simplest extraction methods in which coarse and powdered plant material is soaked in solvents such as methanol, ethanol, ethyl acetate, acetone, hexane etc. It is one of the general and budget techniques used for the extraction of dissimilar bioactive compounds from plant material. Though, maceration procedure has certain limitations such as low extraction yield, lower efficiency and use of big amount of solvents which have some health risks. Furthermore, the selection of suitable solvent is significant along the methodology for the extraction of specific plant extract. Maceration process contains of grinding of plant material into smaller particles to increase the surface area for easy mixing with solvent and efficient extraction of compounds. Before this mixture of plant material and solvent is kept for lengthier time, agitated at different intervals and filtered through a filtration medium. The effectiveness for the removal of bioactive compounds from the plant material depends on the kind of solvent and type of plant material. Maceration can be done with or without heat or agitation to enhance mass transfer. The material is left in the solvent for an extended period of time.

Fig. No. 4.1 Walnut kernel septum membranes

Fig. No. 4.2 Walnut kernel septum membranes powdered

The Walnut fruit was purchased from traditional medicine shopkeeper in local market of Hyderabad, Telangana, INDIA and confirmed by an expert botanist. crushing walnuts in the laboratory using a wooden hammer, by braking of walnut fruit separating the Walnut kernel septum membranes. carefully removing the walnut septa. Each walnut septum sample was a bulk sample that consisted of walnut septa originating from the same tree. The walnut membrane septum was dried by natural method approximately 21 days, in a dark place, in order to remove the moisture initially present. The walnut septum membrane was divided into two parts. In one method the directly walnut septum membrane soak into ethanol and distilled water directly.

Figure No. 4.3 walnut septum membrane soak into ethanol and distilled (F1-F5)

Figure No. 4.4 walnut septum membrane soak into ethanol and distilled (F6-F10)

Another part of Walnut kernel septum membranes was powdered by electric grinder and then The powdered material was soak into ethanol and distilled water. The solvents used were Ethanol and distilled water with different ration, at room temperature with occasionally shaking for 21 days. The solution was holding on in an exceedingly airtight dry, clean and away from light. Afterwards, the mixture was filtered using whatman filter paper and collected the concentrated extract for further evaluation. Obtained extract was stored in a refrigerator for further tests.

4.4 Preparation of Walnut Kernel Septum Membranes solution:

Table. No. 4.2 Kernel Septum Membranes solution with different concentration.

Method	Ratio Alcohol : Water	Alcohol	Water	Formulation No.
walnut septum membrane	100:0	250ml	0	F1
	0:100	0	250ml	F2
	75:25	187.5ml	62.5ml	F3
	50:50	125ml	125ml	F4
	25:75	62.5ml	187.5ml	F5
walnut septum membrane in powder form	100:0	250ml	0	F6
	0:100	0	250ml	F_7
	75:25	187.5ml	62.5ml	F8
	50:50	125ml	125ml	F9
	25:75	62.5ml	187.5ml	F10

All chemicals/reagents used in this study were purchased from SD Fine-Chem Ltd., Mumbai, India. All reagents and solvents used were of analytical grade. Water used in all analyses was ultra-pure distilled water.

4.5 EVALUATION PARAMETER

a. **Determination of Moisture content:** Moisture content is determined via a thermos gravimetric method i.e. by loss on drying. In which, the sample is heated & the weight loss due to evaporation of moisture is recorded.

Requirements:

Apparatus: Porcelain dish, spatula, tongs, digital weighing machine, hot air oven, desiccator.

Chemicals: Walnut kernel septum membranes powder **(0.5gm)**

Procedure:

1. Weigh the empty porcelain dish and note the reading. W1
2. Take the sample of Walnut kernel septum membranes in porcelain dish.
3. Weigh the porcelain dish with sample in it and note the reading. W2
4. Keep the porcelain dish in pre-heated hot air oven for 15 minutes at 100°C-105'C.
5. After heating, keep the porcelain dish in desiccator for 15 minutes.
6. Take out the dish from desiccator, and weigh the porcelain dish with dried sample. W3
7. Repeat the drying in the oven using above steps till a constant weight not obtained.
8. After determining the constant weight, note the reading and calculate the percent moisture content.

Water/Moisture content $W = [(W_2 - W_3)/(W_3 - W_1)]\ 100$

b. Determination of total ash

Principle: Principle involved is that when a known weight of feed is ignited to ash, the weight of ash thus obtained is expressed in terms of percentage.

Apparatus:

- Silica crucible
- Tongs
- Weighing balance
- Electrical bunsen burner
- Muffle furnace
- Desiccator
- Asbestos sheet

Procedure:

1. Find out the weight of a clean dry crucible.
2. Place about 2 g of Walnut kernel septum membranes powder sample and weigh this to find out accurate weight of the sample taken.
3. Carefully place the weighed crucible over electric burner. The crucible should be partially opened.
4. The sample will get charred with initial expulsion of smoke.
5. Place the crucible in a muffle furnace and heat to 600ºC. Keep it for 2 hours. At this temperature all organic matter will be burnt leaving behind minerals.
6. Remove the crucible from the furnace carefully and cool it in a desiccator to room temperature and weight again.

c. Determination of the boiling point of the solution

Materials Required: fusion tube, stand with clamp, capillary tube, tripod, thermometer, and kerosene burner

1. Take a capillary tube and close its one end by holding the end in the flame and rotate it for 2-3 minutes.
2. Transfer a few mL of Walnut kernel septum membranes solution to the fusion tube.
3. Dip the capillary tube into the liquid in the fusion tube keeping the sealed end up.

4. Insert the tube in one of the holes of aluminum block and insert the thermometer in the other hole.
5. Make sure that the liquid is visible in the fusion tube after it is put in the hole.
6. Place the Aluminum block on the tripod.
7. Start heating the block with the help of a kerosene burner.
8. Note down the temperature soon as the regular streams of bubbles are seen out of liquid in the fusion tube.

d. Determination of Specific gravity of the solution

1. Clean thoroughly the specific gravity bottle with chromic or nitric acid.
2. Rinse the bottle at least two to three times with purified water.
3. If required, rinse the bottle with an organic solvent like acetone and dry.
4. Take weight of empty dry bottle with capillary tube stopper (W1).
5. Fill the bottle with distilled water and place stopper; wipe out excess liquid from side tube using tissue paper (W2).
6. Weight bottle with stopper and water on analytical balance (W3).
7. Repeat the procedure for liquid under test by replacing the water after emptying and drying as mentioned in step 4 to 6.
8. Weight bottle with stopper and liquid under test on analytical balance (W4).

Formula for specific gravity: Specific gravity of liquid under test (solution) = weight of liquid under test /weight of water = w5/w4.

e. Determination of density of the solution

1. Clean thoroughly the specific gravity bottle with chromic acid or nitric acid.
2. Rinse the bottle at least two to three times with distilled water.
3. If required, rinse the bottle with an organic solvent like acetone and dry.
4. Take the weight of empty dry bottle with capillary tube stopper (W1).

5. Fill the bottle with Walnut kernel septum membranes solution and place the stopper, wipe out excess liquid from outside the tube using tissue paper.
6. Weight bottle with Walnut kernel septum membranes solution on analytical balance (W2).
7. Calculate weight in grams of Walnut kernel septum membranes solution liquid (W3).

Formula for density: Density of liquid under test (solution) = weight of liquid under test /volume of liquid under test = w3/v

f. Determination of Viscosity of the solution

1. Thoroughly clean the Ostwald viscometer with warm chromic acid and if necessary used an organic solvent such as acetone.
2. Mount viscometer in vertical position on a suitable stand.
3. Fill water in dry viscometer up to mark G.
4. Count time required, in second for water to flow from mark A to mark B.
5. Repeat step 3 at least 3 times to obtained accurate reading.
6. Rinse viscometer with Walnut kernel septum membranes solution and then fill it up to mark A, find out the time required for liquid to flow to mark B.
7. Determination of densities of liquid as mentioned in density determination experiment.

Formula for viscosity

$$\text{Density of test liquid} = \frac{\text{Time required to flow test liquid Viscosity}}{\text{Density of water} \times \text{Time required to flow water}} \times \text{Viscosity of water}$$

pH determination: The pH determination of Walnut kernel septum membranes solution by using two techniques.

a) Glass electrode.

b) pH paper.

Procedure for glass electrode

1. Prepare 30ml buffer of each pH. The volume of the stock solution to be taken. Prepare the buffer by mixing appropriate volume.
2. Allow the solution for 15minutes to establish equilibrium.
3. Measure the pH of Walnut kernel septum membranes solution using a pH meter.

g. Determination of Organoleptic Characters

Organoleptic evaluation resources the study of drugs using organs of senses. It refers to the approaches of analysis like colour, odour, taste, size, shape and special features, such as touch, texture, etc. Clearly, the initial sight of the plant or extract is so specific that it inclines to classify itself. If this is not enough, maybe the plant or extract has a distinguishing odour or taste. The education of form of a crude drug is morphology while explanation of the form is morphography. yet the greatest human form of examination.

h. High performance thin-layer chromatography (HPTLC) in the quality control of herbal products

The introduction of high performance thin-layer chromatography (HPTLC) for quality control of herbal products, using standardized methodology and system suitability tests for the qualification of the plates, has improved reproducibility.

Figure No. 4.5 HPTLC

The use of intensity markers implemented by the Ph. Eur. improved the description and interpretation of the chromatograms. Quantitative information can be retrieved from the electronic images of the chromatograms and used for comprehensive HPTLC fingerprinting: a single HPTLC analysis gives information on identity, purity and content of an herbal drug/preparation/product, simplifying the quality control.

i. HPLC method

a. Chemicals and Reagents

Methanol (MeOH) and acetonitrile (ACN), HPLC grade, were acquired from Merck. Acetic acid 99% and trifluoroacetic acid (TFA) 99% were obtained from Merck. The LiChrolut RP-18 (C18, 3 mL, 500 mg) SPE containers used were supplied by Merck (Darmstadt, Germany). Ultrapure water was provided by a Milli-Q decontamination system (Millipore, Bedford, MA, USA). The flavonoids catechin 98%, rutin 98%,

myricetin 98%, luteolin 98%, quercetin 98%, kaempferol 98%, and apigenin 98% were supplied by Sigma-Aldrich (Steinheim, Germany). Stock standard solutions at 1000 mg/L concentration level were prepared and stored in dark brown glass bottles at -20 °C. Working standard solutions were ready in MeOH after suitable dilution of the stock solutions each laboratory day, before analysis.

b. Instrumentation

A quaternary low-pressure incline HPLC–DAD system by Shimadzu (Kyoto, Japan) was used for examination. The HPLC system consisted of: (a) an FCV-10ALVP mixing system, (b) a Rheodyne 7725i injection valve, and a 20 µL loop for sample injection, (c) an LC- 10ADVP pump equipped with a Shimadzu SCL-10ALVP System Controller, (d) an SPD-M10AVP photodiode array detector. Real time examination monitoring and post run dispensation were carried out using the software Lab Solutions-LC solutions, supplied by Shimadzu. A glass space filtration apparatus, acquired by All tech Associates, and nylon 0.2 µm membrane Filters were utilized for the filtration of the mobile phase, and a DGU-10B de-gassing unit with helium was used for degassing. A vortexer purchased from FALC Instruments was used for sample agitation. Centrifugation was carried out using a HermLe centrifuge, model Z-230. An ultrasonic bath (MRC: DC-150H) by MRC was used for specimen preparation. For disappearance, after SPE extraction, a ReactiVap 9-port evaporator model 18,780 by Pierce was utilized. For sample filtration, prior to the injection in the chromatographic system, Q-Max RR syringe filters (0.45 µm nylon membrane) were purchased from Frisenette ApS.

c. Chromatographic Separation and Analysis

The chromatographic parting of the flavonoids was attained on a C18 Universe column (250 mm 4.6 mm, 5 µm), supplied by Fortis Technologies Ltd. on, A reverse-phase HPLC test was carried out using a gradient scheme with 1 mL/min flow rate, thermos stated at 30 ◦C. The mobile phase consisted of (A) 1% acetic acid in water, and (B) ACN. The gradient elution program begun with 80:20, v/v (A: B), gradually increasing to 50:50, v/v (A: B), in the following 25 min, and then outstanding constant for the next 5 min. The initial circumstances were restored for 10 min, prior to the next injection. The injection volume was 20 µL of solution and the total run

time was less than 25 min for each injection. For peak identification, the Rts of the peaks of the real samples were compared with the Rts of the standard mixtures, along with the spectral information providing by the DAD sensor that operated over the variety 280–400 nm. Peak nursing and quantitation were achieved at the maximum wavelength of each analyte.

d. Sample Collection

walnut septa models were created after crushing walnuts in the workroom using a wooden hammer, and carefully removing the walnut septa. Each walnut septum taster was a bulk sample that contained of ten walnut septa originating from the same tree. In this way, bulk walnut septum samples were created in the laboratory. All the walnut samples were collected during the harvesting period of November 2022.

e. Sample Preparation

The samples were homogenous in a porcelain mortar and stored at 20 °C until analysis. For model preparation, 50 mg of each standardized bulk sample was weighted in 2-mL Eppendorf tubes, and then, 0.5 mL of 0.05% TFA in methanol: water at 60:40 ratios (*v/v*), was added. The combination was vortexed for 1 min and, then ultra-sound assisted extraction acquired place in an ultrasonic bath for 10 min at 25 °C. Each sample was centrifuged for 10 min at 10,000 rpm and, then, the supernatant was collected, according to Kalogiouri et al. The extract was further diluted with water at a final volume of 2 mL. The diluent was purified using a adapted version of the SPE protocol planned by Bajkacz et al. The LiChrolut RP-18 (C18, 3 mL, 500 mg) SPE cartridges were used for this purpose. First, the C18 column was conditioned with 2 mL MeOH, followed by 2 mL of water. Then, the diluted sample extract was passed through the sorbent at a flow rate of roughly 1 mL/min. The analytes were eluted with 3 mL MeOH and the eluates were vanished to dryness with nitrogen. The residues were melted in 1 mL MeOH, and filtered through 0.22 μm nylon syringe filters. Finally, 20 μL was injected into the chromatographic system.

f. Method validation and Quantification

Linearity, choosiness, LODs and LOQs, within-day, and between-day precision and meticulousness were evaluated. Linearity was inspected by testing the lack-of-fit of the calibration curves finished the range 1–10 μg/g. The slopes, intercepts, and the resolve coefficients of each analyte were calculated using last square linear retreat analysis. LODs and LOQs were designed by the equations:

$$\text{LOD} = 3.3 \times Sa/b$$

$$\text{LOQ} = 10 \times Sa/b$$

where, *Sa* is the standard error of the intercept α; and b is the slope of the calibration curve. Precision was evaluated after spiking a wholesale sample at 1, 5, and 10 mg/kg concentration level, and examination was performed in triplicate. Accuracy was articulated as relative recovery, and precision was uttered as relative standard deviation (RSD%). Repeatability, expressed as within-day precision, was assessed in six replicates (n = 6), and reproducibility, articulated as between-days precision, was assessed after examining the spiked bulk samples within three consecutive days (n = 3 3). The analytes were measured using the consistent calibration curves of the standards. For the quantification of the analytes with high meditations that exceeded beyond the linear choice, such as catechin, the extracts were extra diluted with MeOH and re-injected in the chromatographic system to ensure that their calculated attentiveness was within the lined range of the curves

g. Chemometric Analysis

The quantification results were managed with one-way analysis of variance (ANOVA), using the data analysis tool of Microsoft Excel (Microsoft, Redmond, WA, USA). ANOVA was applied to inspect potential statistically significant modifications among the flavonoids' concentrations as well as the many walnut septa variants. The results were assessed using a p-value at a 95% confidence level. When the p-value was less than 0.05., there was a statistically important change between the samples; if the p-value was more than 0.05., there was no statistically significant difference. PCA (Primary Component Analysis) was utilized to investigate the

interactions between the identified flavonoids and the replicas from the different types. Utilizing the Metabo Analyst R package, PCA was generated in R.

h. Procedure for FT-IR studies

The goal of this study was to use FT-IR spectroscopy to ascertain the content and qualitative and quantitative properties of bioactive combinations in native herbal teas that are typically drank.

The FTIR spectra of the samples were verified FTIR instrument (Shimadzu, 8400S). A small quantity of sample was made into pellets using KBr for FTIR analysis. The data of infrared transmission was composed over a wave number ranged from 4000 cm-1to 500 cm 1. The spectra were associated with reference to recognize the characteristic functional groups present. FTIR spectra used for metabolite summarizing of the herbal samples.

i. To study in vitro drug release profile of prepared solution

Osmosis is the phenomena in which solvent particles permit through a semi-permeable membrane (parchment paper) from an area of developed concentration to an area of lesser concentration. The procedure continues until the quantity of liquid is balanced or equalized in both areas, the region of higher concentration and the region of lower concentration of the semipermeable membrane (parchment paper). In other words, osmosis is the diffusion or movement of solution from an area of higher solution potential to a region of lesser water potential.

Fig. No. 4.6 Assembly of drug release appratus

The walnut kernel septum membrane solution was examined in vitro utilizing the osmosis method and a magnetic stirrer instrument with a bead used as the stirrer. The dissolution medium employed was 250 cc of pH 7.4 phosphate buffer, spun at 50 rpm. The experiment was conducted at a constant temperature of 37 ± 0.5°C. For each test, a solution of walnut kernel septum membranes was employed. At predetermined intervals, a syringe equipped with a pre-filter was used to remove 1 milliliter of the dissolving media sample. The sample was then diluted appropriately with phosphate buffer and the absorbance at 360 nm was measured to determine the drug release. A new volume of dissolving liquid was added to the volume removed at each time interval. The amount of Walnut kernel septum membranes solution released was calculated and plotted against time.

Stability Testing:

Consistency The produced walnut kernel septum membrane solution was tested while the sample was reserved at a higher temperature. Ten sections of the final walnut kernel septum membrane solutions, A, B, and C, were held at room temperature and 40 degrees, respectively, at an accelerated temperature. At one-, two-, and three-month intervals, the solution was examined for all physicochemical limitations, homogeneity, and turbidity to appearance for any changes.[112]