International Journal of Advanced Research in Chemical Science (IJARCS)
Volume 2, Issue 4, April 2015, PP 25-32
ISSN 2349-039X (Print) & ISSN 2349-0403 (Online)
www.arcjournals.org

General Techniques Involved in Phytochemical Analysis

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Abstract: *Plants are recognized in the pharmaceutical industry for their broad structural diversity as well as their wide range of pharmacological activities. The biologically active compounds present in plants are called phytochemicals. These phytochemicals are derived from various parts of plants such as leaves, flowers, seeds, barks, roots and pulps. These phytochemicals are used as sources of direct medicinal agents. They serve as a raw material base for elaboration of more complex semi-synthetic chemical compounds. This paper mainly deals with the collection of plants, the extraction of active compounds from the various parts of plants, qualitative and quantitative analysis of the phytochemicals.*

Keywords: *Phytochemicals, Decoction, Infusion, HPLC, HPTLC, OPLC, NMR, X-Ray crystallography.*

1. INTRODUCTION

Phytochemicals are the chemicals that present naturally in plants. Now- a-days these phytochemicals become more popular due to their countless medicinal uses. Phytochemicals play a vital role against number of diseases such as asthma, arthritis, cancer etc. unlike pharmaceutical chemicals these phytochemicals do not have any side effects. Since the phytochemicals cure diseases without causing any harm to human beings these can also be considered as "man-friendly medicines". This paper mainly deals with collection, extraction, qualitative and quantitative analysis of phytochemicals.

2. STEPS INVOLVED IN PLANT COLLECTION

2.1. Collection of Plants

Plants under consideration may be collected either from wild forests or from herbariums. When plants are collected from wild, there is a risk that they have been incorrectly identified. The major advantage of wildlife plants is that they will not contain any pesticides. After the plants are collected from wild or from herbarium they have to be processed for cleaning in order to prevent the deterioration of phytochemicals present in plants.

2.2. Cleaning of Plants

After plants collection they have to be cleaned properly. The cleaning process may involve the following steps. Cleaning, washing, peeling or stripping leaves from stems. Cleaning has to be done by hands in order to get better results.

2.3. Drying

The main purpose of drying is to remove the water content from plants so that the palnts can be stored. Plants have to be dried immediately as soon as the plants collection or this will lead to spoilage of plant materials. The drying consists of two methods. Drying can be done either by natural process or by artificial process.

2.3.1. Natural Process

Natural process includes sun- drying. Sometimes plants are placed on drying frames or on stands, to be air-dried in barns or sheds. But this may take few weeks for complete drying. The time depends on temperature and humidity.

2.3.2. Artificial Drying

Artificial drying can be done with the help of artificial driers. This process will reduce the drying time to several hours or minutes. The common method that is followed in drying medicinal plants is warm-air drying. In this process plants are placed in the plates of drier on which warm air is blown. This method is mainly applicable to fragile flower and leaves and this requires large number of workers since loading and unloading of plants has to be done manually.

2.4. Powdering

After complete drying of plants they have to be powdered well for further analysis

3. METHODS OF EXTRACTION

3.1. Plant Tissue Homogenization

Plant tissue homogenization in solvent has been widely used by researchers. Dried or wet, fresh plant parts are grinded in a blender to fine particles, put in a certain quantity of solvent and shaken vigorously for 5 - 10 min or left for 24 h after which the extract is filtered. The filtrate then may be dried under reduced pressure and re-dissolved in the solvent to determine the concentration. Some researchers however centrifuged the filtrate for clarification of the extract [1].

3.2. Serial Exhaustive Extraction

It is another common method of extraction which involves successive extraction with solvents of increasing polarity from a non polar (hexane) to a more polar solvent (methanol) to ensure that a wide polarity range of compounds could be extracted. Some researchers employ soxhlet extraction of dried plant material using organic solvent. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds [1].

3.3. Soxhlet Extraction

Soxhlet extraction is only required where the desired compound has a limited solubility in a solvent, and the impurity is insoluble in that solvent. If the desired compound has a high solubility in a solvent then a simple filtration can be used to separate the compound from the insoluble substance. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This method cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds [2].

3.4. Maceration

In maceration (for fluid extract), whole or coarsely powdered plant- drug is kept in contact with the solvent in a stoppered container for a defined period with frequent agitation until soluble matter is dissolved. This method is best suitable for use in case of the thermolabile drugs [3].

3.5. Decoction

This method is used for the extraction of the water soluble and heat stable constituents from crude drug by boiling it in water for 15 minutes, cooling, straining and passing sufficient cold water through the drug to produce the required volume [4].

3.6. Infusion

It is a dilute solution of the readily soluble components of the crude drugs. Fresh infusions are prepared by macerating the solids for a short period of time with either cold or boiling water [4].

3.7. Digestion

This is a kind of maceration in which gentle heat is applied during the maceration extraction process. It is used when moderately elevated temperature is not objectionable and the solvent efficiency of the menstrum is increased thereby [4].

3.8. 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 menstrum 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 menstrum 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 menstrum 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 menstrum is added to produce the required volume, and the mixed liquid is clarified by filtration or by standing followed by decanting [5].

3.9. 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 rauwolfi a 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 [5].

4. QUALITATIVE AND QUANTITATIVE ANALYSIS OF PHYTOCHEMICALS

4.1. Preliminary Qualitative Analysis

1. Test for Alkaloids

a. Mayer' s test

To a few ml of plant sample extract, two drops of Mayer's reagent are added along the sides of test tube. Appearance of white creamy precipitate indicates the presence of alkaloids.[6]

b. Wagner's test

A few drops of Wagner's reagent are added to few ml of plant extract along the sides of test tube. A reddish- Brown precipitate confirms the test as positive.[7]

2. Test for Amino acids

The extract (100 mg) is dissolved in 10 ml of distilled water and filtered through Whatmann No. 1 filter paper and the filtrate is subjected to test for Amino acids.

a. Ninhydrin test

Two drops of ninhydrin solution (10 mg of ninhydrin in 200 ml of acetone) are added to 2 ml of aqueous filtrate. Appearance of purple colour indicates the presence of amino acids. [8]

3. Test for Carbohydrates

a. Molish' s test

To 2 ml of plant sample extract, two drops of alcoholic solution of α- naphthol are added. The mixture is shaken well and few drops of concentrated sulphuric acid is added slowly along the sides of test tube. A violet ring indicates the presence of carbohydrates.

b. Benedict' s test

To 0.5 ml of filtrate, 0.5 ml of Benedict's reagent is added. The mixture is heated on a boiling water bath for 2 minutes. A characteristic coloured precipitate indicates the presence of sugar.

4. Test for Fixed oils and Fats

a. Spot test

A small quantity of extract is pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oils.

b. Saponification test

A few drops of 0.5 N alcoholic potassium hydroxide solution is added to a small quantity of extract along with a drop of phenolphthalein. The mixture is heated on a water bath for 2 hours. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.[9]

5. Test for Glycosides

For 50 mg of extract is hydrolysed with concentrated hydrochloric acid for 2 hours on a water bath, filtered and the hydrolysate is subjected to the following tests.

a. Borntrager's test

To 2 ml of filtered hydrolysate, 3 ml of choloroform is added and shaken, choloroform layer is separated and 10% ammomia solution is added to it. Pink colour indicates presence of glycosides.[6]

b. Legal's test

50 mg of extract is dissolved in pyridine, sodium nitroprusside solution is added and made alkaline using 10% NaOH. Presence of glycoside is indicated by pink colour.

6. Test for Phenolic compounds and Tannins

a. Ferric Chloride test

The extract (50 mg) is dissolved in 5 ml of distilled water. To this few drops of neutral 5% ferric chloride solution are added. A dark green colour indicates the presence of phenolic compound.[10]

b. Gelatin test

The extract (50 mg) is dissolved in 5 ml of distilled water and 2 ml of 1% solution of Gelatin containing 10% NaCl is added to it. White precipitate indicates the presence of phenolic compounds.[6]

c. Lead acetate test

The extract (50 mg) is dissolved in of distilled water and to this 3 ml of 10% lead acetate solution is added. A bulky white precipitate indicates the presence of phenolic compounds.

d. Alkaline reagent test

An aqueous solution of the extract is treated with 10% ammonium hydroxide solution. Yellow fluorescence indicates the presence of flavonoids.

e. Magnesium and Hydrochloric acid reduction

The extract (50 mg) is dissolved in 5 ml of alcohol and few fragments of magnesium ribbon and concentrated hydrochloric acid (drop wise) are added. If any pink to crimson colour develops, presence of flavonol glucosides is inferred.[11]

7. Test for phytosterols

a. Libermann-Burchard's test

The extract (50 mg) is dissolved in of 2 ml acetic anhydride. To this, 1 or 2 drops of concentrated sulphuric acid are added slowly along the sides of the test tube. An array of colour change shows the presence of phytosterols.[12]

8. Test for Proteins

The extract (100 mg) is dissolved in 10 ml of distilled water and filtered through Whatmann No. 1 filter paper and the filtrate is subjected to test for proteins.

a. Millon's test

To 2 ml of filtrate few drops of Millon's reagent are added. A white precipitate indicates the presence of proteins.[13]

b. Biuret test

2 ml of filtrate is treated with 1 drop of 2% copper sulphate solution. To this 1 ml of ethanol (95%) is added, followed by excess of potassium hydroxide pellets. Pink colour ethanolic layer indicates the presence of protein.[14]

9. Test for Saponins

The extract (50 mg) is diluted with distilled water and made up to 20 ml. The suspension is shaken in a graduated cylinder for 15 minutes. A two cm layer of foam indicates the presence of saponins.[9]

10. Test for gum and Mucilages

The extract (100 mg) is dissolved in 10 ml of distilled water and to this 2 ml of absolute alcohol is added with constant stirring. White or cloudy precipitate indicates the presence of Gums and Mucilages.[15]

11. Test for volatile oil

For volatile oil estimation 50 mg of powdered material (crude drug) is taken and subjected to hydro- distillation. The distillate is collected in graduate tube of the assembly, wherein the aqueous portion automatically separated out from the volatile oil.[16]

4.2. Qualitative and quantitative Analysis

Qualitative and quantitative analysis of phytochemicals can be done using Gas Chromatography-Mass Spectroscopy (GCMS). GCMS can be applied to solid, liquid and gaseous samples. First the samples are converted into gaseous state then analysis is carried out on the basis of mass to charge ratio. High Performance Liquid Chromatography is applicable for compounds soluble in solvents. High performance thin layer chromatography is applicable for the separation, detection, qualitative and quantitative analysis of phytochemicals.

4.2.1. Gas Chromatogrophy

Gas chromatography is applicable for volatile compounds. In this method, species distribute between a gas and a liquid phase. The gas phase is flowing and the liquid phase is stationary. When the sample molecules are in liquid phase they are stationary. The rate of migration depends on how much of chemical species is distributed into liquid phase. Higher the percentage of material in the gaseous state faster will be the migration. The species which distributes itself 100% in the stationary state will not migrate. If a sample distributes itself in both phases, it will migrate at an intermediate rate. This gas chromatography gives the total amount of vapour. Thus it is most widely used for quantitative analysis.

4.2.2. High Performance Liquid Chromatography: (HPLC)

HPLC is also known as High- Pressure Liquid Chromatography. This separates compounds on the basis of their interaction with solid particles of a tightly packed column and the solvent of the mobile phase. High pressures of up to 400 bars are required to elute the analyte through the column before they pass through detector. HPLC is useful for compounds that cannot be vapourised or that decompose under high temperatures. HPLC provides both quantitative and qualitative analysis in a single operation.

4.2.3. High Performance Thin Layer Chromatography: (HPTLC)

High Performance Thin layer Chromatography is a modified version of thin layer chromatography. High Performance Thin layer Chromatography is planer chromatography where separation of sample components is done on high performance layers with detection and acquisition using an advanced work- station. These high performance layers are pre-coated with a sorbent of particle size 5-7 microns and a layer thickness of 150-200 microns. The reduction in the thickness of the layer and the particle size results in increasing the plate efficiency along with nature of separation. HPTLC is suitable for qualitative, quantitative and micro-preparative chromatography.

4.2.4. Optimum Performance Laminar Chromatography: (OPLC)

OPLC combines the advantages of TLC and HPLC. The system separates about 10-15 mg samples, with simultaneous processing of up to 4 or 8 samples at a time depending on the model. In OPLC a pump is used to force a liquid mobile phase through a stationary phase, such as silica or a bonded-phase medium. The OPLC column housing structure allows flat planar columns to be used in the same way as cylindrical glass or stainless steel ones. The flat column is pressurized up to 50 bars, and mobile phase is forced through it at constant linear velocity via a solvent delivery pump. The work station includes all of the modules required for effective separation of the compound sample of interest, including two 96- well plate sample holders and automated sampling system that withdraws a sample from each well and places it on the OPLC planar sorbent bed, a solvent delivery system including a mobile phase degasser and pump, OPLC purification unit, a four channel diode array detector to monitor the eluent and fraction collector to six 96- well plates to hold the separated compounds.

5. METHODS OF DETECTION

Spectroscopy is used in the detection of phytochemicals. The following are frequently used in the study of phytochemicals.

UV- To find out whether the system is conjugated(the coloured compounds such as β- carotene, crocetin are in system of extensively conjugated pi-electrons).

IR - To identify the functional groups that are present in the compound.

MS - To determine the molecular weight of the compound and to identify the presence of isotopes patterns for Cl and Br.

^{13}C- NMR - To identify how many types of carbon atoms are present in the compound.

^{1}H- NMR - To find out how many types of hydrogen atoms are present in the compound and to find out how the hydrogen atoms are connected.

5.1. UV Spectroscopy

Ultraviolet and visible spectroscopy is the measurement of the attenuation of a beam of light after it passes through a sample or after reflection from a sample surface. Ultraviolet radiation is energetic enough to promote outer electrons to higher energy level and UV spectroscopy is usually applied to molecules or inorganic complexes in solution. This results from transition between the electronic energy levels. Measuring the absorbance at some wavelength by applying Beer- Lambert's law can determine the concentration of the analyte solution. It is useful to characterize the absorption, transmission and reflectivity of a variety of important materials such as pigments and other compounds from plants. This qualitative application requires recording atleast a portion of the UV- Visible spectrum for characterization of the optical or electronic properties of materials.

5.2. IR Spectroscopy

IR spectroscopy is used to determine the functional group present in the sample. Infrared absorption spectroscopy is the measurement of the wavelength and intensity of the absorption of mid-infrared light by a sample. Mid-infrared light is energetic enough to excite molecular vibrations to higher energy levels. The wavelength of many IR absorption bands are characteristics of specific types of chemical bonds, and IR spectroscopy finds its greatest utility for qualitative analysis of organic and organometallic molecules. IR spectroscopy is used to confirm the identity of a particular compound and as a tool to determine the newly synthesized molecule.

5.3. Mass Spectroscopy

Mass spectroscopy is a powerful tool for the identification of materials. Mass spectrometry has become one of the most important tools in the biochemical sciences with capability ranging from small molecule analysis to protein characterization. Mass spectrometry is a powerful analytical technique that is used to identify unknown compounds, to quantify known compounds and to elucidate the structure and chemical properties of molecules. The molecular weight of sample can be determined from MS Spectrum. Structural information can also be generated from certain types of mass spectrometers. This procedure is useful for the structural elucidation of organic compounds,for peptide or oligonucleotide sequencing and for monitoring the existence of previously characterize compounds in complex mixtures with a high specificity by defining both the molecular weight and a diagnostic fragment of the molecule simultaneously.

5.4. Nuclear Magnetic Resonance Spectroscopy: (NMR)

Nuclear Magnetic Resonance Spectroscopy gives physical, chemical and biological properties of matter. Chemists to study chemical structure using simple one-dimensional techniques routinely use NMR spectroscopy. Two dimensional techniques are used to determine the structure of more complicated molecules. These techniques are replacing X-ray crystallography for the determination of protein structure. Time domain NMR spectroscopic techniques are used to probe molecular dynamics in solutions. Solid state NMR spectroscopy is used to determine the molecular structure of solids.^{13}C- NMR is used to identify the types of carbon are present in the compound. ^{1}H- NMR is used to find out types of hydrogen are present in the compound and to find out how the hydrogen atoms are connected.

5.5. X-Ray Crystallography

x-ray crystallography is an experimental technique that exploits the fact that X- ray are diffracted by crystals. X- rays have the proper wavelength(in the Angstrom range 10^{-8}) to be scattered by the electron cloud of an atom of comparable size. Based on the diffraction pattern obtained from X- Ray scattering off the periodic assembly of molecules or atoms in the crystal, the electron density can be reconstructed. Additional phase information can be extracted either from the diffraction data or from supplementing diffraction experiment to complete the reconstruction. A model is then progressively built into the experimental electron density, refined against the data and the result is a quite accurate molecular structure.[17]

REFERENCES

[1] Das K, Tiwari RKS, Shrivastava DK. Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. Journal of Medicinal Plants Research 2010; 4(2): 104-111.

[2] Nikhal SB, Dambe PA, Ghongade DB, Goupale DC. Hydroalcoholic extraction of Mangifera indica (leaves) by Soxhletion. International Journal of Pharmaceutical Sciences 2010; 2 (1): 30-32.

[3] Ncube NS, Afolayan AJ, Okoh AI. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. African Journal of Biotechnology 2008; 7 (12): 1797-1806.

[4] Remington JP. Remington: The science and practice of pharmacy, 21st edition, Lippincott Williams & Wilkins, 773-774.

[5] Handa SS, Khanuja SPS, Longo G, Rakesh DD. Extraction Technologies for Medicinal and Aromatic Plants. International centre for science and high technology, Trieste, 2008, 21-25.

[6] Evans.W.C, "Treaseand Evans Pharmacognosy", Harcourt Brace and company. Asia pvt. Ltd.Singapore, 1997.

[7] Wagner.H, 'Pharmazeutische Biologic", 5 th Edition, AUFI.15 BN 3-437-20 498-X, 1993.

[8] Yasuma. A and Ichikawa. "Ninhydrin-Schiff and alloxan- Schiff staining. A new histochemical staining methods for proteins", J. Lab clin Med, 1953, 41:296-299.

[9] Kokate,C.K, "Practical pharmacognosy" 4 th edition, Vallabh Prakashan Publication, New Delhi, India, 1999.

[10] Mace.M.D, "Histichemical localization of phenols in healthy and diseased tomato roots", Phytopathology, 1963, 16:915-925.

[11] Harborne.J.B, "Phytochemical Methods: A Guide To Modern Techniques of Plant Analysis." 2nd Edition, Chapmann and Hall Publishers, London, 1998.

[12] Finar,I.L, "Stereo Chemistry and the Chemistry of Natural Products" Longman, VOL.2, 1986.

[13] Rasch. E. and H.Swift, "Micro Photometric Analysis of the Cytochemical million eaction", J.Histochem.Cytochem, 1960, 8:4-17.

[14] Gahan. P.B. "Plant Histochemistry and Cytochemistry: An Introduction. Academic Press, Florida, U.S.A, 1984..

[15] Whistler.R.L. and J.N. BeMiller, 'Industrial Gums; Polysaccharides and their Derivatives, Academic Press, London, 1993.

[16] James.E, K.Robbers, m.speedie and E.Varro Tyler, Pharmacognosy and pharmaco Biotechnology, Williams and Willkins, New York, U.S.A, 1996.

[17] N. Raaman, "Phytochemical Techniques ", New India Publishing Agency, New Delhi, ISBN- 81-89422-30-8.

[18] Chapatwala, K.D.; Boykin, M.A. & Rajanna, B. (1982). Effects of intraperitoneally injected cadmium on renal and hepatic glycogenic enzymes in rats. Drug Chemistry and Toxicology. 5:305-317.

[19] Chen, F.W.; Shieh, P., Kuo, D. & Hsieh, C. (2006). Evaluation of the antioxidant activity of Ruellia tuberosa. Food Chemistry. 94: 14-18. Choi, S.W.; Friso, S. & Editors. (2006). Nutrient-Gene Interactions in Cancer.

[20] Boca Raton: Taylor & Francis. Cowan, M.M. (1999): Plant products as antimicrobial agents. Clinical Microbiology Reviews. 12(4): 564 – 582.

[21] Daffre, S.;Bulet, P., Spisni, A., Ehret-sabatier, L., Rodrigues, EG. &Travassos, L.R. (2008). Bioactive natural peptides. In: Atta-ur-Rahman (Ed.) Studies in Natural Products Chemistry, Vol. 35. Elsevier. pp 597- 691.

[22] . Kim HB, Bang HS, Lee HW, Seuk YS, Sung GB (1999) Chemical characteristics of mulberry syncarp. Korean J Med. Crop Sci 47: 3206-3209.

[23] Osman AM, Younes ME, Sheta AE (1974) Triterpenoids of the leaves of Psidium guajava. Phytochem 13: 2015-2016.

[24] Begum S, Hassan SI, Siddiqui BS, Shaheen F, Ghayur MN, et al. (2002) Triterpenoids from the leaves of Psidium guajava. Phytochemistry 61: 399-403.