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A REVIEW OF SOME ANALYTICAL METHODS USED IN NATURAL PRODUCTS RESEARCH

Homa Jilani Khan	Department of Chemistry, Integral University, Lucknow, India, 226 026
Murtaza Abid	Molecular Biology lab, Department of Biochemistry, King George's Medical University, Lucknow, India, 226 003.
T.S. Naqvi	Department of Zoology, Shia P.G. College, Lucknow, U.P., India
Mohammad Waseem	Molecular Biology lab, Department of Biochemistry, King George's Medical University, Lucknow, India, 226 003.
Meenakshi Shukla	Molecular Biology lab, Department of Biochemistry, King George's Medical University, Lucknow, India, 226 003.
A.A. Mahdi	Molecular Biology lab, Department of Biochemistry, King George's Medical University, Lucknow, India, 226 003.

ABSTRACT Recently, medicinal plants are gaining much interest in medicinal research because of their use in ethno medicine treating common disease such as cold, fever and other medicinal claims are now supported with sound scientific evidences. The first step of study on medicinal plants started with extraction procedures (e.g. yield and phytochemicals content) and also to the subsequent assays performed. Nowadays number of different process and a wide range of technologies are available for extraction process. Hence, this review aim to describe and compare the most commonly used methods based on their principle, strength and limitation to help evaluating the suitability and economic feasibility of the methods. The analysis of bioactive phytochemicals present in the plant extracts involving the applications of common phytochemical screening assays, chromatographic techniques such as HPLC and, TLC as well as non-chromatographic techniques such as immunoassay and Fourier Transform Infra Red (FTIR) is discussed. Much research has been carried out towards the evaluation of plant extracts as effective disease- preventing agents since they can act on a specific or multiple molecular and cellular targets. Natural phytochemicals derived from medicinal plants have achieved significant response in the potential management of several human clinical conditions or diseases. The most important of these phytochemicals or bioactive compounds are alkaloids, flavonoid, terpenoids, tannins and phenolic compounds which have great potential in the fight against many diseases.

KEYWORDS : Carcinoid, MEN-1 Syndrome, Gastric tumor.

INTRODUCTION

Natural products, such as plants crude extract or fractions, either as pure compounds or as phytochemicals, offer unlimited opportunities for new drug discoveries because of the unique availability of chemical diversity (Cosa et al., 2006).The qualitative and quantitative studies of bioactive compounds from plant materials mostly depend on the assortment of proper methods. In this section, some of the commonly used methods in natural products research are discussed. The detailed study of medicinal plants starts with the pre-extraction or the extraction procedures, which is an important step in the processing of the bioactive constituents from plant materials. Conventional methods such as maceration process and Soxhlet extraction are commonly used at the small research surroundings level. The significance advancement have been made in the processing of medicinal plants such as the modern extraction methods; Soxhlet, Sonification and maceration process, in which these advances are aimed to increase yield at lower cost. Moreover, many modifications on the developed methods are continuously increased. With such variety of methods present, selection of proper extraction method needs meticulous evaluation and further laid to isolation of compounds with the developed methods. This review describes the theory, strength and limitation of the commonly used methods with examples in recent years to help in the assortment of proper methods (Duraipandiyan et al., 2006).

EXTRACTION

Extraction is the primary step for the study of medicinal plant and plays a significant and key role in the final outcome of the study. Frequently affecting factors for extraction processes are the environmental condition of the plant, part of the plant used, the solvents used, temperature and time took for extraction. After extraction, the further possible process can be successfully attained such as separation, identification, and characterization of bioactive compounds. Bioactive compounds from plant materials can be extracted by a variety of classical extraction techniques. The frequently used classical techniques are soxhlet extraction, maceration, and hydrodistillation to obtain a crude extract which is then concentrated using a rotary evaporator (Azmir et al., 2013). The recent modern extraction techniques include solid-phase micro-extraction, supercritical-fluid extraction, pressurized-liquid extraction, microwave-assisted extraction, solid-phase extraction, and surfactant-mediated techniques, which possess convinced advantages. These are the reduction in organic solvent consumption and in sample degradation, elimination of additional sample clean-up and concentration steps before chromatographic analysis, improvement in extraction efficiency, selectivity, and/ kinetics of extraction. The ease of automation for these techniques also favors their usage for the extraction of plants materials (Huie, 2002). As the target compounds may be non-polar to polar, the suitability of the methods of extraction must be considered. A brief summary of the experimental conditions for the various methods of extraction is shown in Table 1.

Table 1. A brief summary of the experimental conditions for various methods of extraction for plants material.

	Soxhlet extraction	Sonification	Maceration
Common Solvents used	Methanol, ethanol, or mixture of alcohol and water	Methanol, ethanol, or mixture of alcohol and water	Methanol, ethanol, or mixture of alcohol and water
Temperature (°C)	Depending on solvent used	Can be heated	Room temperature
Pressure applied	Not applicable	Not applicable	Not applicable

Time required	3–18 hour	1 hour	3-4 days
Volume of solvent required (ml)	100-200	50-150	Depending on the sample size

Identification and characterization

Naturally plant extracts usually occur with the combination of various types of bioactive compounds or phytochemicals with different solubility and polarities, their separation still remains a big challenge for the process of identification and characterization of bioactive compounds. For the isolation of these bioactive compounds, number of different separation techniques such as TLC, column chromatography, flash chromatography, Sephadex chromatography and HPLC, have been used to obtain pure compounds. The pure compounds obtained are then used for the determination of structure and biological activity. Besides that, non-chromatographic techniques such as immunoassay, which use monoclonal antibodies (Mabs), phytochemical screening assay, Fourier-transform infrared spectroscopy (FTIR), can also be used to obtain and facilitate the identification of the bioactive compounds.

CHROMATOGRAPHIC TECHNIQUES

COLUMN CHROMATOGRAPHY

In column chromatography, the stationary phase (a solid adsorbent) is positioned in a vertical glass column and the mobile phase (a liquid) is added to the top and flows downward through the column (by either gravity or external pressure). Column chromatography is normally used as a purification technique to isolate preferred compounds from a mixture or extracts of the medicinal plants (Sasidharan et al., 2011). Silica gel (SiO_2) and alumina (Al_2O_3) are the two basic adsorbents which are commonly used for column chromatography. The polarity of the solvent which is passed through the column depends on the nature of the compounds and extracts used and affects the relative rates at which compounds move through the column (Harvey, 2000).

THIN LAYER CHROMATOGRAPHY

Thin layer chromatography (TLC) is often used to analyze the number of compounds present in the fractions obtained from column chromatography (Kenkel, 2003). The separation of compounds of the particular fractions by TLC depends on the relative resemblance of compounds towards stationary and mobile phase. Thus, by the TLC process separation of components in the mixture is achieved. Once separation occurs, the individual components are visualized as spots on the plate after staining with iodine vapor, UV light, Diluted H_2SO_4 solution depending upon the nature of the compounds present in the mixture (Harvey, 2000).

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography (HPLC) is a well known versatile, robust, and extensively used technique for the isolation of natural products (Cannell, 1998). In present scenario for fingerprinting study of the phytochemicals for the quality and quantity of the medicinal plants, this technique is gaining popularity among various analytical techniques (Fan et al., 2006). Isolation of phytochemicals from the evaluation of a relatively crude extract of medicinal plants is processed on biological assay in order to fully characterize the active entity. The biologically active phytochemical is often present only as minor constituent in the crude extract and the resolving power of HPLC is ideally suited to the rapid processing of such multi-component samples on both an analytical and preparative scale (S Sasidharan et al., 2010). Nowadays modular design HPLC instruments are available and comprise with a solvent delivery pump, a sample introduction device such as an auto-sampler or manual injection valve, an analytical column, a guard column, detector and a recorder or a printer.

Table 2. A brief summary of phytochemical screening of secondary metabolites.

Test of Phytochemicals	Methodology	Result
Alkaloids	Mayer's test: Crude extract was mixed with Mayer's reagent (Potassium mercuric iodide solution) Cream color ppt. was formed showing the presence of alkaloids.	Orange spot
	Hager's Test: To the 2-3 ml of filtrate, Hager's reagent was added. The yellow precipitate was formed showing the presence of alkaloids.	Orange spot
	Dragendroff's Test: Spot a drop of extract on a small piece of precoated TLC plate. Spray the plate with Dragendorff's reagent.	Orange spot
Glycosides	Kellar kiliani Test: Add 2ml filtrate with 1ml of glacial acetic acid, 1ml ferric chloride and 1ml concentrated sulphuric acidGreen blue colorReducing sugarFehling Test: Add 25ml of diluted sulphuric acid (H_2SO_4) to 5ml of water extract in a test tube and boil for 15mins. Then cool it and neutralize with 10% sodium hydroxide to pH 7 and 5ml of Fehling solution.Brick red precipitateSteroidsLiebermann-Burchardt Test: To 1ml of methanolic extract, add 1ml of chloroform, 2–3ml of acetic anhydride, 1 to 2 drops of concentrated sulphuric acid.Dark green colorTanninFeCl3 Solution Test: On addition of 5% $FeCl_3$ solution to the crude extract, the deep blue-black color appeared and indicated the presence of tannins.Dark green or greenish grey FlavonoidShinoda Test: Crude extract was mixed with few fragments of magnesium ribbons and conc. hydrochloric acid was added dropwise. Pink scarlet color appears after Zinc hydrochloride test to the test solution, add a mixture of Zinc dust and conc. Hydrochloric acid. It gives red color after few minutes. Pink red colorSaponinsAbout 1g of the powdered sample was boiled in 20 ml of distilled water in a water bath and filtered. 10ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously. Persistent of frothing	

Different compounds have different migration rates given on a particular column and mobile phase because of this property of compounds their chemical separations can be accomplished by using HPLC. The amount of chemical separation is mostly determined by the choice of stationary phase and mobile phase (Zygmunt and Namiesnik, 2003). Usually, the identification and separation of phytochemicals can be accomplished using isocratic system (using single unchanging mobile phase system). Identification of compounds is very tough by HPLC assay for this a detector must first be selected (Tona et al.., 1998). Once the detector is selected and is set to optimal detection settings, a separation assay must be developed. The parameters of this assay is comparable with a clean peak of the known sample is observed from the chromatograph. The identified peak of the performed assay should have a reasonable retention time and should be well separated from extraneous peaks. Among all the detectors available for HPLC, UV detectors are popular because they offer high sensitivity and also because majority of naturally occurring phytochemicals encountered have some UV absorbance at low wavelengths (190–210 nm) (Cannell, 1998).

NON-CHROMATOGRAPHIC TECHNIQUES

PHYTOCHEMICAL SCREENING ASSAY

Naturally occurring large number of secondary metabolic compounds found in plants are known as phytochemicals. Phytochemical screening assay is a simple, rapid, and inexpensive method that gives the researcher a quick answer that different types of phytochemicals are present in a mixture and its very important method in analysis of bioactive compound (Kumar et al., 2007; Chanda et al., 2006; Onwukaeme et al., 2007). A brief summary of the investigational procedures for the secondary metabolites for the various phytochemical screening methods is shown in Table 2. Phytochemical screening analysis can be carried out of the crude extract or active fraction obtained from plant material, with the appropriate tests as shown in the Table 2 to get an idea regarding the type of phytochemicals existing in the extract mixture or fraction.

FOURIER-TRANSFORM INFRARED SPECTROSCOPY (FTIR)

FTIR has been established an important instrument for the characterization and identification of phytochemicals or functional groups (chemical bonds) present in an unidentified mixture of plants crude extract (Eberhardt et al., 2007; Hazra et al., 2007). Fingerprinting of phytochemicals or pure compounds are usually carried out by GC-MS/MS analysis, HPLC in addition to these methods, FTIR spectra is also used for fingerprinting of compounds and the spectrum of an unknown compound can be identified by comparing with the library of known compounds feed in the memory of instrument.

CONCLUSION

Since bioactive crude extracts of medicinal plants are mixtures of the number of phytochemicals, their separation and character ization still create problems for the researcher. Practically most of them have to be purified by the combination of several chromatographic techniques and various other purification methods to isolate the bioactive compounds.

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