Analytical Techniques for Identification and Characterization of Bioactive Compounds Extracted from Medicinal Plants

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Vandana, Suresh Kumar, Atul Arya, Dolly Kain

Abstract

Natural herbal products, both in the form of pure compounds and crude extracts, offer limitless opportunities for new avenues of drugs due to the unparalleled availability of chemical diversity. Due to the increasing demand of chemical diversity in search of therapeutic drugs from natural products, in particular the interest in edible plants, has developed all over the world. Botanicals and herbal preparations for medicinal use contain various types of bioactive compounds. Phytochemical screening assays and spectroscopic techniques such as UV-Visible, infrared, NMR and Mass Spectroscopy are important analytical tools that help in characterization of unknown bioactive molecules present in crude extract of medicinal plants. Purification of bioactive compounds from crude extract of medicinal plants can be achieved by using different chromatographic techniques including thin layer

Vandana
Associate Professor, Department of Chemistry, Dyal Singh College, University of Delhi, Delhi - 110003
Suresh Kumar*
Professor, Department of Botany, Ramjas College, University of Delhi, Delhi - 110007
***Corresponding Author's e-mail: suresh.kumar@ramjas.du.ac.in**
Atul Arya
Medicinal Plant Research Laboratory, Department of Botany, Ramjas College, University of Delhi, Delhi - 110007
Dolly Kain
Medicinal Plant Research Laboratory, Department of Botany, Ramjas College, University of Delhi, Delhi - 110007

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chromatography (TLC), column chromatography (CC) and high-performance liquid chromatography (HPLC) most importantly.

Keywords

Phytochemical analysis, UV-Visible spectroscopy, Infrared spectroscopy, NMR spectroscopy, Mass spectroscopy, Thin layer chromatography, Column chromatography, HPLC

1. Introduction

Natural products as pure compounds and crude extracts, offer limitless opportunities for the discovery of new drugs due to the unparalleled availability of chemical diversity (Cos et al. 2006). According to the World Health Organization (WHO), more than 80% of the world's population depends on traditional medicine for their primary health needs. The use of herbal remedies in Asia represents a long history of human interactions with the environment. Plants used for traditional medicine contain a wide range of substances that can be used to treat chronic and infectious diseases (Duraipandiyan et al. 2006). Due to the development of adverse effects and microbial resistance to chemically synthesized drugs, men have turned to ethnopharmacognosy. Majority of bioactive compounds from medicinal plants were safe and used effectively without any side effects. Many beneficial biological activities have been reported such as antitumor, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activities. In many cases, people claim the benefits of certain natural or herbal products. However, clinical studies are needed to prove the efficacy of a bioactive compound in order to verify this traditional claim. Clinical tests aimed at understanding the pharmacokinetics, bioavailability, efficacy, safety, and drug interactions of newly isolated bioactive compounds and their formulations (extracts) require careful consideration. Knowledge of the chemical constituents of medicinal plants is desirable not only for the discovery of bioactive agents, but also because this information can be of great value in revealing new sources of inexpensive bioactive compounds for the synthesis of drugs. Herbal plants act as sources of phytochemical compounds continue to play a prominent role in the maintenance of human health. Ethnopharmacological reports available on green plants represent a reservoir of

effective chemotherapeutic agents which are non-phytotoxic, more systemic and readily biodegradable. These scientific research trends in Pharmacopoeia, paved the way for the development of a wide range of isolation and analytical techniques. The qualitative and quantitative analysis of medicinal plant extract is primarily focused on the use of phytochemical, chromatographic and spectroscopic techniques (Figure 1). Various phytochemical assays reveal the major profiling of plant secondary metabolites. Isolation of bioactive compound from crude extract is one of the most tedious tasks but can be achieved by different chromatographic techniques including thin layer chromatography (TLC), column chromatography (CC) and high-performance liquid chromatography (HPLC). Spectroscopic analytical techniques such as UV–Visible spectroscopy, Infrared spectroscopy, Nuclear magnetic resonance spectroscopy and Mass spectroscopy are employed to characterize the bioactive compounds.

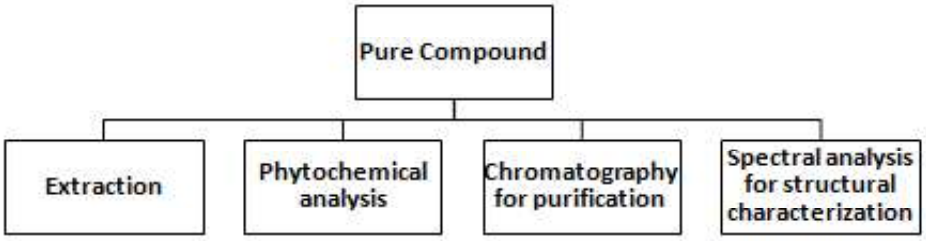

Figure 1: General approaches for medicinal plant extract analysis

2. Structure Determination of Bioactive Organic Molecules Present in Medicinal Plants

Bioactive organic molecules or compound present in medicinal plant are extracted and purified by different techniques. After purification, we need to go for structure determining of organic compounds which are responsible for a particular kind of therapeutic uses. Every plant and its part like flower, seed, fruit contains different types of organic compounds. So, plants used traditionally becomes very important to analyse the bioactive compounds by various modern analytical techniques. The analytical techniques can be categories as:

1. Phytochemical analysis
2. Chromatographic techniques
3. Spectroscopic analysis

2.1. Phytochemical screening assay for determining bioactive organic molecules present in medicinal plants

Phytochemicals are chemicals derived from plants and the term is often used to describe the large number of secondary metabolic compounds present in plants. The phytochemical screening assay is a very simple, inexpensive and rapid procedure that provides a rapid response to various types of phytochemicals in a mixture. It is an important tool in the analysis of bioactive compounds. A brief summary of the experimental procedures for the different phytochemical screening methods for secondary metabolites is shown in Table 2. After obtaining the crude extract or the active fraction of the plant material, the phytochemical screening can be performed with the appropriate tests such as shown in Table 1 to get an idea of the type of phytochemicals present in the mixture or extraction fraction.

Table 1: A brief summary of phytochemical screening of secondary metabolites

S. No.	Secondary metabolites	Name of the test	Methodology	Results	References
1.	Alkaloids	Wagner's test	Add 2ml extract +1% HCl + steam + 1ml of the solution with 6 drops of Wagner's reagent	Brownish red ppt	Chanda et al. 2006
2.	Cardiac Glycosides	Kellar Killiani test	50mg methanolic extract + 2ml of chloroform + H2SO4 to form a layer	Brown ring at interphase	Onwukaeme et al. 2007
3	Flavonoids	NaOH test	Extract + dilute NaOH + dilute HCl	Yellow solution on adding NaOH turns colorless after adding HCl	Onwukaeme et al. 2007
4	Phenolic compounds	Lead acetate test	To the test solution, a few drops of 10% lead acetate solution were added	Formation of white ppt.	Bag et al. 2013
5	Saponin	Frothing test	0.5ml extract + 5ml distilled water and shake well	Persistence of frothing	Parekh and Chanda 2007
6	Tannin	Braemer's test	10% alcoholic $FeCl_3$ +2-3ml of methanol extract (1:1)	Dark blue or greenish grey coloration	Parekh and Chanda 2007

7	Terpenoids	Salkowski test	5ml extract + 2ml Chloroform + 3ml conc. H_2SO_4	Reddish brown color at interface	Edeoga et al. 2005
8	Anthroquinone	Ammonia test	Add 1ml of dilute (10%) ammonia to 2ml of chloroform extract	A pink-red color in the ammoniacal (lower) layer	Onwukaeme et al. 2007
9	Phlobatannin	HCl test	Extract was boiled with 2 ml of 1 % HCl	Formation of red ppt	Bag et al. 2013
10	Starch	Iodine test	The aqueous extract 5ml was treated with the reagent of the starch (iodine)	Blue color indicates the presence of starch	Zohra et al. 2012

2.2. Chromatographic techniques for purification of compounds

Chromatographic techniques used for purification of compounds on the basis of R_f value (R_f= distance travelled by solute/ distance travelled by solvent). Chromatography relies on the basic principle of solubility difference in the two phases i.e., mobile phase and stationary phase. Some widely used chromatographic technique includes TLC, CC and HPLC (Figure 2). TLC is used to separate the mixture of compounds present in plant samples and also provides the direction for column chromatographic separation of compounds. CC is used to collect separated compounds on large scale for further characterization of active compound(s) (Enyoh et al. 2019). HPLC is the most important technique with high pressurized packed column which increases the efficiency of separation of compounds many folds as compared to conventional liquid chromatography (Bansal et al. 2010). In HPLC, mobile phase can be a single solvent (isocratic elution) or combination of solvent with increasing polarity (gradient elution) (Rogatsky 2016; Dixon and Stoll 1976). HPLC have been used for isolation, purification and identification of compounds on the basis of characteristic peak.

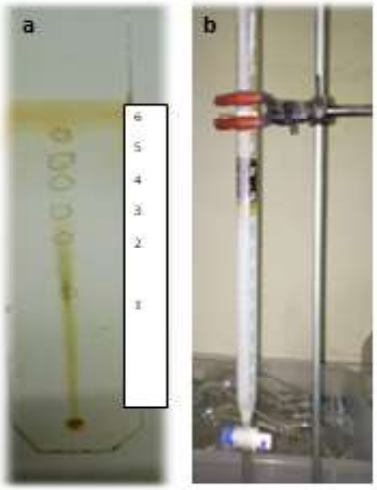

Figure 2: (a) TL chromatogram (b) CC apparatus

2.3. Spectroscopic analysis of bioactive organic molecules present in medicinal plants

Spectroscopic techniques play an important role in identification of bioactive organic molecules present in medicinal plants. Spectroscopy is a technique in which we study the interaction of electromagnetic radiations with the organic compounds. Spectroscopic techniques extensively use in identification, characterization and biochemical studies. The characterization of bioactive compounds is based on their spectral properties. The different types of electromagnetic radiations are ultraviolet visible rays, infrared rays, γ-rays, X-rays, radio waves and microwaves.

The natural product structure determination utilizes data from a wide variety of spectroscopic techniques such as UV-Visible, Infrared (IR), Nuclear magnetic resonance (NMR) and Mass spectroscopy. In spectroscopic studies, molecules interact with radiations of various regions over a wide range of wavelengths. Different special types of instruments are used to examine the spectra which give information about the structure of unknown compounds. The various types of techniques commonly used are as follows:

2.3.1. UV Visible spectroscopy

UV-Visible spectroscopy (UV-VIS) has been extensively used technique and can be performed for qualitative analysis and for the identification of certain classes of compounds in pure and biological mixtures. It is an analytical technique that measures the amount of discrete wavelengths of UV or visible light. UV or visible light are absorbed or transmitted through a sample compared to a reference or blank sample. Preferably, visible UV spectroscopy can be used for quantitative analysis because aromatic molecules are powerful chromophores in the UV range. The natural compounds can be determined by UV-VIS (Kemp1991).

2.3.1.1. UV-VIS spectroscopy analysis, absorption spectrum and absorbance units

In UV-VIS spectroscopy, compounds absorb light of particular wavelength(s) which defines its absorption spectra. The UV-Vis spectroscopy includes excitation of ground state electrons to the excited state i.e., transitions occurring determining wavelength of light absorbed. Therefore, the absorption peaks give relative information about the molecular structures.

UV-Vis spectroscopy information may be presented as a graph of absorbance, optical density or transmittance as a function of wavelength. However, the information is more often presented as a graph of absorbance on the vertical *y* axis and wavelength on the horizontal *x* axis. This graph is typically referred to as an absorption spectrum.

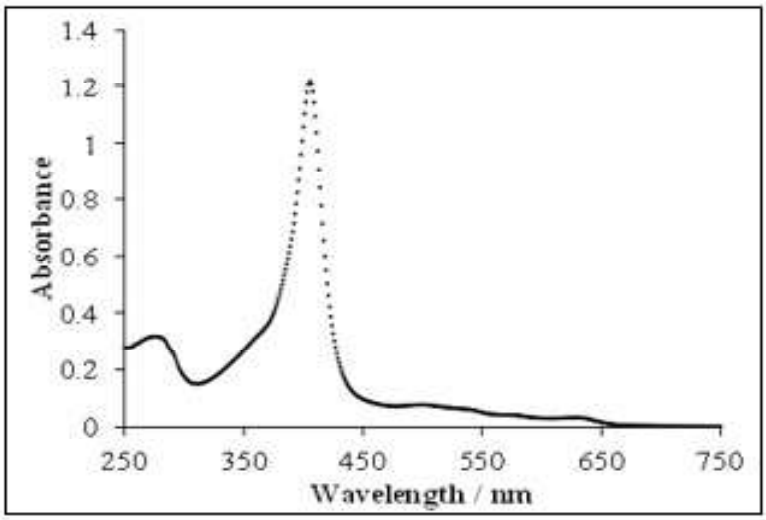

Figure 3: An example absorption spectrum taken from a UV-VIS spectrophotometer

The relationship between concentration of compound and the extent of absorption of light is based on two important laws:

Lambert's laws

Beer's Law

When a beam of monochromatic light (light of one wavelength) passes through a homogenous solution, it may be transmitted as such or a fraction of it may be absorbed. According to Lambert's law, light absorbed by a solution is directly proportional to the length of light path through the solution

$$A=\log_{10}(I_o/I)=\varepsilon. L$$

Where A = absorbance

I_o = Intensity of light before passing through the sample i.e., incident light

I = Intensity of light after passing through the solution

ε = Molar absorbance coefficient

L = Light path or width of liquid in cell or cuvette in cm

Beer's law stated that the amount of light absorbed is directly proportional to the concentration of absorbing solute in the solution. So,

$A = \log_{10} (I_o /I) = \varepsilon. c$

c = concentration of absorbing solute in moles per liter

Combining both the laws

$A = \log_{10} (I_o /I) = \varepsilon. C.l$

If we used a standard cuvette with light path of 1cm than the above equation become

$A = \log_{10} (I_o /I) = \varepsilon. c$

According to this equation, absorption of light by a solution is dependent on the molar absorbance coefficient and concentration (moles per liter) of the absorbing solute.

The UV-VIS analysis was carried out for the identification of the bioactive molecules present in the methanolic extract of *Mentha spicata*. The UV-visible spectra were done to identify the compounds having π-bonds, σ-bonds, and lone pair of chromophores, electrons, and aromatic rings. The observed absorption bands relating to the *Mentha spicata* plant extract are shown in Figure1. The UV VIS spectra of *Mentha spicata* was taken at the wavelength of 300 nm to 800 nm due to the peaks sharpness and proper baseline. The spectrum of the *M. spicata* extract shows two peaks at the 353, 407, 504, 535, 609 and 665 nm with the absorption 1.309, 1.463, 0.1, 0.066, 0.108 and 0.625, respectively (Jain et al. 2016).

The appearance of one or more peaks in the 200-400 nm regions is a clear indication of the presence of unsaturated groups and heteroatoms such as S, N, O. The spectrum of the *M. spicata* extract shows two peaks at the 353 nm and 407 nm positions. This confirms the presence of organic chromophores in the *M. spicata* extract (Figure 4) (Jain et al. 2016). However, the use of the visible UV spectrophotometer in the analysis of complex media is limited by the inherent difficulties in assigning absorption peaks to a particular constituent of the system. Therefore, UV-VIS results should be integrated with another analytical technique such as GC/MS, etc., to enable proper characterization of the extract and identification of constituents (Jain et al. 2016).

UV-VIS spectroscopy is significantly useful in other specialized research. By analysing changes in the wavelength corresponding to the peak absorbance is useful in examining specific structural protein change. Shifts in peak absorbance wavelengths can also be useful in more modern applications such as characterization of very small nanoparticles. The applications of this technique are varied and seemingly endless.

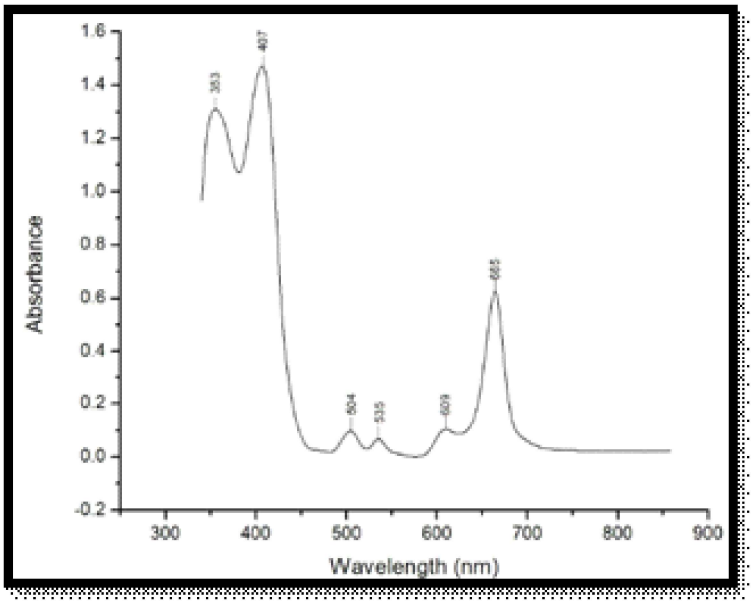

Figure 4: UV-VIS spectra of *Mentha spicata* extract

2.3.2. Fourier transformed infrared spectroscopy (FTIR)

Fourier transformed infrared spectroscopy is most widely used techniques by researchers. Infra-red radiations are the electromagnetic radiations used for FTIR spectroscopy technique. The Infra-red radiations can be categories into near IR region (1-2μm), infrared (2-25μm) and far IR (25-250μm) regions. The infrared (2-25μm) region is most significant for analytical purposes. Infra-red spectroscopy gives us information about functional groups present in extracted medicinal plants. These functional groups give characteristics peaks in infrared spectrum. These functional groups include aldehydic, ketonic, hydroxyl, phenolic, esters, carboxylic acid, amide, anhydride, amines, hydrocarbons, nitro compounds, nitrile, isonitrile etc. In IR spectrum characteristics peaks are observed due to stretching and bending vibrations of bonds. Particularly unsymmetrical stretching vibrations of bonds have characteristics peaks.

FTIR spectroscopy is considered as a valuable tool for the characterization and identification of compounds or functional groups present in a crude mixture of herbal extracts (Eberhardt et al. 2007). In addition, the FT-IR spectra of pure compounds are generally so unique that they resemble a molecular "fingerprint". The spectrum of an unknown compound can be identified against a library of known compounds.

There are different ways for the preparation of FT-IR samples. For liquid samples, the easiest way is to place a drop of sample between two sodium chloride plates. The drop of a sample forms a thin film between the sodium chloride plates. Solid samples can be ground with potassium bromide (KBr) and then compressed into a fine pellet that can be analysed. Alternatively, solid samples can be dissolved in a solvent such as methylene chloride and the solution then placed on a single salt plate. A thin film of the original material is left on the plate after evaporation of solvent. The IR bands at 3472 cm^{-1}, 3155 cm^{-1} corresponding to $\nu(NH_2)$, ν(NH). IR spectrum exhibits bands at 1695 cm^{-1}, 1590 cm^{-1} which have assigned to ν(C=O) of amide group, ν(C=N) imine group, respectively (Chandra et al. 2015). The FTIR of the plant *A. millefolium* was obtained using a spectrometer (Shimadzu) in the range from 400–4,000 cm^{-1} (Figure 5) (Kain et al. 2021). FTIR of *A. millefolium* plant inflorescence revealed the presence of the major functional groups including C-Cl, CN, NO, S=O, CH, C=O, C=C, CH and NH (Kain et al. 2021).

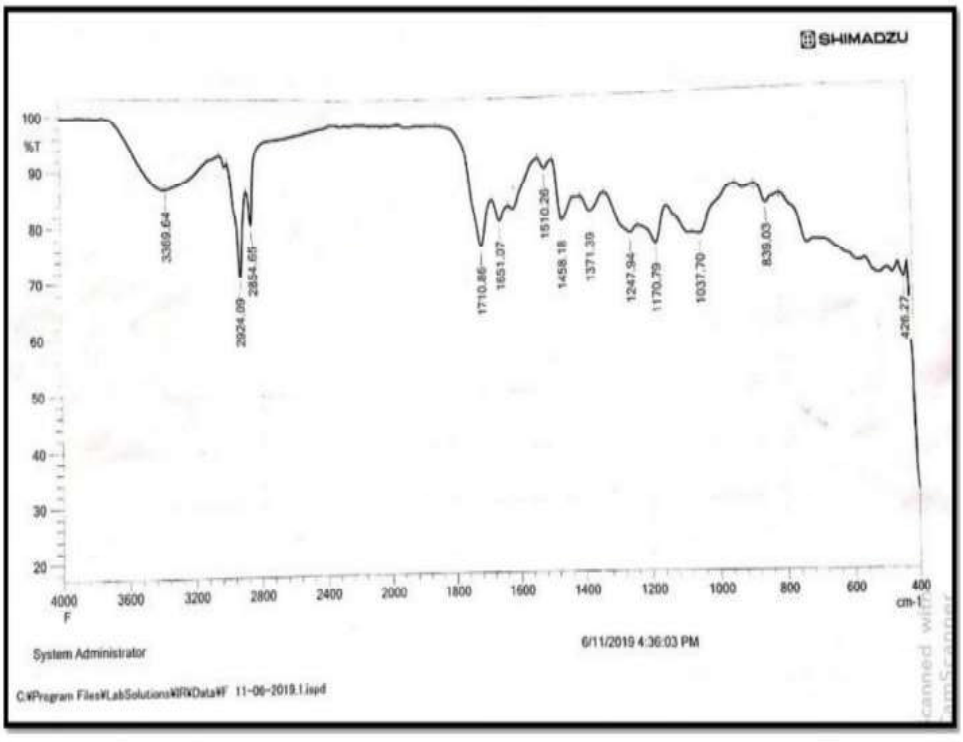

Figure 5: FT-IR Graph of inflorescence of plant *Achillea millefolium* (Kain et al. 2021)

Table 2: FT-IR of inflorescence of plant *A. millefolium* (Kain et al. 2021)

Peak (cm^{-1})	Stretching type	Functional Groups
839.03	C-Cl stretching	Halo compound
1037.70	S=O stretching	Sulfoxide
1170.79	C-H stretching	Alkane compound
1247.94	C-N stretching	Amine
1510.26	N-O stretching	Nitro compound
1651.07	C=C stretching	Alkene
1710.86	C=O stretching	Aliphatic ketone, carboxylic acid, conjugated aldehyde

2.3.3. Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy known as NMR spectroscopy is an important analytical technique to characterize organic structure and identify hydrogen skeleton and carbon skeleton of organic compounds. NMR spectroscopy use radio waves, electromagnetic radiations to interact with the matter and the NMR spectrum provides physical, chemical and biological properties of matter. Radiowaves have long wavelengths; consequently, will have low energy and frequency. ^{1}H NMR and ^{13}C NMR are two common types of NMR spectroscopy and are used to characterize and identify organic structure. ^{1}H NMR is used to determine the type and number of H atoms in a molecule. ^{13}C NMR is used to determine the type of carbon atoms in the molecule. When long wavelengths radio waves interact with an organic molecule, they can change the nuclear spins of some elements, including ^{1}H and ^{13}C. Nucleus of a hydrogen atom (proton) behaves as a spinning bar magnet because it possesses both electric and magnetic spin. All the electromagnetic radiations also possess both electric and magnetic spin. Nucleus of hydrogen atom generates a magnetic field when kept in external static magnetic field and behaves as a spinning charged body. In NMR spectroscopy, interaction between magnetic energy of hydrogen nucleus and some other type of nuclei with the oscillating magnetic field of electromagnetic radiation takes place. At the point of resonance, the sample absorbs electromagnetic radiations in radiowave region at different frequencies or energies because absorption of radiowaves depends upon the type of protons or certain nuclei contained in the sample. The different types of protons being present and surrounded by different chemical environment will absorb different frequencies of radio waves. An NMR spectrometer automatically integrates the

area under the peaks, and prints out a stepped curve (integral) on the spectrum. The area under the peak is proportional to the height of each step, which in turn is proportional to the number of absorbing protons. In modern era, NMR spectrometers automatically calculate and plot the value of each integral in arbitrary units. The ratio of integrals to one another gives the ratio of absorbing protons in a spectrum. Note that this gives a ratio, and not the absolute number of absorbing protons. One dimensional technique is most commonly used. But the complicated structure of the molecules can be achieved by two-dimensional NMR techniques.

^{1}H-NMR fingerprint peak for the methanol extracts of the different parts of *Peganum harmala* revealed the presence of total 23 metabolites out of which 19 are primary and 4 are secondary (Yinping et al. 2018). The 19 primary metabolites were five organic acids, three carbohydrate compounds, eight amino acids, and the last three were compounds of other types. The four secondary metabolites were vasicinone, harmine, vasicine, and harmaline. These twenty-three compounds were identified in methanol extracts of *P. harmala* by comparing chemical shifts and coupling separation values in nuclear magnetic spectra and relevant information from two-dimensional NMR spectra to related literature data and standard spectra of the amino acids, organic acids, and sugar compounds in the Human Metabolome Database (HMDB) (http://www.hmdb.ca/). The ^{1}H-NMR spectra of methanol extracted from different organs of *P. harmala* and the characteristic signals of the identified metabolites are shown in Figure 6 (Yinping et al. 2018).

The major amino acids identified in the organic acid and amino acid region (δ_H 0.5–3.0 ppm) were isoleucine, valine, threonine, alanine, proline, lysine, 4-hydroxyisoleucine and asparagine. The major organic acids identified in this region included acetic, succinic, and malic acids. The signals of sugar compounds in the δ_H 3.0–6.0 ppm range overlapped considerably. However, the diagnostic anomeric proton signal of sucrose and glucose could be easily identified. During this time, signals characteristic of nitrogen-containing metabolites, such as choline, phosphorylcholine and betaine, were clearly observed in this region (Yinping et al. 2018).

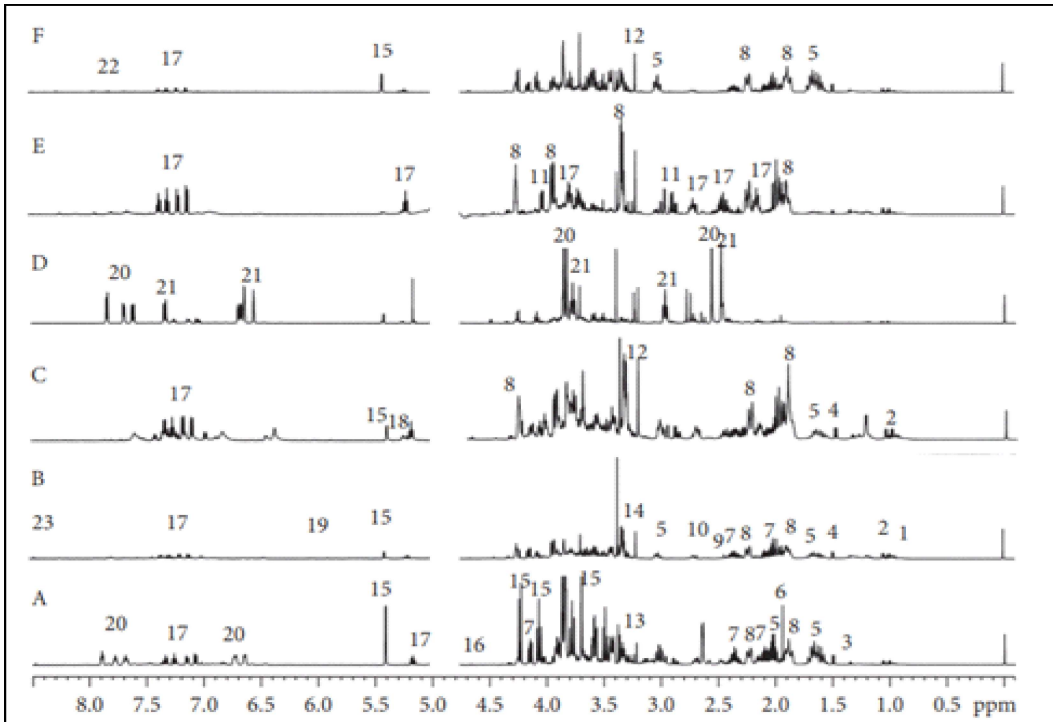

Figure 6: Typical 500 MHz ^{1}H-NMR spectra of the metabolites of the different parts of *P. harmala*: (A) root, (B) leaf, (C) flower, (D) seed, (E) testa, and (F) stem. Key metabolites: 1: leucine; 2: valine; 3: threonine; 4: alanine; 5: lysine; 6: acetic acid; 7: proline; 8: 4 hydroxyisoleucine; 9: succinic acid; 10: malic acid; 11: asparagine; 12: choline; 13: phosphorylcholine; 14: betaine; 15: sucrose; 16: β-glucose; 17: vasicine; 18: α-glucose; 19: maleic acid; 20: harmine; 21: harmaline; 22: vasicinone; 23: formic acid (Yinping et al. 2018)

2.3.4. Mass spectrometry

Mass spectrometry is one of the best techniques to determine the molecular mass (molecular weights) of the compound or substance and their moieties (fragments or constituent ions) (Ingle et al. 2017). This method is mainly used for the structural elucidation of organic compounds, sequencing of peptides or oligonucleotides, and monitoring the existence of previous characterizes compounds in complex mixtures. The fragments or constituent ions are formed from the molecule by fragmentation i.e., wherever breakage of bonds takes place due to various chemical or physical properties. Unknown compounds can be identified by comparing their mass spectra with libraries of digitalized mass spectra of known compounds. In MS, organic

molecules are bombarded with high energy electrons and converted to highly energetic positively charged ions (molecular ions or parent ions). The parent Ion ($M^{+\cdot}$) formed when a molecule (M) loses an electron ($M \rightarrow M^{+\cdot}$). The molecular ion is known as parent ion and is usually designated as M^+. It is positively charged molecule with an unpaired electron with the bombardment of high energy electrons the molecules get ionised and broken up into many smaller ions which are known as fragment ions or daughter ions. The process of formation of fragment ions or daughter ions is known as fragmentation.

$$M^{+\cdot} \rightarrow m_1^{+\cdot} + m_1$$

In the Mass spectrum, the set of fragment ions or daughter ions are analysed in such a way that a signal is obtained for each value of m/e that is represented. The intensity of each signal represents the relative abundance of the ion producing the signal. The base peak is the largest peak in the structure which has 100% abundance and intensity is also 100 for base peak. The intensities of other peaks are represented relative to the base peak. The molecular ion peak is also known as parent ion. Depending on the structure molecular ion peak can or cannot be base peak. Mass spectrum of a compound is a plot which represents the intensities of the signals at various m/e values. Mass spectra have vast usefulness. It helps in identification of compound and also elucidate the structure unknown compound. Mass spectrum can tell us exact molecular mass of unknown compound. Mass spectrum helps in determining the presence of certain structural units in a molecule.

One of the most widely used version of MS is MALDI-TOF (Matrix assisted laser desorption ionization-time of flight). In this technique, the sample is mixed in the matrix consists of crystallized molecules. On exposure to radiation like UV lasers such as nitrogen lasers (337 nm) and frequency-tripled and quadrupled Nd: YAG lasers (355 nm and 266 nm), the sample get ionized. The positive ions then travelled in the flight tube and separated on the basis of difference in time-of-flight (TOF) as heavier ions will take more time to reach detector as compared to lighter ions (Croxatto et al. 2012). The detector then produce a MS spectrum of the sample (Figure 7).

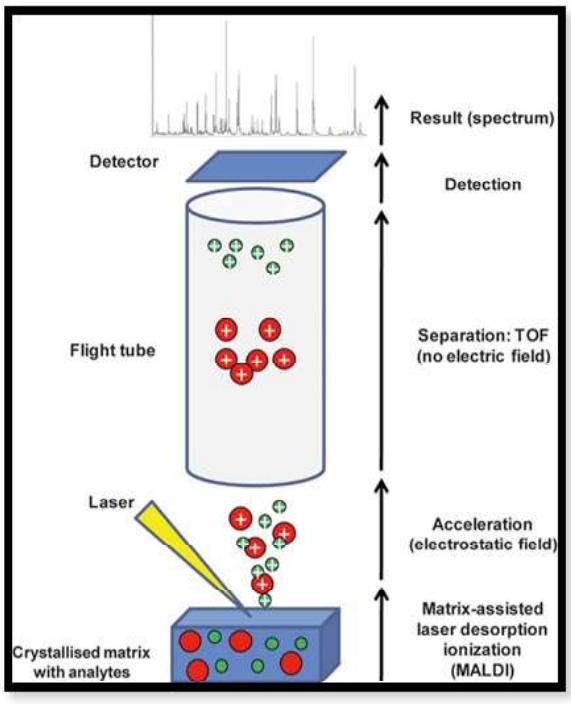

Figure 7: Diagrammatic representation of MALDI- TOF (Croxatto et al. 2012)

3. Conclusion

Phytochemical, chromatographic and spectroscopic analysis are important analytical techniques in the characterization of unknown bioactive components in extracts obtained from plant material. Spectroscopic analysis requires only small quantity of purified compounds. Sometimes, the compounds can be reused. The quantity can be taken into mg for spectroscopic analysis and results are accurate. Serial dilutions are used to determine MICs for various biological activities.

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