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Research Article

Ethnophamacological Approach in Extraction, Isolation and Characterization of Bioactive Compounds

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ABSTRACT

From the natural products of medicinal plants, either as pure or standard compounds, give unlimited chances to new medication heads in view of the unmatched accessibility of substance assorted qualities. Because of an expanding interest for chemical diversity in screening projects, looking for therapeutic drugs from characteristic items, engage especially in palatable plants has developed all through the world. Botanicals and herbal preparation for therapeutic utilization contain different sorts of bioactive compounds. The focus of this paper is on the logical systems, which incorporate the extraction, isolation and characterization from botanicals and herbal preparation. The basic issues and key difficulties in the extraction, confinement and characterization of dynamic add-ins in botanicals and herbal preparations are talked about. As extraction is the most imperative venture in the examination of constituents present in botanicals and herbal preparations, the qualities and shortcomings of diverse extraction strategies are talked about. The investigation of bioactive mixes exhibit in the plant concentrates including the applications of basic phytochemical screening procedures, chromatographic systems, for example, HPLC/LCMS and, TLC and additionally nonchromatographic methods, for example, immunoassay and Fourier Transform Infra Red (FTIR) are discussed in details.

Key words: Bioactive compound, Plant Extraction, Isolation, Herbal preparations, Natural products

INTRODUCTION

Plant extracts obtained from Natural products, either as pure compounds or as standard extracts, gives unlimited opportunities to new medication disclosures as a result of the unmatched accessibility of substance assorted qualities (Cos et al., 2006). As per the World Health Organization (WHO), more than 80% of the world's population depends on conventional pharmaceutical (traditional medicine) for healthcare needs. Plants utilized for conventional pharmaceutical contain an extensive variety of substances that can be utilized to treat ceaseless and additionally irresistible ailments (Duraipandiyan et al., 2006). Because of the improvement of antagonistic impacts and microbial imperviousness to the artificially blended medications, men turned to ethnopharmacognosy. They discovered actually a large number of phytochemicals from plants as protected and extensively compelling choices with less unfavourable impact. Numerous helpful organic movement, for example, anticancer, antimicrobial, cancer prevention agent, antidiarrheal, pain relieving and wound mending action were accounted for. By and large the individuals assert the great profit of certain regular or home grown items. On the other hand, clinical trials are important to exhibit the viability of a bioactive compound to check this customary care. Clinical trials coordinated towards understanding the pharmacokinetics, bioavailability, viability, wellbeing and medication cooperation of recently created bioactive mixes and their details (concentrates) oblige a cautious assessment. Clinical trials are painstaking wanted to protect the wellbeing of the members and answer particular exploration addresses by assessing for both quick and long haul symptoms and their results are measured before the medication is broadly connected to patients. according to the World Health Organization (WHO), about 20,000 therapeutic plants exist in 91 nations including 12 mega biodiversity nations. The head steps to use the naturally dynamic compound from plant assets are extraction, pharmacological screening, isolation and characterization of bioactive compound, toxicological assessment and clinical assessment. A short summary of the general methodologies in extraction, disengagement and characterization of bioactive compound from plants concentrate can be found in Figure 1. This paper gives points of interest in extraction, disengagement and characterization of bioactive compound from plants separate with normal phytochemical screening test, chromatographic systems, for example, HPLC, and HPLC/MS and Fourier Transform Mass Spectrometry (FTMS).

METHODS

The essential operation included steps like prewashing, drying of plant materials or stop drying, crushing to acquire a homogenous specimen and regularly enhancing the energy of diagnostic extraction. Basic operation includes active constituents are not lost, mutilated or crushed amid the arrangement of the concentrate from

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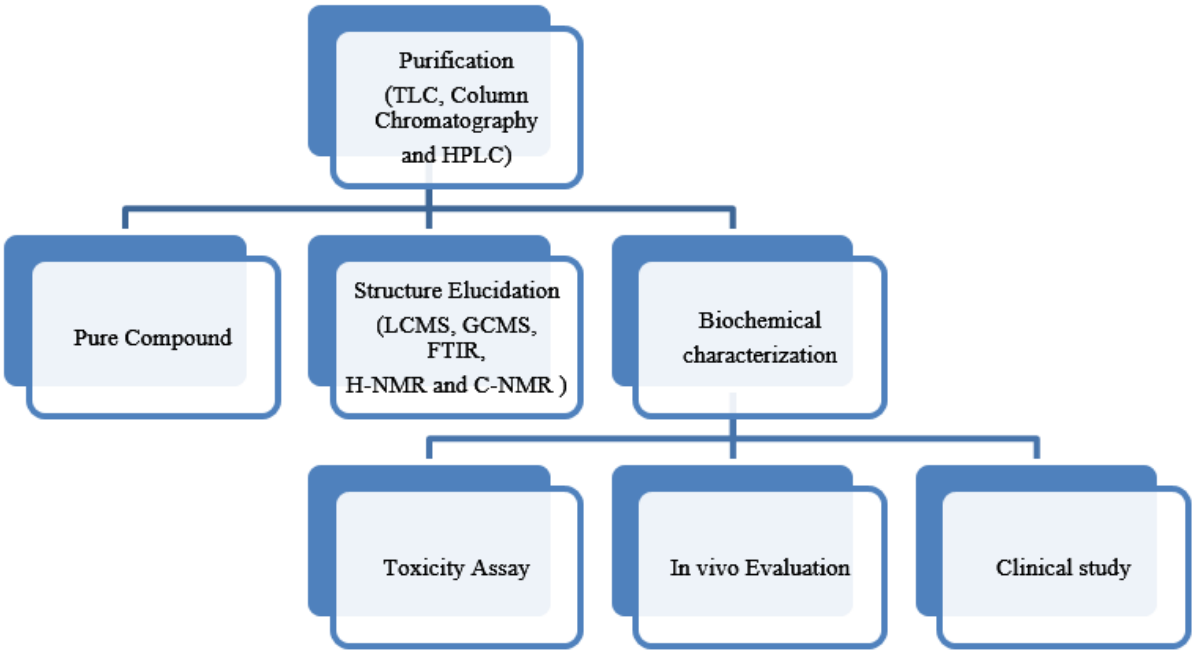

Figure 1: A brief scheme of extraction, isolation and characterization of bioactive compound from plants extract

Table 1: A brief summary of the experimental conditions for various methods of extraction for plants materials

	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 (^{0}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)	150-200	50 -100	Depending on the sample size

plant examples. In the event that the plant was chosen on the premise of customary uses (Fabricant and Farnsworth, 2001), then it is required to set up the concentrate as depicted by the conventional healer to copy as nearly as would be prudent the customary "home grown" drug. The determination of dissolvable framework to a great extent relies on upon the particular nature of the bioactive compound being focused on. Diverse dissolvable frameworks are accessible to concentrate the bioactive compound from regular items. The extraction of hydrophilic mixes utilizes polar solvents, for example, methanol, ethanol or ethyl-acetic acid derivation. For extraction of more lipophilic mixes, dichloromethane or a mixture of dichloromethane/methanol in proportion of 1:1 are utilized. In a few cases, extraction with hexane is utilized to uproot chlorophyll (Cos et al., 2006). As the target mixes may be non-polar to polar and thermally labile, the suitability of the systems for extraction must be considered. Different systems, for example, sonification, warming under reflux, soxhlet extraction and others are usually utilized (United States Pharmacopeia and National Formulary, 2002; Pharmacopeia of the People's Republic of China, 2000; The Japanese Pharmacopeia, 2001) for the plant specimens extraction. What's more, plant concentrates are additionally arranged by maceration or permeation of new green plants or dried powdered plant material in water and/or natural dissolvable frameworks. A short synopsis of the exploratory conditions for the different techniques for extraction is indicated in Table 1. The other present day extraction systems incorporate robust stage micro-extraction, supercritical-liquid extraction, pressurized-fluid extraction, microwave-supported extraction, strong stage extraction, and surfactant-intervened methods, which have specific points of interest. These are the decrease in natural dissolvable utilization and in specimen debasement, disposal of extra example clean-up and fixation steps before chromatographic investigation, change in extraction effectiveness, selectivity, and/ energy of extraction. The simplicity of robotization for these systems additionally supports their use for the extraction of plants materials (Huie, 2002).

Identification and characterization

Due to the fact that plant extracts usually occur as a

combination of various type of bioactive compounds or phytochemicals with different polarities, their separation still remains a big challenge for the process of identification and characterization of bioactive compounds. It is a common practice in isolation of these bioactive compounds that a number of different separation techniques such as TLC, column chromatography, flash chromatography, Sephadex chromatography and HPLC, should be used to obtain pure compounds. The pure compounds are then used for the determination of structure and biological activity. Beside 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

Thin-layer chromatography (TLC) and Bio-autographic methods

TLC is additionally used to backing the personality of a compound in a mixture when the Rf of a compound is ompared with the Rf of a known compound. Extra tests include the showering of phytochemical screening reagents, which cause shade changes as indicated by the phytochemicals existing in a plants remove; or by survey the plate under the UV light. This has additionally been utilized for affirmation of immaculateness and character of confined mixes.

Bio-autography is a helpful method to focus bioactive compound with antimicrobial action from plant concentrate. TLC bioautographic strategies join chromatographic partition and in situ movement determination encouraging the confinement and target-coordinated disconnection of dynamic constituents in a mixture. Customarily, bioautographic method has utilized the development restraint of microorganisms to recognize hostile to microbial segments of concentrates chromatographed on a TLC layer. This strategy has been considered as the most viable test for the identification of hostile to microbial mixes (Shahverdi, 2007).bio-autography limits antimicrobial movement on a chromatogram utilizing three methodologies: (i) coordinate bio-autography, where the micro-creature develops straightforwardly on the slim layer chromatographic (TLC) plate, (ii) contact bio-autography, where the antimicrobial mixes are exchanged from the TLC plate to a vaccinated agar plate through immediate contact and (iii) agar overlay bio-autography, where a seeded agar medium is connected specifically onto the TLC plate (Hamburger and Cordell, 1987; Rahalison et al., 1991). The hindrance zones created on TLC plates by one of the above bioautographic method will be use to imagine the position of the bioactive compound with antimicrobial action in the TLC unique mark with reference to Rf values (Homans and Fuchs, 1970). Preparative TLC plates with a thickness of 1mm were readied utilizing the same stationary and portable stages as above, with the destination of confining the bioactive parts that showed the antimicrobial action against the test strain. These zones were scratched from the plates, and the substance eluted from the silica with ethanol or methanol. Eluted examples were further filtered utilizing the above preparative chromatography system. At last, the segments were recognized by HPLC, LCMS and GCMS. In spite of the fact that it has high affectability, its relevance is restricted to micro-organic entities that effectively develop on TLC plates. Different issues are the requirement for complete evacuation of remaining low unpredictable solvents, for example, n-Buoh, trifluoroacetic corrosive and alkali and the exchange of the dynamic mixes from the stationary stage into the agar layer by dispersion (Cos et al., 2006). Since bio-autography permits limiting antimicrobial exercises of a concentrate on the chromatogram, it backs a brisk quest for new antimicrobial executors through bioassay-guided disengagement (Cos et al., 2006). The bioautography agar overlay system is worthwhile in that, firstly it utilizes next to no measure of example when contrasted with the ordinary plate dissemination strategy and subsequently, it can be utilized for bioassay-guided segregation of mixes. Also, since the rough concentrate is determined into its distinctive segments, this method disentangles the procedure of ID and separation of the bioactive mixes (Rahalison et al.,1991).

High performance liquid chromatography

High performance liquid chromatography (HPLC) is a flexible, powerful, and generally utilized strategy for the confinement of regular items (Cannell, 1998). Presently, this system is picking up notoriety among different investigative strategies as the principle decision for fingerprinting study for the quality control of natural plants (Fan et al., 2006). Regular items are every now and again disengaged after the assessment of a moderately unrefined concentrate in an organic test to completely describe the dynamic element. The organically dynamic element is frequently introduce just as minor part in the concentrate and the determining force of HPLC is preferably suited to the quick handling of such multicomponent tests on both an investigative and preparative scale. Numerous seat top HPLC instruments now are measured in outline and involve a dissolvable conveyance pump, a specimen presentation gadget, for example, an auto-sampler or manual infusion valve, an investigative segment, a gatekeeper section, locator and a recorder or a printer. Compound divisions can be fulfilled utilizing HPLC by using the way that certain mixes have distinctive
movement rates given a specific section and versatile stage. The degree or level of partition is generally dictated by the decision of stationary stage and portable stage. For the most part the recognizable proof and partition of phytochemicals can be fulfilled utilizing isocratic framework (utilizing single perpetual portable stage framework). Inclination elution in which the extent of natural dissolvable to water is changed with time may be attractive if more than one specimen segment is, no doubt examined and vary from one another essentially in maintenance under the conditions utilized.

Cleansing of the compound of enthusiasm utilizing HPLC is the methodology of dividing or concentrating the target compound from other (potentially structurally related) mixes or contaminants. Each one compound ought to have

a trademark top under certain chromatographic conditions. Contingent upon what needs to be divided and how nearly related the examples are, the chromatographer may pick the conditions, for example, the best possible versatile stage, stream rate, suitable finders and segments to get an ideal detachment. Distinguishing proof of mixes by HPLC is a vital piece of any HPLC measure. Keeping in mind the end goal to distinguish any compound by HPLC, a locator should first be chosen. Once the identifier is chosen and is situated to ideal discovery settings, a detachment measure must be created. The parameters of this examine ought to be such that a clean crest of the known example is seen from the chromatograph. The recognizing crest ought to have a sensible maintenance time and ought to be generally differentiated from incidental crests at the identification levels which the test will be performed. UV finders are famous among all the indicators on the grounds that they offer high affectability (Lia et al., 2004) furthermore in light of the fact that lion's share of regularly happening mixes experienced have some UV absorbance at low wavelengths (190-210 nm) (Cannell, 1998). The high affectability of UV identification is reward if a compound of investment is just present in little sums inside the example. Other than UV, other identification strategies are likewise being utilized to catch phytochemicals among which is the diode cluster identifier (DAD) coupled with mass spectrometer (MS) (Tsao and Deng, 2004). Fluid chromatography coupled with mass spectrometry (LC/MS) is additionally an effective system for the examination of complex plant concentrates (Cai et al., 2002; He, 2000). It gives inexhaustible data to structural clarification of the mixes when pair mass spectrometry (Msn) is connected. Consequently, the combo of HPLC and MS encourages fast and precise recognizable proof of substance mixes in restorative herbs, particularly when an immaculate standard is inaccessible (Ye et al., 2007).the transforming of an unrefined source material to give an example suitable to HPLC dissection and the decision of dissolvable for specimen reconstitution can have a noteworthy bearing on the general accomplishment of regular item detachment. The source material, e.g., dried powdered plant, will at first need to be dealt with in such a route as to guarantee that the compound of investment is productively freed into arrangement. On account of dried plant material, a natural dissolvable (e.g., methanol, chloroform) may be utilized as the introductory extractant and after a time of maceration, robust material is then evacuated by tapping off the concentrate by filteration. The filtrate is then packed and infused into HPLC for partition. The use of gatekeeper segments is fundamental in the dissection of rough concentrate. Numerous common item materials contain critical level of firmly tying segments, for example, chlorophyll and different endogenous materials that may in the long haul bargain the execution of systematic sections. Hence, the watchman sections will altogether secure the lifespan of the diagnostic segments.

Non-chromatographic techniques

Immunoassay

Immunoassays, which use monoclonal antibodies against drugs and low molecular weight natural bioactive compounds, are becoming important tools in bioactive compound analyses. They show high specificity and sensitivity for receptor binding analyses, enzyme assays and qualitative as well as quantitative analytical techniques. Enzyme-linked immunosorbent essay (ELISA) based on MAbs are in many cases more sensitive than conventional HPLC methods. Monoclonal antibodies can be produced in specialized cells through a technique known as hybridoma technology (Shoyama et al., 2006). The following steps are involved in the production of monoclonal antibodies via hybridoma technology against plant drugs:

(i) A rabbit is immunized through repeated injection of specific plant drugs for the production of specific antibody, facilitated due to proliferation of the desired B cells.

(ii) Tumors are produced in a mouse or a rabbit.

(iii) From the above two types of animals, spleen cell (these cells are rich in B cells and T cells) are cultured separately. The separately cultured spleen cells produce specific antibodies against the plants drug, and against myeloma cells that produce tumors.

(iv) The production of hybridoma by fusion of spleen cells to myeloma cells is induced using polyethylene glycol (PEG). The hybrid cells are grown in selective hypoxanthine aminopterin thymidine (HAT) medium.

(v) The desired hybridoma is selected for cloning and antibody production against a plant drug. This process is facilitated by preparing single cell colonies that will grow and can be used for screening of antibody producing hybridomas.

(vi) The selected hybridoma cells are cultured for the production of monoclonal antibodies in large quantity against the specific plants drugs.

(vii) The monoclonal antibodies are used to determine similar drugs in the plants extract mixture through enzyme-linked immunosorbent essay (ELISA).

Phytochemical screening assay

Phytochemicals are chemicals derived from plants and the term is often used to describe the large number of secondary metabolic compounds found in plants. Phytochemical screening assay is a simple, quick, and inexpensive procedure that gives the researcher a quick answer to the various types of phytochemicals in a mixture and an important tool in bioactive compound analyses. A brief summary of the experimental procedures for the various phytochemical screening methods for the secondary metabolites is shown in Table 2. After obtaining the crude extract or active fraction from plant material, phytochemical screening can be performed 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 ended up being an important apparatus for the characterization and distinguishing proof of mixes or utilitarian gatherings (synthetic bonds) display in an obscure mixture of plants concentrate (Eberhardt et al., 2007; Hazra et al., 2007). What's more, FTIR spectra of unadulterated mixes are normally so novel that they are similar to an atomic "unique finger impression". For most

regular plant exacerbates, the range of an obscure compound can be recognized by correlation to a library of known mixes. Tests for FTIR can be arranged in various ways. For fluid specimens, the most straightforward is to place one drop of example between two plates of sodium chloride. The drop structures a meager film between the plates. Robust examples can be processed with potassium bromide (KBr) to and afterward compacted into a slender pellet which can be broke down. Something else, strong specimens can be broken down in a dissolvable, for example, methylene chloride, and the arrangement then put onto a solitary salt plate. The dissolvable is then vanished off, leaving a slender film of the first material on the plate.

Table2 : A brief summary of phytochemical screening of secondary metabolites

Secondary metabolites	Name of test	Methodology	Results	References
Alkaloid	Dragendorff's test	Spot a drop of extract on a small piece of precoated TLC plate. Spray the plate with Dragendorff's reagent.	Orange spot	(Kumar et al., 2007)
	Wagner test	Add 2ml filtrate with 1% HCl + steam. Then add 1ml of the solution with 6 drops of Wagner's reagent.	Brownish-red precipitate	(Chanda et al., 2006).
	TLC method 1	Solvent system: Chloroform: methanol: 25% ammonia (8:2:0.5). Spots can be detected after spraying with Dragendorff reagent	Orange spot	(Tona et al., 1998)
	TLC method 2	Wet the powdered test samples with a half diluted NH4OH and lixiviated with EtOAc for 24hr at room temperature. Separate the organic phase from the acidified filtrate and basify with NH4OH (pH 11-12). Then extract it with chloroform (3X), condense by evaporation and use for chromatography. Separate the alkaloid spots using the solvent mixture chloroform and methanol (15:1). Spray the spots with Dragendorff's reagent.	Orange spot	(Mallikharjuna et al., 2007).
Anthraquinone	Borntrager's test	Heat about 50mg of extract with 1ml 10% ferric chloride solution and 1ml of concentrated hydrochloric acid. Cool the extract and filter. Shake the filtrate with equal amount of diethyl ether. Further extract the ether extract with strong ammonia.	Pink or deep red coloration of aqueous layer	(Kumar et al., 2007)
	Borntrager's test	Add 1 ml of dilute (10 %) ammonia to 2 ml of chloroform extract.	A pink-red color in the ammoniacal (lower) layer	(Onwukaeme et al., 2007).
Cardiac glycosides	Kellar – Kiliani test	Add 2ml filtrate with 1ml of glacial acetic acid, 1ml ferric chloride and 1ml concentrated sulphuric acid.	Green-blue coloration of solution	(Parekh and Chanda, 2007).
	Kellar – Kiliani test	Dissolve 50 mg of methanolic extract in 2 ml of chloroform. Add $H2SO_4$ to form a layer.	Brown ring at interphase	(Onwukaeme et al., 2007).
	TLC method	Extract the powdered test samples with 70% EtOH on rotary shaker (180 thaws/min) for 10hr. Add 70% lead acetate to the filtrate and centrifuge at 5000rpm/10 min. Further centrifuge the supernatant by adding 6.3% Na2CO3 at 10000 rpm/10min. Dry the retained supernatant and redissolved in chloroform and use for chromatography. Separate the glycosides using EtOAc-MeOH-H_2O (80:10:10) solvent mixture.	The color and hRf values of these spots can be recorded under ultraviolet (UV254 nm) light	(Mallikharjuna et al., 2007).

Flavonoid	Shinoda test	To 2-3ml of methanolic extract, add a piece of magnesium ribbon and 1ml of concentrated hydrochloric acid.	Pink red or red coloration of the solution	(Kumar et al., 2007).
	TLC method	Extract 1g powdered test samples with 10ml methanol on water bath (60°C/ 5min). Condense the filtrate by evaporation, and add a mixture of water and EtOAc (10:1 mL), and mix thoroughly. Retain the EtOAc phase and use for chromatography. Separate the flavonoid spots using chloroform and methanol (19:1) solvent mixture.	The color and hRf values of these spots can be recorded under ultraviolet (UV254nm) light	(Mallikharjuna et al., 2007).
	NaOH test	Treat the extract with dilute NaOH, followed by addition of dilute HCl.	A yellow solution with NaOH, turns colourless with dilute HCl	(Onwukaeme et al., 2007).
Phenol	Phenol test	Spot the extract on a filter paper. Add a drop of phoshomolybdic acid reagent and expose to ammonia vapors.	Blue coloration of the spot	(Kumar et al., 2007);
Phlobatannin	-	2 ml extract was boiled with 2 ml of 1% hydrochloric acid HCl.	Formation of red precipitates	(Edeoga et al., 2005).
Pyrrolizidine alkaloid	-	Prepare 1ml of oxidizing agent, consisting of 0.01ml hydrogen peroxide (30% w/v) stabilized with tetrasodium pyrophosphate (20mg/ml) and made up to 20ml with isoamylacetate, and add to 1ml of plant extract. Vortex the sample and add 0.25ml acetic anhydride before heating the sample at 60°C for 50-70s.Cool the samples to room temperature. Add 1ml of Ehrlich reagent and place the test tubes in water bath (60°C) for 5min. Measure the absorbance at 562nm. The method of Holstege et al. (1995) should be used to confirm results of the screening method	Peaks were compared with the GC–MS library	(McGaw et al., 2007; Mattocks, 1967; Holstege et al., 1995)
Reducing sugar	Fehling test	Add 25ml of diluted sulphuric acid (H2SO4) 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 precipitate	(Akinyemi et al., 2005)
Saponin	Frothing test / Foam test	Add 0.5ml of filtrate with 5ml of distilled water and shake well.	Persistence of frothing	(Parekh and Chanda, 2007).
	TLC method	Extract two grams of powdered test samples with 10 ml 70% EtOH by refluxing for 10 min. Condense the filtrate, enrich with saturated *n*-BuOH, and mix thoroughly. Retain the butanol, condense and use for chromatography.Separate the saponins using chloroform, glacial acetic acid, methanol and water (64:34:12:8) solvent mixture. Expose the chromatogram to the iodine vapours.	The colour (yellow) and hRf values of these spots were recorded by exposing chromatogram to the iodine vapours	(Mallikharjuna et al., 2007).
Steroid	Liebermann-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 coloration	(Kumar et al., 2007).
	-	To 1 ml of extract, add 2 ml acetic anhydride and 2 ml concentrated sulphuric acid H_2SO_4.	Color change to blue or green	(Edeoga et al., 2005).

	TLC method	Extract two grams of powdered test samples with 10ml methanol in water bath (80°C/15 min). Use the condensed filtrate for chromatography. The sterols can be separated using chloroform, glacial acetic acid, methanol and water (64:34:12:8) solvent mixture. The color and hRf values of these spots can be recorded under visible light after spraying the plates with anisaldehydesulphuric acid reagent and heating (100°C/6 min)	The color (Greenish black to Pinkish black) and hRf values of these spots can be recorded under visible light	(Mallikharjuna et al., 2007).
Tannin	Braemer's test	10% alcoholic ferric chloride will be added to 2-3ml of methanolic extract (1:1)	Dark blue or greenish grey coloration of the solution	(Kumar et al., 2007); (Parekh and Chanda, 2007).
Terpenoid	Liebermann-Burchardt test	To 1ml of methanolic extract, add 1ml of chloroform, 2-3ml of acetic anhydride, 1 to 2 drops of concentrated sulphuric acid.	Pink or red coloration	(Kumar et al., 2007).
	Salkowski test	5 ml extract was added with 2 ml of chloroform and 3 ml of concentrated sulphuric acid H2SO4.	Reddish brown color of interface	(Edeoga et al., 2005).
Volatile oil	-	Add 2 ml extract with 0.1 ml dilute NaOH and small quantity of dilute HCl. Shake the solution.	Formation of white precipitates	(Dahiru et al., 2006).

CONCLUSION

Since bioactive compounds occurring in plant material consist of multi-component mixtures, their separation and determination still creates problems. Practically most of them have to be purified by the combination of several chromatographic techniques and various other purification methods to isolate bioactive compound(s).

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