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PHYTOCHEMICAL TECHNIQUES - A REVIEW

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ABSTRACT

Plant and plant products are an important part of the human diet and a major source of biologically active substances such as vitamins, dietary fiber, antioxidants, and cholesterol-lowering compounds. Despite a large amount of information on this topic, the nutritional quality of plants has not been defined. Historically, the value of many plant nutrients and health-promoting compounds was discovered by trial and error. By the turn of the century, the application of chromatography, mass spectrometry, infrared spectrometry, and nuclear magnetic resonance allowed quantitative and qualitative measurements of a large number of plant metabolites. Approximately 50000 metabolites have been elucidated in plants, and it is predicted that the final number will exceed 200000. Most of them have unknown function. Metabolites such as carbohydrates, organic and amino acids, inorganic elements, vitamins, hormones, flavonoids, phenolics, and glucosinolates are essential for plant growth, development, stress adaptation, and defense. Besides the importance for the plant itself, such metabolites determine the nutritional quality of food, color, taste, smell, antioxidative, anticarcinogenic, antihypertension, anti-inflammatory, antimicrobial, immunostimulating, and cholesterol-lowering properties. This review is focused on major plant metabolites and the methods of their analysis.

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INTRODUCTION

Phytochemicals (from the Greek word phyto, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients (Hasler and Blumberg,1999). They protect plants from disease and damage and contribute to the plant's color, aroma and flavor. In general, the plant chemicals that protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack are called as phytochemicals (Mathai, 2000). Recently, it is clearly known that they have roles in the protection of human health, when their dietary intake is significant. More than 4,000 phytochemicals have been cataloged and are classified by protective function, physical characteristics and chemical characteristics (Meagher and Thomson,1999). Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs.

Medicinal plants are the nature's gift to human being to make disease free healthy life. It plays a vital role to preserve our health. India is one of the most medico culturally diverse countries in the world where the medicinal plant sector is part of a time honored tradition that is respected even today. Traditional medicines derive their scientific heritage from rich experiences of ancient civilization. Hence, it is not surprising that traditional medicines claim comes for several "difficult to cure" diseases (Satyavati, 1982). India is well known for its rich traditional systems of medicine i.e. Siddha, Ayurveda, Unani and Amchi (Tibetan) besides a vast reservoir of living traditions in ethnomedicine. The earliest mention of the use of plants in medicine is found in the Rigveda, which was written between 4500 and 1600 BC. During British period due to Western culture, our traditional art of natural healing is disappeared. Now it is reappearing due to realization of its importance in curing diseases without any side effects.

The World Health Organization (WHO) estimates that 80 % of the people of developing countries rely on traditional medicines, mostly plant derived drugs, for their primary health needs. Medicinal plants are commonly used in treating and preventing specific ailments and are considered to play a significant role in health care. Use of plants in traditional medicinal systems is an indispensable source of medicinal preparations. Hundreds of species are recognized as having medicinal value. Indeed, 'Phytomedicines' are beginning to link traditional and modern medicines (WHO, IUCN and WWF, 1993).

Research regarding medicinal plant is a highlighted issue today. Medicinal plants are the nature's gift of human being to make disease free healthy life. It plays a vital role to preserve our health. Plants synthesize an array of chemical compounds that are not involved in their primary metabolism. These 'secondary compounds' instead serve a variety of ecological functions, ultimately to enhance the plant's survival during stress. In addition, these compounds may be responsible for the beneficial effects of fruits and vegetables on an array of health related measures (Dahanukar, 2000).

Many of today's synthetic drugs originated from the plant kingdom but, historically, medicinal herbalism went into decline when pharmacology established itself as a leading and effective branch of medical therapeutics. In much of the English-speaking world, herbalism virtually vanished from the therapeutic map of medicine during the last part of the 19th and early part of the 20th century. However, in many third world countries various forms of ethnic herbalism prevail to the present day (e.g., Ayurvedic medicine in India, Kampo medicine in Japan and Chinese herbalism in China). In some developed countries (e.g. Germany and France), medical herbalism continues to co-exist with modern pharmacology, albeit on an increasingly lower key (Gao *et al*., 1999).

Owing to the global trends towards improved 'quality of life', there is a considerable evidence of an increase in demand for medicinal plant (Kotnis *et al*., 2004). Use of plants for treating various ailments of both man and animal is as old practice as man himself. India is the richly endowed with a wide variety of plants having medicinal value. These plants are widely used by all sections of the society whether directly as folk remedies or indirectly as pharmaceutical preparation of modern medicine (Bhagwati Uniyal, 2003). In recent times, focus on plants research has increased all over the world and a large body of evidence collected to show immense potential of medicinal plants used in various traditional systems (Ayurveda, Siddha and Unani) (Dahanukar, 2000).

Medicinal plants are assuming greater importance in the primary health care of individuals and communities in many developing countries. There has been an increase of demand in international trade because of very effective, cheaply available, supposedly have no side effects and used as alternative to allopathic medicines. Medicinal plants are believed to be much safer and proved elixir in the treatment of various ailments (Ashis, 2003).

Plants have basic nutritional importance by their content of protein, carbohydrate, fats and oils minerals, vitamins and water responsible for growth and development in man and animals. Phytochemical simply means plant chemicals. "Phyto" is the Greek word for plant. Phytochemicals are classified as primary or secondary constituents, depending on their role in plant metabolism. Primary metabolism is important for growth and development of plants include the common sugars, aminoacids, proteins, purines and pyrimidines of nucleic acids, chlrophyll's etc. Secondary metabolism in a plant plays a major role in the survival of the plant in its environment. Attractions of pollinators, natural defense system against predators and diseases, etc., are examples of the roles of secondary metabolites (Velavan *et al*., 2007).

The secondary metabolites formed also are an important trait for our food plants (taste, colour, scent, etc.) and ornamental plants. Moreover, numerous plant secondary metabolites such as flavonoids, alkaloids, tannins, saponins, steroids, anthocyanins, terpenoids, rotenoids etc. have found commercial application as drug, dye, flavour, fragrance, insecticide, etc. Such fine chemicals are extracted and purified from plant materials. Plant produces these chemicals to protect itself but recent research demonstrates that many phytochemicals can protect humans against diseases including cancer, cardiovascular, arthritis, diabetic, aging etc (Velavan, 2011).

Selection of Plant Materials

The selection, collection, and identification of plant material are essential for phytochemical research. Carelessness at this stage of an investigation may greatly reduce the scientific value of the overall study.

Saples may be selected using a number of approaches

1.The investigation of plants traditionally used by humans for food, medicine, or poison based on review of the literature or interviews conducted as part of the investigation

2.The random or systematic collection of a biodiverse set of plant samples, typically from an ecological region that is comparatively uncharted as regards secondary metabolite production.

3.The selection of species based on phylogenetic relationship to a species known to produce a compound or compound class of interest.

4.The study of species based on reports of biological activity in the literature (including chemical ecology, toxicology, and veterinary reports).

Collection and Identification of Plant Materials

The collections of plant material from the field have been addressed elsewhere.

1.Review the flora of the region; to compile a list of which species, genera, or families are of particular interest; and to determine which taxa are to be avoided.

2.It is advisable to attempt field identification of the samples collected (at least to the level of genus).

3.To aid taxonomic experts in confirming or refining the field identification, and as a permanent scientific record, voucher specimens (including reproductive organs, when feasible) should be prepared and deposited in herbaria, including at least one major institution and, if applicable, in a local herbarium in the source country.

Drying and Grinding of Plant Materials

Plant material should be dried at temperatures below 300C to avoid decomposition of thermolabile compounds. Likewise, it should be protected from sunlight because of the potential for chemical transformations resulting from exposure to ultraviolet radiation.

1.To prevent the buildup of heat and moisture, air circulation around the plant material is essential. Hence, it should not be compacted, and it may be necessary to use a fan or other means to provide air flow around or through the drying sample.

2.Plant material can be milled using an electric grinder or spice mill, or in a mortar and pestle.

3.Grinding improves the efficiency of extraction by increasing the surface area of the plant material. It also decreases the amount of solvent needed for extraction by allowing the material to pack more densely.

4.Milling plant material to a fine powder would be ideal, if the particles are too fine, solvent cannot flow easily around them. Furthermore, the friction of milling generates heat (the finer the particle produced, the more heat), potentially causing volatile onstituents to be lost, and thermolabile components to degrade and oxidize.

Choice of solvents

Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate. The factors affecting the choice of solvent are quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractants. The choice of solvent is influenced by what is intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay. The choice will also depend on the targeted compounds to be extracted (Table 1).

The various solvents that are used in the extraction procedures are:

1.Water: Water is universal solvent, used to extract plant products with antimicrobial activity. Though traditional healers use primarily water but plant extracts from organic solvents have been found to give more consistent antimicrobial activity compared to water extract. Also water soluble flavonoids (mostly anthocyanins) have no antimicrobial significance and water soluble phenolics only important as antioxidant compound.

2.Acetone: Acetone dissolves many hydrophilic and lipophilic components from the two plants used, is miscible with water, is volatile and has a low toxicity to the bioassay used, it is a very useful extractant, especially for antimicrobial studies where more phenolic compounds are required to be extracted. A study reported that extraction of tannins and other phenolics was better in aqueous acetone than in aqueous methanol. Both acetone and methanol were found to extract saponins which have antimicrobial activity.

3.Alcohol: The higher activity of the ethanolic extracts as compared to the aqueous extract can be attributed to the presence of higher amounts of polyphenols as compared to aqueous extracts. It means that they are more efficient in cell walls and seeds degradation which have unpolar character and cause polyphenols to be released from cells. More useful explanation for the decrease in activity of aqueous extract can be ascribed to the enzyme polyphenol oxidase, which degrade polyphenols in water extracts, whereas in methanol and ethanol they are inactive. Moreover, water is a better medium for the occurrence of the micro-organisms as compared to ethanol. The higher concentrations of more bioactive flavonoid compounds were detected with ethanol 70% due to its higher polarity than pure ethanol. By adding water to the pure ethanol up to 30% for preparing ethanol 70% the polarity of solvent was increased. Additionally, ethanol was found easier to penetrate the cellular membrane to extract the intracellular ingredients from the plant material (Bimakr, 2010). Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction (Cowan, 1999). Polar solvents such as methanol, ethanol, either and their aqueous mixtures, are mostly recommended for the extraction of phenolics from a plant matrix. The use of mixture alcohol and water present the advantage of modulating the polarity of alcohol solvents, also adding that solubility of polyphenols depends mainly on the hydroxyl groups, the molecular size and the length of hydrocarbon. Addition of water to alcohol improves extraction rate. Methanol is the best solvent for polyphenol extraction (Adil *Et al*., 2007). Methanol is more polar than ethanol but due to its cytotoxic nature, it is unsuitable for extraction in certain kind of studies as it may lead to incorrect results.

4.Chloroform: Terpenoid lactones have been obtained by successive extractions of dried barks with hexane, chloroform and methanol with activity concentrating in chloroform fraction. Occasionally tannins and terpenoids will be found in the aqueous phase, but they are more often obtained by treatment with less polar solvents.

5.Ether: Ether is commonly used selectively for the extraction of coumarins and fatty acids.
6.Dichloromethanol: It is another solvent used for carrying out the extraction procedures. It is specially used for the selective extraction of only terpenoids
.

Table 1 shows the solvents used for extraction of active components

SOLVENTS						
Aqueous	**Methanol**	**Hydro-alcoholic (70%) (Methanol)**	**Ethanol**	**Chloroform**	**Ether**	**Acetone**
Terpenoids	Polyphenols	Polyphenols	Polyphenols	Terpenoids	Alkaloids	Phenol
Starches	Terpenoids	Terpenoids	Flavonol	Flavonoids	Terpenoids	Flavonols
Saponins	Saponins	Saponins	Tannins		Coumarins	
Anthocyanins	Tannins	Tannins	Terpenoids		Fatty acids	
Polypeptides	Xanthoxylines	Amino acid	Sterols			
Lectins	Totarol	Carbohydrate	Alkaloids			
Tannins	Quassinods	Quassinods	Polyacetylenes			
	Lactones	Glycoside				
	Flavones	Flavonoids,				
	Phenones	Phenones				
	Anthocyanins	Anthocyanins, Anthroquinone				
		Sterols				
		Alkaloids				

Factors affecting selection of an extraction process
1.**Character of Herb:-** The knowledge of pharmacognosy of the herb is essential to select the right method of extraction process. The maceration process is used when the herb is soft, unorganized unpowderable and to avoid powdering of it. The percolation process is used when the herb is hard and tough.
2.**Therapeutic value of the herb:-** When the herb has considerable therapeutic value, the maximum extraction is required, so the percolation process is used e.g. Belladonna. In case the herb has little therapeutic value, the efficiency of extraction is unimportant, and maceration process can be used to extract the herb.
3.**Stability of herb:-** Continuous hot extraction process should be avoided when the constituents of the herb are thermo labile in nature; in that case maceration or percolation process may be used to extract the active constituents of the herb.
4.**Solvent:-** If water is used as a solvent the maceration process should be recommended. The percolation process should be preferred if non-aqueous solvents are used for extraction.
5.**Concentration of product:-** The dilute products such as tinctures can be made by using maceration or percolation process, depending on the other factors. For semi-concentrated preparations, such as concentrated infusions, double or triple maceration process can be used. The liquid extracts or dry extracts which are concentrated preparations are prepared by using percolation process.

EXTRACTION PROCESS

Extraction (as the term is pharmaceutically used) is the separation of medicinally active portions of plant (and animal) tissues using selective solvents through standard procedures. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form, and are intended for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered extracts.

The general techniques of medicinal plant extraction include maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction (with hydrofluorocarbon solvents). For aromatic plants, hydrodistillation techniques (water distillation, steam distillation, water and steam distillation), hydrolytic maceration followed by distillation, expression and enfleurage (cold fat extraction) may be employed. Some of the latest extraction methods for aromatic plants include headspace trapping, solid phase micro-extraction, protoplast extraction, microdistillation, thermo microdistillation and molecular distillation (Handa *et al*., 2008).

Extraction methods used pharmaceutically involves the separation of medicinally active portions of plant tissues from the inactive/inert components by using selective solvents. During extraction, solvents diffuse into the solid plant material and solubilize compounds with similar polarity.

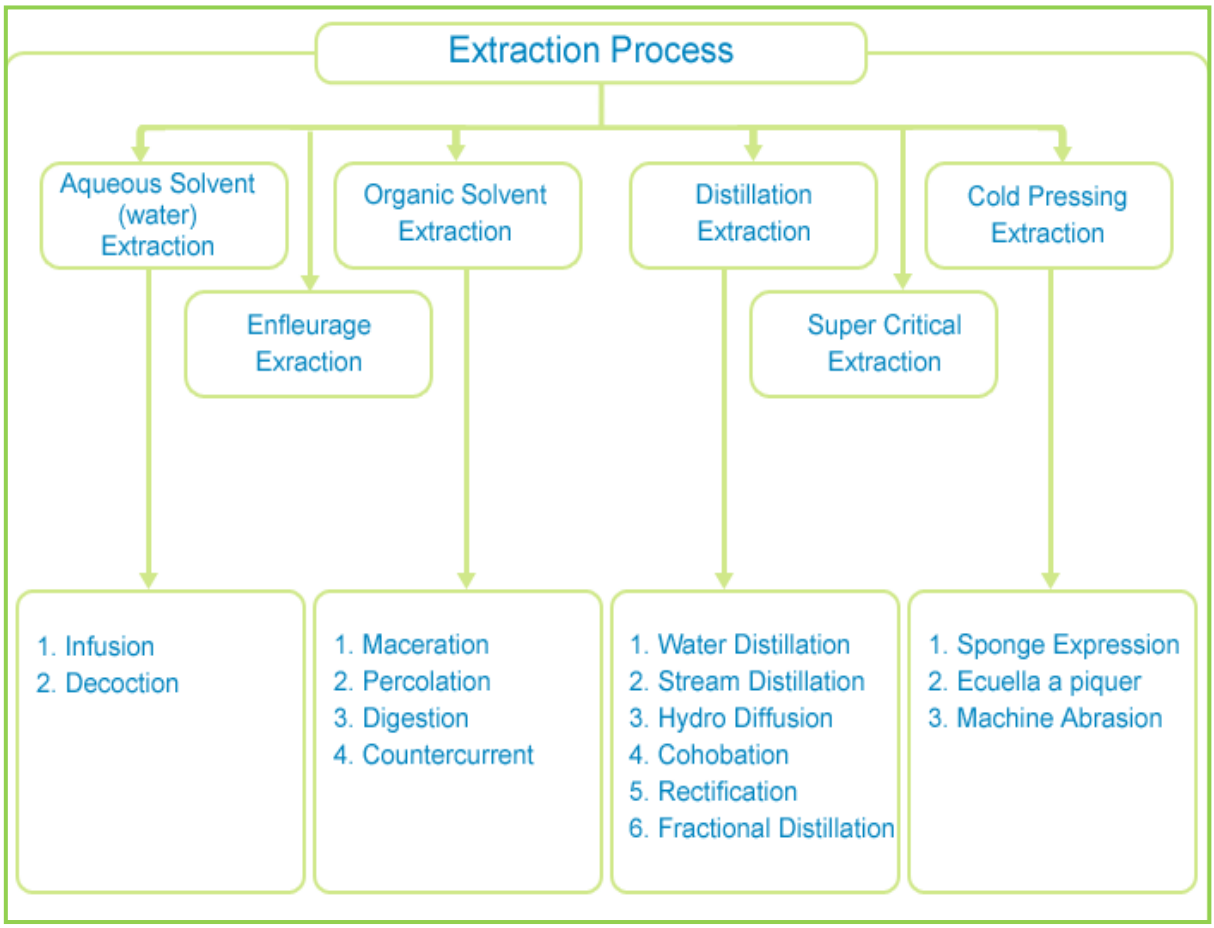

Fig 1 shows the methods of extraction process

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 redissolved in the solvent to determine the concentration. Some researchers however centrifuged the filtrate for clarification of the extract.

Serial exhaustive extraction: It is another common method of extraction which involves 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 compound 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.

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.

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.

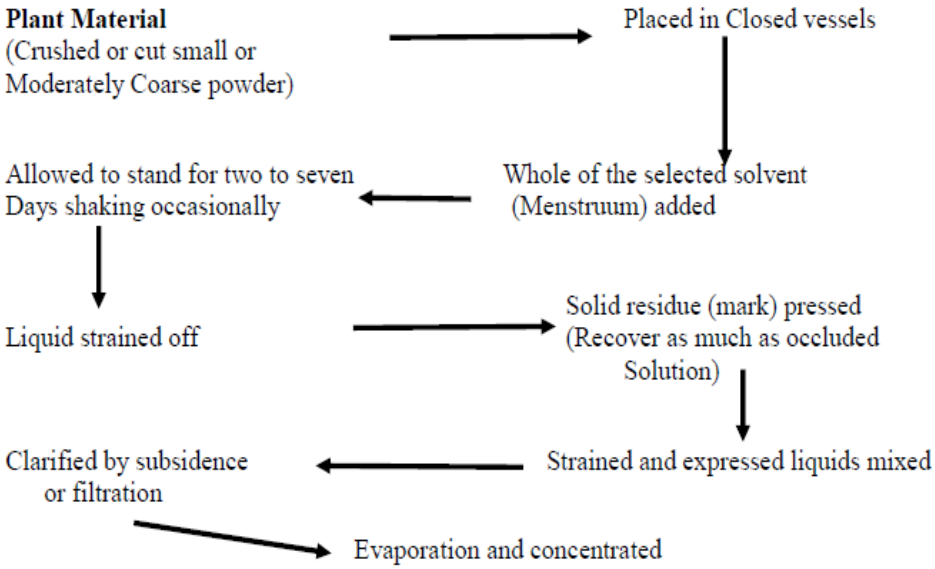

Fig 1. Maceration Process (Steady- State Extraction)

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.

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.

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.

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.

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.

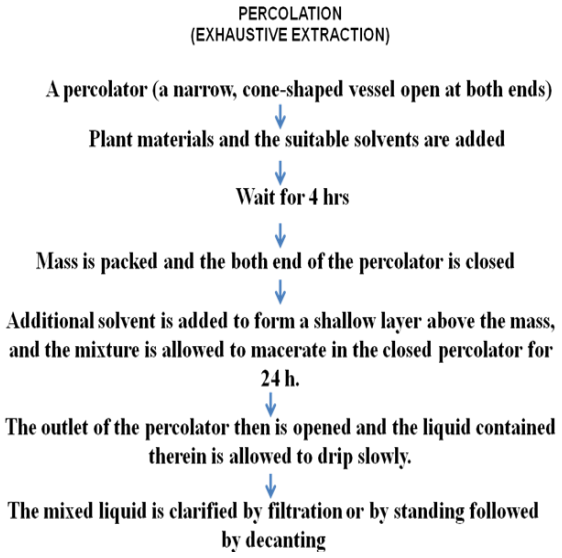

Fig 2. Percolation Process

Determination of extraction yield

The yield of evaporated dried extracts based on dry weight basis was calculated from equation shown below

Yield (%) = (W1 X 100) / W2

Where W1 was the weight of extract after evaporation of solvent. W2 was the dry weight of the sample

PHYTOCHEMICALS SCREENING

It is a series of tests that determines the presence or absence of certain chemical substances present in a plant. Chemical tests were carried out by using standard procedures to identify the preliminary phytochemical screening following the methodology of Sofowara (1993), Trease and Evans (1989), Savithramma *et al*. (2011), Rasal, (2005) Kokate, (2003) and Harborne (1973).

QUALITATIVE ANALYSIS OF PRIMARY METABOLITES

Test for carbohydrates

1.Benedict's test: To 0.5 ml of the filtrate, 0.5 ml of Benedict's reagent was added. The mixture was heated on boiling water bath for 2 min. A characteristic red colored precipitate indicates the presence of sugar.

2.Molisch's test: Crude extract was mixed with 2ml of Molisch's reagent and the mixture was shaken properly. After that, 2ml of concentrated H_2SO_4 was poured carefully along the side of the test tube. Appearance of a violet ring at the interphase indicated the presence of carbohydrate.

Test for Starch

0.01gms of Iodine and 0.075gms of KI were dissolved in 5ml of distilled water and 2-3ml of extract was added. Formation of blue color indicated the presence of starch.

Test for Proteins

1.1ml of plant sample was taken and added 1ml of 40% Sodium hydroxide and added slowly in the sides of test tubes of few drops of copper sulphate. Appearance of violet or pink colour indicates that the presence of protein.

2.Crude extract when mixed with 2ml of Millon's reagent, white precipitate appeared which turned red upon gentle heating that confirmed the presence of protein.

Test for Amino acid:

1.One ml of the extract was treated with few drops of Ninhydrin reagent. Appearance purple colour shows the presence of amino acids.

2.To 2 ml of extract, 3 drops of nitric acid were added by the side of the test tube. Absence of yellow colouration indicated the absence of proteins and free amino acids.

Fatty Acids: 0.5 ml of extract was mixed with 5 ml of ether. These extract was allow it for evaporation on filter paper and dried the filter paper. The appearance of transparence on filter paper indicates the presence of fatty acids

MISCELLANEOUS

Test for resins (Precipitation test): The extract (0.2 g) was extracted with 15 ml of 95% ethanol. The alcoholic extract was then poured into 20 ml of distilled water in a beaker.

Test for Fixed Oils and Fats: To 1ml of the extract, add few drops of 0.5 N alcoholic Potassium hydroxide along with a drop of phenolphthalein. Heat the mixture on a water bath for 1-2 hours. The formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

Test for Gums and Mucilage: Add about 10ml of aqueous extract slowly to 25ml of absolute alcohol with constant stirring. Filter the precipitate and dry in air. Examine the precipitate for its swelling properties and for the presence of carbohydrate.

QUALITATIVE ANALYSIS OF SECONDARY METABOLITES

Test for anthraquinones: Five ml of the extract solution was hydrolysed with diluted Conc. H_2SO_4 extracted with benzene. 1 ml of dilute ammonia was added to it. Rose pink coloration suggested the positive response for anthraquinones.

Test for alkaloids

1.Mayer's test (Evans, 1997): To a few ml of the filtrates, a drop of Mayer's reagent was added by the side of the test tube. A creamy or white precipitate indicates the test is positive.

2.Five ml of the extract was added to 2 ml of HCl. To this acidic medium, 1 ml of Dragendroff's reagent was added. An orange or red precipitate produced immediately indicates the presence of alkaloids.

3.Each extract was boiled (15 minutes) in HCl (25.0 ml, 1%). Equal volumes of the resulting suspension were filtered into two test tubes (A and B). To A, 5 drops of freshly prepared Dragendorrf's reagent was added. Formation of a precipitate indicated the presence of alkaloids. To confirm the results, B was treated with saturated sodium carbonate solution until a drop of the solution turned the Universal Indicator paper blue, (pH 8-9). The resulting solution was dissolved in $CHCl_3$ (4 ml) and allowed to stand. The aqueous layer was collected and acetic acid added to it drop wise, until the solution turned Universal Indicator paper yellow-brown (pH 5).

Test for Polyphenols

1.Ethanol (10.0 ml) was added to each extracts and the resulting solution (3.0 ml) was transferred in test tubes and warmed in a water bath (15 minutes). Three drops of freshly prepared ferric cyanide solution were added to the extract solution. Formation of a blue green colour indicated the presence of polyphenols.

2.Yellow precipitates were obtained by the addition of 3 drops of lead acetate solution (5%) indicated the phenolic compounds.

3.3 ml of 0.1% of gelatin solution was added to 5ml of ethanolic extract. Precipitation indicated.

Test for Tannins: About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue-black colouration.

Test for Phlobatannins: Deposition of a red precipitate when an aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatinins.

Test for Saponin: About 2 g 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 for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

Test for Flavonoids:

1.Three methods were used to determine the presence of flavonoids in the plant sample (Sofowara, 1993; Harbrone, 1973). 5 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H_2SO_4. A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colouration disappeared on standing.

2.Few drops of 1% aluminium solution were added to a portion of each filtrate. A yellow colouration was observed indicating the presence of flavonoids.

3.A portion of the powdered plant sample was in each case heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration was observed indicating a positive test for flavonoids.

Test for Catechins:

A 3 ml of test solution in alcohol was added with Ehrlich reagent and a few drops of Concentrated HCl. Formation of pink color indicates the presence of catechins.

Test for flavonols and flavones

3 ml of the filtrate was mixed with 4 ml of 1% aluminium chloride in methanol in a test tube and the colour was observed. Formation of yellow colour indicated the presence of flavonols amd flavones

Test for Chalcones

2 ml of Ammonium hydroxide was added to 0.5 g extract sample. Appearance of reddish colour showed the presence of chalcones

Test for phytosterol:

1.Two ml of acetic anhydride was added to 0.5 g ethanolic extract of each sample with 2 ml H_2SO_4. The colour changed from violet to blue or green in some samples indicating the presence of steroids.

2.The extract was refluxed with solution of alcoholic potassium hydroxide till complete saponification takes place. The mixture was diluted and extracted with ether. The ether layer was evaporated and the residue was tested for the presence of phytosterol. The residue was dissolved in few drops of diluted acetic acid; 3 ml of acetic anhydride was added followed by few drops of Conc. H_2SO_4. Appearance of bluish green colour showed the presence of phytosterol.

Test for Terpenoids (Salkowski test): Five ml of each extract was mixed in 2 ml of chloroform, and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the inter face was formed to show positive results for the presence of terpenoids.

Test for triterpenoids: Ten mg of the extract was dissolved in 1 ml of chloroform; 1 ml of acetic anhydride was added following the addition of 2 ml of Conc. H_2SO_4. Formation of reddish violet colour indicates the presence of triterpenoids.

Test for Cardiac glycosides (Keller-Killani test): Five ml of each extracts was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayed with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

Test for Anthocyanins: 2 ml of aqueous extract is added to 2 ml of 2N HCl and ammonia. The appearance of pink-red turns blue-violet indicates the presence of anthocyanins.

Test for Leucoanthocyanins: 5 ml of aqueous extract added to 5 ml of isoamyl alcohol. Upper layer appears red in colour indicates for presence of leucoanthocyanins

Test for Coumarins: 3 ml of 10% NaOH was added to 2 ml of aqueous extract formation of yellow colour indicates the presence of coumarins.

Test for Emodins: 2 ml of NH_3OH and 3 ml of Benzene was added 4 to the extract. Appearance of red colour indicates the presence of emodins.

Test for Acidic Compounds

1. To the 2ml of alcoholic extract, 1ml sodium bicarbonate solution was added. The effervescence produced indicated the presence of acidic compounds.

2. 2ml of alcoholic extract was taken in warm water and filtered. The filtrate was then tested with litmus paper and methyl orange. The appearance of blue color.

QUALITATIVE ANALYSIS OF VITAMINS

Test for Vitamin- A

Dissolve 250mg of powder sample in 5 ml of chloroform and filtered. Add 5ml of antimony trichloride solution. A transient blue colour is produced immediately.

Test for Vitamin-C

Dilute 1 ml of aqueous sample solution with 5 ml of water and add 1drop of freshly prepared 5% w/v solution of sodium nitroprusside and 2 ml of dilute sodium hydroxide solution. Add 0.6 ml of HCl drop wise and stir, the yellow colour turns blue.

Test for Vitamin -D

Dissolve a 500mg of plant sample in 10ml of chloroform and filtered. Add 10ml of antimony trichloride solution. a pinkish -red colour appears at once.

Test for Vitamin -E

500mg of the sample powder was macerated with 10ml of ethanol for 5 minutes and then filtered. Few drops of 0.1% ferric chloride in ethanol and 1ml of 0.25% of 2'-2'dipyridyl to 1ml of the filtrate. Bright-red colour was formed on a white background. The background gradually assumes a pink (Pearson, 1976; Patel, 2005).

QUALITATIVE ANALYSIS OF INORGANIC ELEMENTS

Ash of drug material (500mg) was prepared and treated with HNO_3 and HCl (3:1 v/v) for 1 hour. After the filtration, the filtrate was used to perform the following tests (Khandelwal 2006):

Calcium: One drop of dil. ammonium hydroxide and saturated ammonium oxalate solution was added to 10ml of the above filtrate. White precipitates of calcium oxalate,

soluble in hydrochloric acid but insoluble in acetic acid, were formed.

Magnesium: White calcium oxalate precipitate was separated by filtering the above solution. The filtrate was heated and cooled. Solution of sodium phosphate in dilute ammonia solution was added. White crystalline precipitate was observed.

Sodium: Little uranyl magnesium acetate reagent was added to 2ml of the test solution, shaken well and kept for few minutes. Yellow crystalline precipitate of sodium magnesium uranyl acetate was observed.

Potassium: Few drops sodium cobalt nitrite solution was added to 2-3ml of the test solution. Yellow precipitate of potassium cobalt nitrite was observed.

Iron: Few drops of 2% potassium ferrocyanide were added to 5ml of the test solution. Dark blue coloration was observed.

Sulphate: To 5ml of the test solution, lead acetate reagent was added. A white precipitate, soluble in sodium hydroxide, was formed.

Phosphate: 5ml of test solution was prepared in nitric acid and a few drops of ammonium molybdate solution were added. It was heated for about 10 minutes and left to be cooled. A yellow crystalline precipitate of ammonium molybdate was observed.

Chloride: 3 to 5ml of lead acetate solution was added to about 5 to 7ml of the filtrate. A white precipitate soluble in hot water was observed.

Nitrates: Ferrous sulphate solution was added to 5ml of the test solution. No brown colour was produced, but when sulphuric acid was added (slowly from the side of the test tube), a brown colored ring was produced at the junction of two liquids.

QUANTITATIVE ANALYSIS

Quantitative determination of the chemical constituency

Preparation of fat free sample: 2 g of the sample were defatted with 100 ml of diethyl ether using a soxhlet apparatus for 2 h.

Estimation of flavonoids

1.Total flavonoid contents were measured with the aluminum chloride colorimetric assay (Kumar *et al*., 2008) Aqueous and ethanolic extracts that has been adjusted to come under the linearity range i.e. (400µg/ml) or Aliquots of extract solutions were taken and made up the volume 3ml with methanol and different dilution of standard solution of Quercetin (10-100µg/ml) were added to 10ml volumetric flask. To the above mixture, 0.3ml of 5% $NaNO_2$ was added. After 5 minutes, 0.3ml of 10% $AlCl_3$ was added. After 6 min, 2ml of 1 M NaOH was added and the total volume was made up to 10ml with distill water. Then the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 520 nm. Total flavonoid content of the extracts was expressed as percentage of Quercetin equivalent per 100 g dry weight of sample.

2.Flavonoid determine by the method of Bohm and Kocipai-Abyazan (1994). 10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through whatman filter paper No 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight.

Determination of total phenols by spectrophotometric method:

The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. 5 ml of the extract was pipetted into a 50 ml flask, then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amylalcohol were also added. The samples were made up to mark and left to react for 30 min for colour development. This was measured at 505 nm.

Determination of Alkaloid

Alkaloid determine by the method of Harborne (1973). 5 g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

Tannin determination by Van-Burden and Robinson (1981) method:

500 mg of the sample was weighed into a 50 ml plastic bottle. 50 ml of distilled water was added and shaken for 1 h in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtered was pipetted out into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.1N HCl and 0.008 M potassium ferro cyanide. The absorbance was measured at 120 nm within 10 min.

Determination of Saponin:

The method used was that of Obadoni and Ochuko (2001). The samples were ground and 20 g of each were put into a conical flask and 100 cm3 of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 550C. The mixture was filtered and the residue re-extracted with another 200 ml 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90 0C. The concentrate was transferred into a 250 ml separatory funnel and 20 ml of diethyl ether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated. 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant.

QUANTITATIVE DETERMINATION OF VITAMINS

Determination of Riboflavin

Riboflavin was determined as per the method given by Okwu (2004). 5 gms of the individual plant sample was extracted with 100 ml of 50% ethanol solution and shaken for 1 hr. This was filtered into a 100 ml flask;

10 ml of the extract was pipetted into 50 ml volumetric flask. 10 ml of 5% potassium permanganate and 10 ml of 30% H2O2 were added and allowed to stand over a hot water bath for about 30 min. 2 ml of 40% Sodium sulphate was added. This was made up to 50 ml and the absorbance was measured at 510 nm in a spectrophotometer.

Determination of Niacin

Niacin was determined as per the method given by AOAC (1987). 5 gms fresh or 2 gms dried form of each plant samples was homogenized in sodium hydroxide and distilled water. The mixture was heated for 1 hour over a boiling water-bath, cooled and pH was adjusted to 4.5. 17g of ammonium sulphate was added. Color development achieved by reaction of the extract with cyanogen bromide was observed and then was measured at 450nm in spectrophotometer.

Determination of Thiamin:

Thiamin was determined as per the method given by Okwu (2004). 0.5gm of sample was homogenized in 50 ml ethanolic sodium hydroxide. Its 10 ml filtrate was added to 10 ml potassium dichromate and absorbance was recorded at 360 nm after development of color.

Determination of Ascorbic acid:

Ascorbic acid was determined as per the method given by Barkat *et al*., (1973). 5gm of sample was taken into 100 ml EDTA/ TCA (2:1) and mixed well. This mixture was centrifuge at 3000 rpm for 20 min. It was transferred to 100ml volumetric flask and volume was made up. 20ml of this mixture with 1% starch solution was titrated with 20% CuSO4 till the appearance of dark end point.

Determination of Vitamin A

Determination of vitamin A by the method of (Bayfield and Cole, 1980). Grind 1to5 gm of the sample material to a fine paste and add 1.0ml of saponification mixture. Reflex the tubes gently for 20minutes at 600C and cool the tubes at room temperature added 20ml water and mix well. Extract vitamin with 10ml of petroleum ether in a separating funnel twice. Pool the extract and added sodium sulphate to remove the moisture for 30-60minutes evaporate 5ml aliquot of the ether extract to dryness at 600C dissolve the dried residue in 1.0ml of chloroform. Make up the volume in each test tube to 1.0ml with chloroform. Added 2.0ml of TCA solution from a fast delivery pipette, rapidly mixing the contents of the tube. Read at620nm immediately in a spectrophotometer.

Ultraviolet/Visible (UV/VIS) Spectroscopy

Spectroscopy is the branch of science dealing with the study of interaction between Electromagnetic radiation and matter. It is a most powerful tool available for the study of atomic and molecular structure/s and is used in the analysis of wide range of samples. Optical spectroscopy includes the region on electromagnetic spectrum between 100 and 400 µm. UV-Visible spectrophotometry is one of the most frequently employed technique in pharmaceutical analysis. It involves measuring the amount of ultraviolet or visible radiation absorbed by a substance in solution. Instrument which measure the ratio, or function of ratio, of the intensity of two beams of light in the U.V-Visible region are called Ultraviolet-Visible spectrophotometers. In qualitative analysis, organic compounds can be identified by use of spectrophotometer, if any recorded data is available, and quantitative spectrophotometric analysis is used to ascertain the quantity of molecular species absorbing the radiation. Spectrophotometric technique is simple, rapid, moderately specifc and applicable to small quantities of compounds. Te fundamental law that governs the quantitative spectrophotometric analysis is the Beer - Lambert law (Davidson, 2002).

UV/Vis spectroscopy is in its simplest form, a sample is placed between a light source and a photo detector, and the intensity of a beam of light is measured before and after passing through the sample. These measurements are compared at each wavelength to quantify the sample's wavelength dependent extinction spectrum. The data is typically plotted as extinction as a function of wavelength. Each spectrum is background corrected using a "blank" - a cuvette filled with only the dispersing medium to guarantee that spectral features from the solvent are not included in the sample extinction spectrum.

The application of standardized UV (or UV Vis) spectroscopy has years been used in analyses of flavonoids. The various flavonoid classes can be recognized by their UV spectra and UV-spectral characteristics of individual flavonoids including the effects of number of aglycone hydroxyl groups, glycosidic substitution pattern, and nature of aromatic acyl groups have been reviewed (Markham, 1982). All the flavonoids contain at least one aromatic ring and consequently absorb UV light (Mabry *et al*., 1970). The typical UV-Vis spectra of flavonoids include two absorbance bands maxima in the range of λ240~280 nm (Band I) and λ300~550 nm (Band II) composed of three rings structure (A, B, and C) with various substitutions. Changes in the substitution of the A-ring tend to reflected in the band II absorption while alterations in the substitution of the B- and C-rings tend to be more apparent from band I absorption (Markham, 1982). Additional oxygenation (especially hydroxylation) generally causes a shift of the appropriate band the longer wavelengths. Based on the UV-visible absorbance spectra, the flavonoid class can be predicted for each chromatographic peak separated.

Fourier Transforms - Infrared Spectroscopy (FT-IR) Analysis

FTIR is most useful for identifying chemicals that are either organic or inorganic. It can be utilized to quantitate some components of an unknown mixture. It can be applied to the analysis of solids, liquids, and gasses. The term Fourier Transform Infrared Spectroscopy (FTIR) refers to a fairly recent development in the manner in which the data is collected and converted from an interference pattern to a spectrum. Today's FTIR instruments are computerized which makes them faster and more sensitive than the older dispersive instruments. FTIR can be used to identify chemicals from spills, paints, polymers, coatings, drugs, and contaminants. FTIR is perhaps the most powerful tool for identifying types of chemical bonds (functional groups). The wavelength of light absorbed is characteristic of the chemical bond as can be seen in this annotated spectrum (Eberhardt *et al*., 2007; Hazra *et al*., 2007).

By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. FTIR

spectra of pure compounds are generally so unique that they are like a molecular "fingerprint". While organic compounds have very rich, detailed spectra, inorganic compounds are usually much simpler. For most common materials, the spectrum of an unknown can be identified by comparison to a library of known compounds. To identify less common materials, IR will need to be combined with nuclear magnetic resonance, mass spectrometry, emission spectroscopy, X-ray diffraction, and/or other techniques.

THIN LAYER CHROMATOGRAPHY

Thin layer Chromatography is based upon the principles of column and partition Chromatography. A thin layer of the stationary phase is formed on a suitable flat surface, such as glass and plastic plate. Separation of a mixture in this case is achieved over a thin layer of alumina or silica gel to which they are absorbed by different physical forces (Harborne, 1984, 1973; Stahl, 1969).

Procedure

A thin-layered plate is prepared by spreading aqueous slurry of Silica gel G on the clean surface of a glass or rigid plastic. Calcium carbonate or starch is also added to the adsorbent to increase adhesion. The plate is then heated in an oven for about 30 mins at 105°C to activate the plate. It is then cooled inside the oven itself. Test samples (1mg/ml of all extracts in respective solvents) were applied in the form of spots using capillary tube. The choice of solvents depends upon the nature of compound to be separated and also on the adsorbent used. The solvent is poured into the chamber and closed tightly and the chamber is saturated for a few hours before running the chromatogram.

The extracts were drawn with capillary tubes and applied as spots on a stationary phase (silica-gel coated plate) about 1 cm from the base. The plate was then dipped into a suitable solvent system (mobile phase). The plate is then placed in a container with enough solvent in a well covered tank. The solvent migrates up the plate. As the solvent rising through thin layer separates different components of the mixture at different rates which appear as spots in the thin layer. After the solvent has reached almost the top edge of the plate, nearly 3/4th of the plate, the plate is removed from the tank and dried briefly at moderate temperatures 60-120°C. The presences of secondary metabolites in the extracts were detected by TLC using suitable spraying reagents. Detection of spots by using spraying reagents

Colored substances can be seen directly when viewed against the stationery phase whilst colorless species were detected by spraying the plate with appropriate reagent, which produced coloured areas in the regions, which they occupy (Harborne, 1973). The following spraying systems were used:

1.The presence of alkaloids in the developed chromatograms was detected by spraying the freshly prepared Dragendorff"s reagent. A positive reaction in the chromatogram (orange brown) was confirmatory evidence that the alkaloid was present in the extract (Harborne, 1984, 1973).

2.The presence of steroids in the developed chromatogram was detected by spraying the (Folin phenol ciocaltu's reagent). After the plates were heated at 100oC for 6 minutes, a positive reaction was formation of blue colour spot (Harborne, 1984,1973).

3.The presence of flavonoid was detected by the formation of colour in the plate a positive reaction was formation of yellow colour spot by exposure of ammonia (Harborne, 1984, 1973).

4.The presence of terpenoids was detected by Vanillin reagent method.10% vanillin was dissolved in Ethanoic acid – concentrated sulphuric acid in ratio of 2:1 mixed and sprayed onto the plates and then they were put in the oven for 15mins. Presence of terpenoids was indicated by the separation into different colours; brown, dark green and purple colour (Harborne, 1984, 1973).

Rf Value: It is a ratio of distance travelled by the sample and distance travelled by the solvent.

$$Rf = \frac{\text{Distance of the sample (solute) from the origin}}{\text{Distance of the solvent from origin}}$$

The separated constituents were recovered by scraping off the adsorbent at the appropriate places on the developed plate, and the powder was reconstituted in methanol, followed by centrifugation (Eppendorf tube) at 7,000 rpm for 15 min. This step was carried out twice to ensure complete removal of the adsorbent. The supernatant was used for the estimation of flavonoids.

CONCLUSION

Nature is a unique source of structures of high phytochemical diversity, many of them possessing interesting biological activities and medicinal properties. The choice of the solvent is very essential to extraction of various phytochemicals from plants. Non standardized procedures of extraction may lead to the degradation of the phytochemicals present in the plants and may lead to the variations thus leading to the lack of reproducibility. Efforts should be made to produce batches with quality as consistent as possible (within the narrowest possible range) and to develop and follow the best extraction processes.

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