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A review of qualitative and quantitative phytochemicalanalysis for plant extracts

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ABSTRACT

Plants and their derivatives constitute a significant component of the human diet. They serve as a primary reservoir of biologically active compounds such as vitamins, dietary fiber, antioxidants, and cholesterol-lowering agents. Despite the abundance of information in this domain, the nutritional profile of plants remains inadequately characterized. Historically, identifying many plant nutrients and health-promoting substances relied on a trial-and-error approach. As the 21st century unfolded, advanced analytical techniques, including chromatography, mass spectrometry, infrared spectrometry, and nuclear magnetic resonance, revolutionized the field by enabling quantitative and qualitative assessments of a myriad of plant metabolites. The application of these methodologies has unveiled approximately 50,000 metabolites in plants, with projections anticipating the eventual discovery of over 200,000. Despite thiswealth of information, the functions of a substantial proportion of these metabolites remain unknown. Crucial for plant growth, development, stress adaptation, and defense, metabolites such as carbohydrates, organic and amino acids, inorganic elements, vitamins, hormones, flavonoids, phenolics, and glucosinolates play pivotal roles. Beyond their significance for plant biology, these metabolites profoundly influence the nutritional quality of food, impacting aspects such as color, taste, and smell, and conferring antioxidative, anticarcinogenic, antihypertensive, anti-inflammatory, antimicrobial, and cholesterol-lowering properties. This review concentrates on elucidating major plant metabolites and detailing the methodologies employed in their analysis.

Keywords: Plant Extracts, Plant Metabolites, Phytochemical Screening, Qualitative and Quantitative.

INTRODUCTION

Term Phytochemicals, derived from the Greek word "phyto" signifying plant, represent a diverse group of naturally occurring chemical compounds found in plants. These biologically active substances extend health benefits to humans beyond those associated with macronutrients and micronutrients (Hasler and Blumberg, 1999). Serving as a natural defense mechanism, phytochemicals safeguard plants from diseases and environmental damage, also contributing to the plant's distinctive colour, aroma, and flavor. Broadly, these plant compounds, crucial for shielding plant cells from various environmental stressors like pollution, UV exposure, and pathogens, are collectively termed phytochemicals (Mathai,

2000).

Recent insights have underscored the pivotal role of phytochemicals in promoting human health when incorporated into the diet significantly. With over 4,000 catalogued phytochemicals, they are categorized based on protective functions, physical characteristics, and chemical attributes (Meagher and Thomson, 1999). This wealth of phytochemical diversity has garnered attention due to its versatile applications, particularly in the realm of medicinal plants. These plants serve as an abundant bio-resource, contributing to traditional medicine, modern pharmaceuticals, nutraceuticals, food supplements, folk remedies, and as sources for pharmaceutical intermediates and synthetic drugs. The growing interest in plant-derived substances reflects their potential across diverse fields, highlighting their importance in both traditional and contemporary medicinal practices.

Medicinal plants, bestowed by nature, contribute to disease-free, healthy living, playing a crucial role in maintaining our well-being. India, renowned for its cultural diversity, boasts a rich tradition of medicinal plants deeply embedded in time-honored practices. Traditional medicine systems like Siddha, Ayurveda, Unani, and Amchi (Tibetan) thrive alongside vibrant ethnomedicine traditions. Drawing on the scientific heritage of ancient civilizations, these traditional medicines have shown efficacy in addressing challenging illnesses (Satyavati, 1982). India's historical roots in herbal medicine trace back to the Rigveda, written between 4500 and 1600 BC. Despite a decline during the British period, the resurgence of interest in natural healing methods is evident today, driven by the recognition of their efficacy in treating ailments without adverse effects.

The World Health Organization (WHO) reports that 80% of people in developing countries primarily rely on traditional medicines, particularly plant-derived drugs, for their essential health needs. Medicinal plants are integral to treating and preventing specific ailments, playing a crucial role in healthcare. They serve as an indispensable source for medicinal preparations, with hundreds of species recognized for their medicinal value. The concept of 'Phytomedicines' is bridging the gap between traditional and modern medicine (WHO, IUCN, and WWF, 1993).

Today, medicinal plant research is a prominent focus, recognizing these plants as nature's gift for fostering a disease-free, healthy life and preserving our well-being. Plants produce a diverse array of chemical compounds, known as 'secondary compounds,' which serve various ecological functions, enhancing plant survival during stress. Additionally, these compounds may contribute to the beneficial effects of fruits and vegetables on various health-related measures (Dahanukar, 2000).

While many synthetic drugs today find their origins in the plant kingdom, the historical decline of medicinal herbalism coincided with the ascendance of pharmacology as a dominant and effective branch of medical therapeutics. In the English-speaking world, herbalism largely disappeared from the medical landscape during the late 19th and early 20th centuries. Nonetheless, in numerous third-world countries, diverse forms of ethnic herbalism persist to the present, exemplified by practices like Ayurvedic medicine in India, Kampo medicine in Japan, and Chinese herbalism in China. In some developed nations such as Germany and France, medical herbalism coexists with modern pharmacology, albeit with diminishing prominence (Gao *et al.*, 1999).

Global trends toward an improved "quality of life" have led to a notable surge in the demand for medicinal plants (Kotnis *et al*., 2004). The use of plants in treating various ailments has ancient roots, with India being richly endowed with a diverse array of plants possessing medicinal value. These plants are extensively utilized across society, either directly as folk remedies or indirectly as pharmaceutical preparations in modern medicine (Bhagwati Uniyal, 2003). Contemporary global attention on plant research has uncovered substantial evidence showcasing the immense potential of medicinal plants utilized in various traditional systems such as Ayurveda, Siddha, and Unani (Dahanukar, 2000). Medicinal plants are assuming heightened importance in primary healthcare for individuals and communities in many developing countries. The international trade in these plants has surged due to

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their perceived effectiveness, affordability, purported lack of side effects, and use as alternatives to allopathic medicines. These plants are considered much safer and proven elixirs in the treatment of various ailments (Ashis, 2003).

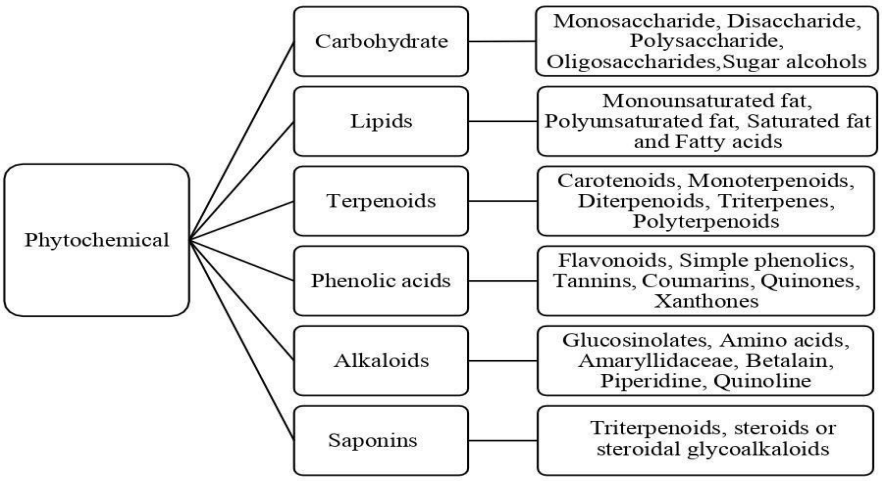

Figure 1: Categorization of Phytochemical

Plants have basic nutritional importance by their content of protein, carbohydrates, 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, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll'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 systems 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 applications as drugs, dyes, flavour, fragrances, insecticides, 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).

PLANT MATERIALS SELECTION

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.

Samples may be selected using several approaches

1. The investigation of plants traditionally used by humans for food, medicine, or poison basedon 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).

Plant Materials: Collection and Identification

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 permanentscientific 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 materialto 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 constituents to be lost, and thermolabile components to degrade and oxidize.

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).

Table 1: Various solvents used for extraction of active components

VARIOUS SOLVENTS						
Ethanol	**Methanol**	**Hydro-alcoholic (70%) (Methanol)**	**Ether**	**Acetone**	**Chloroform**	**Aqueous**
Polyphenols	Polyphenols	Polyphenols	Alkaloids	Phenol	Terpenoids	Terpenoids
Flavonol	Terpenoids	Terpenoids	Terpenoids	Flavonols	Flavonoids	Starches
Tannins	Saponins	Saponins	Coumarins			Saponins

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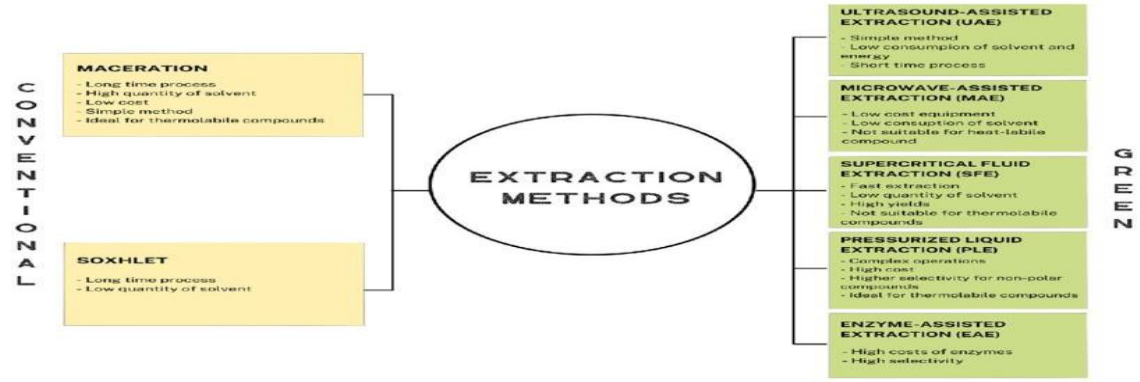
Terpenoids	Tannins	Tannins	Fatty acids			Anthocyanins
Sterols	Xanthoxylines	Amino acid				Polypeptides
Alkaloids	Totarol	Carbohydrate				Lectins
Polyacetylenes	Quassinods	Quassinods				Tannins
	Lactones	Glycoside				
	Flavones	Flavonoids,				
	Phenones	Phenones				
	Anthocyanins	Anthocyanins, Anthroquinone				
		Sterols				
		Alkaloids				

FACTORS AFFECTING SELECTION OF AN EXTRACTION PROCESS

1. Character of Plant Material: - The knowledge of the pharmacognosy of the herb is essential to select the right method of the extraction process. The maceration process is used when the herb is soft, unorganized unpowderable and to avoid powdering ofit. The percolation process is used when the herb is hard and tough.
2. Therapeutic value of the Plant Material: - 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 isunimportant, and maceration process can be used to extract the herb.
3. Stability of Plant Material: - Continuous hot extraction process should be avoided whenthe 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 concentratedpreparations 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.



Source: https://www.researchgate.net/figure/Advantages-and-limitations-of-conventional-and-green-extraction-methods_fig1_367318046

Fig 2. Extraction Process

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, hydro distillation 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).

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.

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).

PRIMARY METABOLITES: QUALITATIVE ANALYSIS

Test for carbohydrates

1. Benedict‟s test: To 0.5 ml of the filtrate, 0.5 ml of Benedict‟s reagent was added. The mixturewas 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 H2SO4 was poured carefully along the side ofthe test tube.

Appearance of a violet ring at the interphase indicated the presence of carbohydrate.

Test for Starch

1. 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.

Test for Fatty Acids

1. 0.5 ml of extract was mixed with 5 ml of ether. These extracts were allowed it for evaporation on filter paper and dried the filter paper. The appearance of transparence on filter paper indicatesthe presence of fatty acids

OTHER TEST

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.

SECONDARY METABOLITES: QUALITATIVE ANALYSIS

Test for anthraquinones

1. Five ml of the extract solution was hydrolyzed 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 addedby 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‟sreagent was added. An orange or red precipitate produced immediately indicates the presenceof 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 Dragendorff‟s reagent was added. Formation of a precipitate indicated the presence ofalkaloids. To confirm the results, B was treated with saturated sodium carbonate solution untila 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 collectedand 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 extract and the resulting solution (3.0 ml) was transferredin 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

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

Test for Phlobatannins

1. 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

1. 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 astable 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 ofthe aqueous filtrate of each plant extract followed by addition of concentrated H2SO4. A yellowcolouration 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:

1. 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

1. 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

1. 2 ml of Ammonium hydroxide was added to 0.5 g extract sample. Appearance of reddish colourshowed the presence of chalcones

Test for phytosterol:

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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 coloration 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 color indicates the presence of triterpenoids.

Test for Cardiac glycosides (Keller-Killani test): Five ml of each extracts was treated with 2 ml of glacialacetic 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 redin 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 color 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 effervescenceproduced indicated the presence of acidic compounds.
2. 2ml of alcoholic extract was taken in warm water and filtered. The filtrate was then tested withlitmus paper and methyl orange. The appearance of blue color.

VITAMINS: QUALITATIVE ANALYSIS

Test for Vitamin- A

1. 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

1. Dilute 1 ml of aqueous sample solution with 5 ml of water and add 1drop of freshly prepared5%

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

1. 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

1. 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.
2. The bright-red colour was formed on a white background. The background gradually assumesa pink (Pearson, 1976; Patel, 2005).

INORGANIC ELEMENTS: QUALITATIVE ANALYSIS

Ash of drug material (500mg) was prepared and treated with HNO3 and HCl (3:1 v/v) for 1 hour. After the filtration, the filtrate was used to perform the following tests (Khandelwal 2006):

1. Calcium: One drop of dil. ammonium hydroxide and saturated ammonium oxalate solution wasadded to 10ml of the above filtrate. White precipitates of calcium oxalate, soluble in hydrochloric acid but insoluble in acetic acid, were formed.
2. 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.
3. Sodium: Little uranyl magnesium acetate reagent was added to 2ml of the test solution, shakenwell and kept for few minutes. Yellow crystalline precipitate of sodium magnesium uranyl acetate was observed.
4. Potassium: Few drops sodium cobalt nitrite solution was added to 2-3ml of the test solution. Yellow precipitate of potassium cobalt nitrite was observed.
5. Iron: Few drops of 2% potassium ferrocyanide were added to 5ml of the test solution. Dark blue coloration was observed.
6. Sulphate: To 5ml of the test solution, lead acetate reagent was added. A white precipitate, soluble in sodium hydroxide, was formed.
7. 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.
8. Chloride: 3 to 5ml of lead acetate solution was added to about 5 to 7ml of the filtrate. A whiteprecipitate soluble in hot water was observed.
9. Nitrates: Ferrous sulphate solution was added to 5ml of the test solution. No brown colour wasproduced, 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 (Kumaret al., 2008) Aqueous and ethanolic extracts that has been adjusted to come under the linearityrange i.e. (400µg/ml) or Aliquots of extract solutions were taken and made up the volume 3mlwith 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% NaNO2 was added. After 5 minutes, 0.3ml of 10% AlCl3 was added. After 6 min, 2ml of 1 M NaOH was added and thetotal volume was made up to 10ml with distill

water. Then the solution was mixed well and theabsorbance was measured against a freshly prepared reagent blank at 520 nm. The total
flavonoid content of the extracts was expressed as a percentage of Quercetin equivalent per 100g 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 evaporated into dryness over a water bath and weighed to a constant weight.

Determination of total phenols by the 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 amyl alcohol 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 was determined 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 dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitate 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 cm^3 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 sulfate 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 sample 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 a 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.

TLC (THIN LAYER CHROMATOGRAPHY)

Thin-layer Chromatography is based on the principles of column and partition Chromatography. A thin layer of the stationary phase is formed on a suitable flat surface, such as glass or 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.

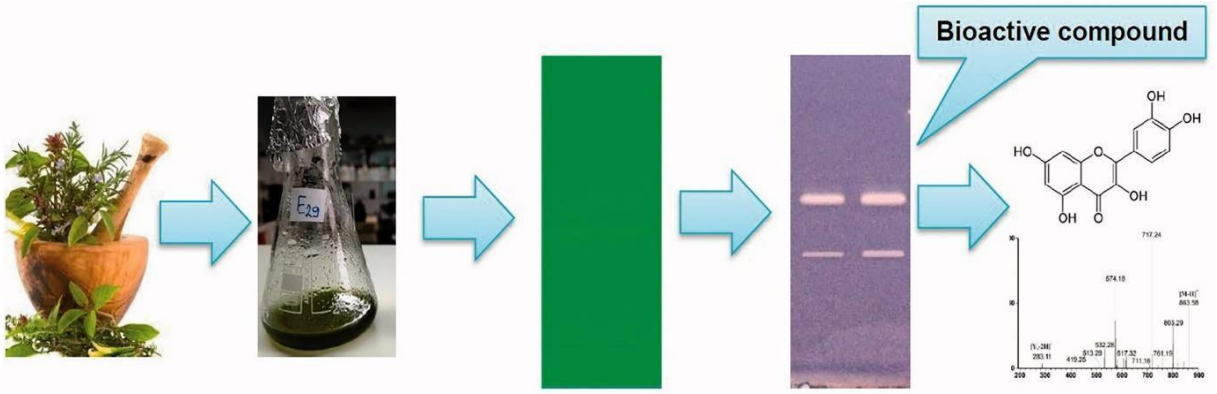

Figure 3: TLC for Bioactive Compound

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 stationary phase whilst colorless species were detected by spraying the plate with the appropriate reagent, which produced colored areasin the regions, they occupied (Harborne, 1973). The following spraying systems were used:

1. The presence of alkaloids in the developed chromatograms was detected by spraying the freshlyprepared 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 platesand 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 = Distance of the sample (solute) from the origin/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

The ecosystem stands as an unparalleled reservoir of structurally diverse phytochemicals, with many exhibiting compelling biological activities and medicinal potential. The selection of an appropriate solvent is a critical factor in extracting various phytochemicals from plants. Non-standardized extraction procedures pose a risk of degrading the phytochemical composition within plants, resulting in variations and a lack of reproducibility. It is imperative to undertake efforts to ensure the production of batches with consistent quality, aiming for the narrowest possible range, and to establish and adhere to optimal extraction processes.

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