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Plant secondary metabolites: Extraction, screening, analysis and their bioactivity

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Abstract
The plant secondary metabolites (PSMs) are novel sources for the development of pharmaceuticals, food additives, and other industrial products. Plants are a ubiquitous source of potential novel medicinal compounds. In recent years, the commercial value of these secondary metabolites has sparked a lot of interest in their production and finding ways to improve it using tissue culture technologies. Secondary metabolites of the plant are currently the focus of lot of research, but extracting them for phytochemical or biological studies poses unique obstacles that must be handled throughout the extraction process. A thorough evaluation of the applicable literature for which procedures are suited for a given class of chemicals or plant species, is essential for successful extraction. Secondary metabolites are bioactive compounds recognized for healing a variety of ailments and possessing antioxidant properties, which leads to the isolation of new and unique compounds. This review gives an in-detail overview of extraction, screening, and analysis of the plant secondary metabolites concerning their bioactivity.

Keywords: Plant bioactive compounds, biosynthesis pathways, bioactive properties, extraction methods, antioxidant assay, analytical techniques

Introduction
Secondary metabolites (SMs) are natural products primarily produced by bacteria, fungi, and plants. Plant secondary metabolites are low molecular weight compounds with various chemical structures and biological activities. Secondary metabolites were named after the initial observation that their production is not required for organism growth and reproduction, as compared to primary metabolites [1]. Plants can produce a wide range of organic compounds, which can be divided into two categories: primary metabolites and secondary metabolites. Every living system requires primary metabolite for its growth and survival, and it is biosynthesized by a specific biochemical pathway. These metabolites are part of basic metabolic processes like respiration and photosynthesis. The secondary metabolites are a metabolic intermediate or product that are required not only for plant growth but also play a vital role in plant survival. It is required for plant environment interaction and in response to deal with biotic and abiotic stress. Secondary metabolites play an important role to protecting plants from predators (herbivores) and microbes. Some secondary metabolites assist plants in communicating with other organisms, while others protect plants from abiotic stress, such as UV-B radiation [2]. The metabolites synthesized by plant secondary metabolism are essential because they have various human health benefits. Secondary metabolites that have received the most attention are phenolic compounds, flavonoids, and anthocyanins [3]. The secondary metabolism precisely regulates plant growth and development by acting as an essential source of phytochemicals that protect plants from environmental constraints [4].
Plant SMs research intersects with various biological and medical disciplines because secondary metabolites have numerous properties that can be used in pharmaceuticals, crop protection, dyes, flavours, and fragrances [5]. Secondary metabolites have been shown to have various biological effects, providing a scientific foundation for the use of herbs in traditional medicine in many ancient communities. They are antibiotic, antifungal, and antiviral and thus capable of protecting plants from pathogens [6]. Polyphenols are secondary metabolites produced by higher plants that play various important roles in plant physiology and may have health benefits in humans, primarily as antioxidants, anti-allergic, anti-inflammatory, anticancer, antihypertensive, and antimicrobial agents [7]. These compounds are synthesized by the plant shikimate pathway. Flavonoids are a group of phenolic compounds that contribute to the antioxidant capacity produced naturally by plants.

Plant secondary metabolite is classified into four major classes: terpenes, phenolics, glycosides, and nitrogen/sulfur-containing compounds alkaloids (Figure 1 gives a schematic representation of the biosynthesis pathway of all four major types of secondary metabolites). The shikimic acid pathway synthesizes the products that form phenolics which imparts the defensive ability to plants. Most nitrogen and sulfur-containing compounds are biosynthesized from amino acids [2]. The mevalonic acid pathway and DOX/MEP pathway are primary pathway involved in biosynthesis of terpenoids.

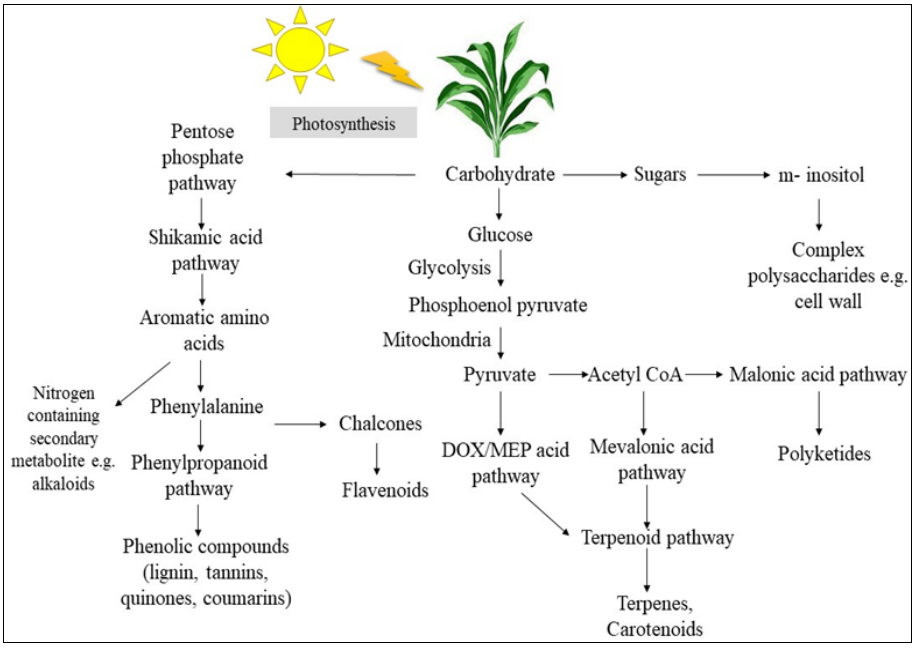

Fig 1: Schematic representation of secondary metabolite biosynthesis in plants

2. Classification and biosynthesis of plant secondary metabolites

2. 1 Alkaloids

Alkaloids are complex organic molecules with a heterocyclic nitrogen ring that have been widely used for their various pharmacological properties [8]. They have a heterogeneous chemical structure. Plant tissues contain alkaloids as water-soluble salts of organic acids [9]. Alkaloids are a diverse chemical class of organic products with a wide array of biological activities and many of them having critical pharmaceutical applications. Plants are thought to be the oldest source of alkaloids, and some of the most well-known alkaloids, including morphine, quinine, strychnine, and cocaine, are derived from plants [10]. The L- amino acids such as ornithine, lysine, phenylalanine, tyrosine, tryptophan, histidine, aspartic acid, and anthranilic acid are precursors of most alkaloids. Tryptophan or aspartic acid can be used to make nicotinic acid. Alkaloid biosynthesis can take a variety of forms that are difficult to classify.

2.1. Glycosides

The plant glycosides are secondary metabolites made up by linkage between a sugar portion and a non-sugar moiety. A glycoside's non-sugar moiety is derived from aglycones. The link between the sugar and the aglycones is a hemiacetal between the sugar's aldehyde or keto group and an alcoholic or phenolic hydroxyl group of the aglycone [11]. Glycosides classified as 3 different types saponins, cardiac glycoside and cyanogenic glycosides.

2.2. Saponins

Saponins are bioorganic compounds which occur naturally, and they have glycosidic linkage (C-O-sugar bond) at carbon-3 that links an aglycone and a sugar chain [12]. The name saponins are derived because of their ability to form firm, soap-like foams in aqueous solutions. Saponins are glycosides with a carbohydrate moiety that is glycoside linked to a triterpenoid or steroids [13]. The biosynthesis of saponins involves two significant pathways the mevalonate pathway (MVA) and the methylerythritol 4-phosphate (MEP) pathways. The precursor for MVA and MEP pathways are Acetyl CoA and glyceraldehyde 3-phosphate, respectively [14]. Dimethylallyl diphosphate (DMAPP) is the product of these pathways, which is further used for the synthesis of squalene through the subsequent action of three enzymes, namely geranyl diphosphate synthase (GPS), farnesyl diphosphate synthase (FPS), and squalene synthase (SQS). Squalene is further converted into 2, 3-oxidosqualene via the action of squalene epoxidase catalysis (SE). Different oxidosqualene cyclases then cyclize 2, 3-oxidosqualene, yielding products that undergo a series of cyclization, hydroxylation, and glycosylation reactions, eventually yielding different types of steroidal saponin glycosides [15].

2.3. Cardiac glycosides

Cardiac glycosides are an aglycone component of sterol glycosides that contains the steroid nucleus linked with an unsaturated lactone ring at carbon 17. Digoxin (derived from the foxglove plant is a type of cardiac glycoside. It inhibits the Na+-K+ pump and raises intracellular Na+ levels [16]. The biosynthetic pathway of plant cardiac glycosides is divided into three stages: terpenoid backbone biosynthesis, steroid biosynthesis, and cardenolide biosynthesis [17].

2.5. Cyanogenic glycosides

Cyanogenic glycosides are secondary metabolites found in over 2600 plant species, including up to 26 economically important crops. They are secondary plant compounds that contain nitrile and produce cyanide (cyanogenesis) [18]. They are a natural source of hydrogen cyanide (HCN), which primarily provides an immediate chemical defence response to herbivores and pathogens that cause plant tissue damage

[19]. Cyanogenic glycosides are plant secondary metabolites derived from amino acids. Despite their structural diversity, the precursor for cyanogenic glycosides is six different amino acids: valine, isoleucine, leucine, phenylalanine, tyrosine, and cyclopentenyl glycine (a nonprotein amino acid). Cyanogenic glycosides play critical roles in plant protection and plant-insect interactions [18].

2.6. Terpenoids

Terpenoids are the most abundant type of plant secondary metabolite. The chemical structures of these naturally occurring chemical compounds vary greatly. Although there have been many excellent studies of terpenoids, the majority of them have concentrated on compounds composed entirely of isoprene units) [20]. Terpenoids, also known as isoprenoids, are the most widespread and structurally diverse compounds found in a wide range of plants [21]. Terpenes are synthesized by MVP (Mevalonic acid pathway) and MEP (Methylerythritol phosphate) Pathway. The Mevalonic acid pathway (MVP), also recognized as the isoprenoid pathway or 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), is an anabolic pathway that generates metabolites for numerous cellular processes [22]. The first two steps of the MVP pathway, three molecules of acetyl–CoA are condensed to synthesize MVP. The MVP pathway was the first recognized pathway for isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) biosynthesis [23]. That consists of six enzymatic steps that convert acetyl–CoA to IPP. Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are formed by another condensation reaction by geranylgeranyl pyrophosphate synthase after the sequential condensation reaction of DMAPP with two units of IPP [24]. The MEP Pathway, also known as the mevalonic acid-independent pathway, occurs in plant plastids. The first step of this pathway involves the formation of DXP from pyruvate and glyceraldehyde-3-phosphate, which is why it is known as the DXP pathway or the pyruvate/glyceraldehyde-3-phosphate pathway. As a result, MEP is regarded as the pathway's first intermediate, and the name MEP pathway is widely accepted.

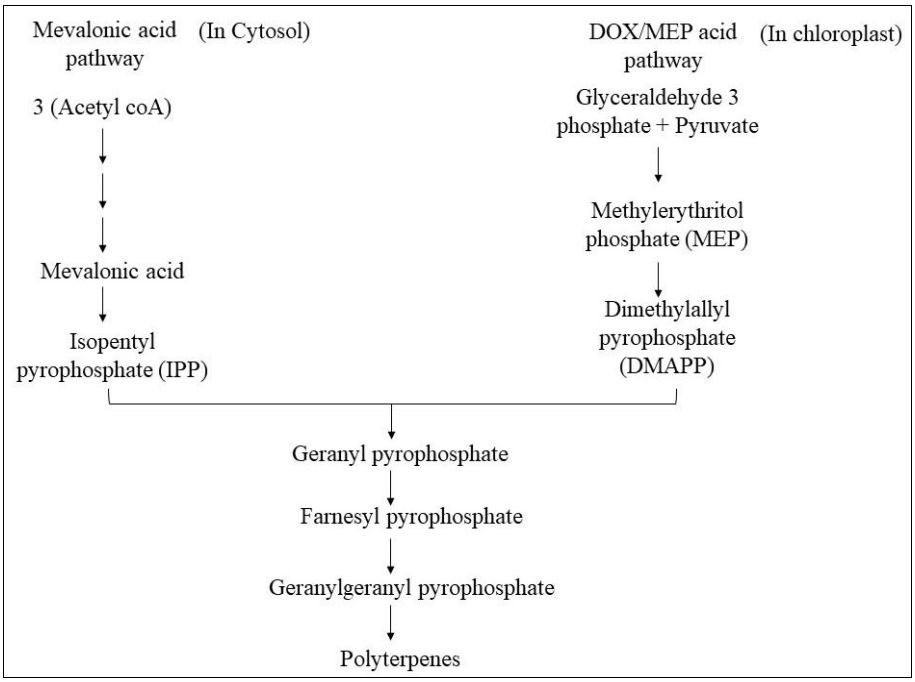

Fig 2: Schematic representation of the mevalonic acid pathway (MVA) and methylerythritol pathway (MEP)

2.7. Tannins

Tannins are the second most abundant plant polyphenol after lignin; they play an essential role in protecting plants from the attack of pests and abiotic stresses like drought, heat, and high UV (ultra violet) radiation. They are divided into two types: hydrolyzable tannins (produced solely by angiosperms) and condensed tannins or proanthocyanidins (produced by both gymnosperms and angiosperms) [25]. The condensed tannins are polyphenol compounds derived from the polyhydroxy-flavan-3-ol units commonly linked via C- C bonds between carbon-4 and carbon 8 and occasionally between C-4 and C-6. Sugar (mostly d-glucose or similar polyols) and phenolic acids are the two primary units of hydrolyzable tannins (HT). Tannins are quantitatively significant components of a variety of plant parts [26]. Tannins are a heterogeneous group of macromolecules which classified as gallotannin or ellagitannin. Tannins are a class of secondary metabolites that belong to phenolics. All phenolic compounds, whether primary or secondary phenolics are formed via the shikimic acid pathway (phenylpropanoid pathway). Isoflavones, coumarins and lignin are also produced via the same pathway.

2.8. Phenolics (polyphenols)

The phenolic is a significant group of plant secondary metabolites with a wide range of structures, from relatively simple compounds, such as phenolic acids, to polyphenols, which include flavonoids, which consist of numerous groups, to polymeric compounds based on these various classes [27]. Polyphenols are recognized as the most generous and distant group of compounds in the plant kingdom, with over 8000 phenolic compounds identified. The phenolic compounds are classified based on their chemical structure into simple compounds like phenolic acids to highly polymerized compounds like tannins [28]. They are composed of benzene rings along with one or more hydroxyl substituents [29]. The shikimic acid pathway is linked to carbohydrate and aromatic amino acid metabolism. The shikimate pathway comprises seven steps that begin with the condensation of phosphoenolpyruvate and erythrose-4-phosphate [30]. The phenolic acids synthesize by plants through the

shikimate/phenylpropanoid pathway form l-phenylalanine, and l-tyrosine are two aromatic amino acids. The Biosynthesis of phenolic acids involves three processes: deamination, hydroxylation, and methylation. In brief, deamination of the amino acids phenylalanine and tyrosine are produced cinnamic acid (not a phenolic acid) and p-coumaric acid, respectively. Benzoic acid (C6–C1) is produced by removing the ethyl side chain from cinnamic acid (C6–C3). Phenolic acids are structurally hydroxylated derivatives of cinnamic acid or benzoic acid [31].

2.9. Flavonoids

Flavonoids are secondary metabolites related to polyphenols and can be found in many fruits and vegetables in the form of aglycones or glycosides [32]. Flavonoids have a 15-carbon skeleton with two benzene rings linked by a three-carbon linking chain. As a result, they are represented as C6-C3-C6 compounds. According to their chemical structure, the unsaturation of the linking chain (C3), and the degree of oxidation, flavenoids are classified into the following groups, namely flavonols, flavones, flavanones, anthocyanidins, and isoflavones (Fig. 3) [33, 34]. A structural dissimilarity between each group is partly due to the degree and pattern of hydroxylation, methoxylation, prenylation, or glycosylation. The phenylpropanoid metabolic pathway is the main pathway associated with the biosynthesis of flavonoids. Through the phenylpropanoid pathway, Amino acid phenylalanine is converted into a 4-coumaroyl-CoA which, further combined with malonyl-CoA, produces the true backbone of flavonoids which is a class of compound known as chalcones that contain two phenyl rings. Conjugating a close-ring class of compound chalcones results in a flavone's well-known three-ring structure. This pathway undergoes a series of enzymatic modifications to produce flavanones, dihydroflavonols and anthocyanins. The product of this pathway includes flavonols, flavan-3-ols, proanthocyanidins (tannins), and a variety of other polyphenolics [35].

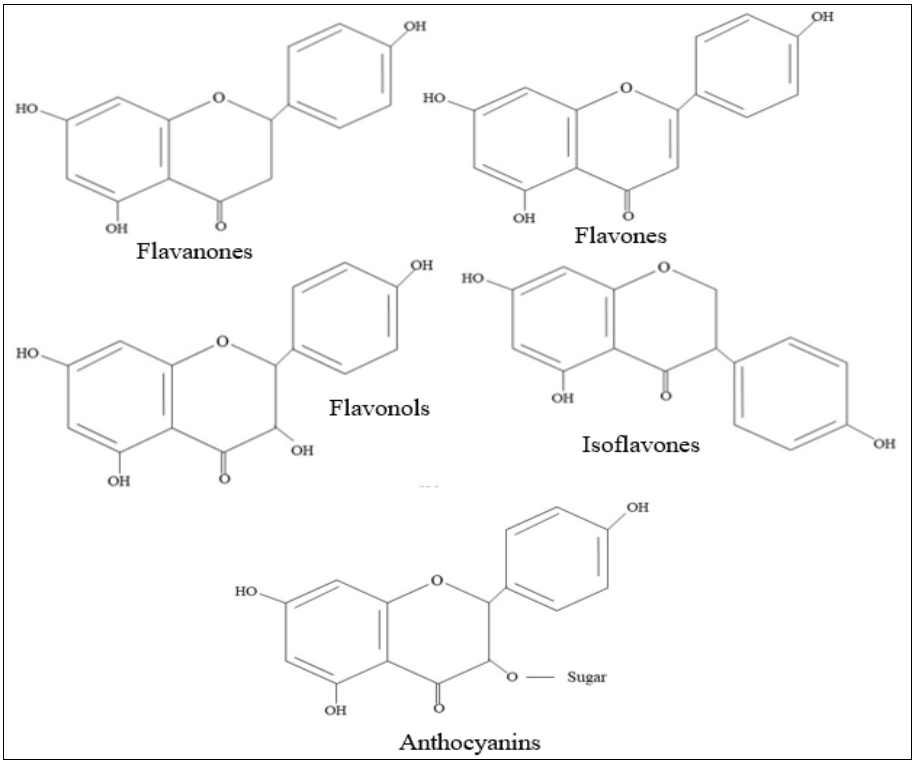

Fig 3: Structure of different classes of flavonoids

2.10. Anthocyanin

Anthocyanins are glycosides that contain anthocyanidin as an aglycone moiety as well as one or more glycosidically bonded mono- or oligosaccharide units [36]. Anthocyanins are belonged to flavenoids they are polyphenolic compounds they are part of plant secondary metabolism. The red, violet and blue pigmentation in plant are due to the presence of anthocyanins. In and aqueous solution these pigments are exist in various forms and pH dependent in equilibria [37]. Anthocyanins are naturally occurring phenolic pigments that have biological activity. They are known for their potent antioxidant and anti-inflammatory activity, there are variety of biological property are reported: anti-diabetic and anti-cancer activity, as well as their role in cardiovascular and neuroprotective prevention [38]. The majority of anthocyanins found in nature are derived from six aglycones: pelargonidin, cyanidin, delphinidin, pelargonidin, petunidin, peonidin, and malvidin [39].

2.11. Coumarins

Coumarins are a type of phenolic substance that is made up of fused benzene and -pyrone rings. There are over 1300 different coumarins known. Coumarins are antithrombotic, anti-inflammatory, and vasodilatory [40]. Coumarins (1,2-benzopyrones) are found in all higher plants and are derived from the phenylpropanoid pathway [41]. Coumarins are exist either free or as glycosides they are cis-O-hydroxycinnamic acid lactones [42]. The coumarin structure is synthesised from cinnamic acid structure through ortho-hydroxylation, lactonization and trans-cis isomerization of the side-chain double bond. The structure exists in two forms cis form and transforms compared to cis transform is more stable and therefore cannot cyclize. Because the cis form is very unstable, it will tend to shift to the trans-configuration [43].

3. Extraction of plant secondary metabolite

Extraction is the separation of plant bioactive fractions by using selective solvents system and extraction procedures. Plant products are relatively mixtures of metabolites in liquid, semisolid, or dry powder form. The solvents that are used for extraction are diffuse into solid plant matter and solubilize compounds of similar polarity during extraction process, the selection of solvent system is primarily determined by the precise nature of the bioactive compounds being targeted. Depending on their relative polarity solvents such as methanol, ethanol, or ethyl acetate are used in the extraction of hydrophilic compounds. Dichloromethane is used to extract more lipophilic compounds. Hexane is used in extraction to eliminate the chlorophyll and oil [44].

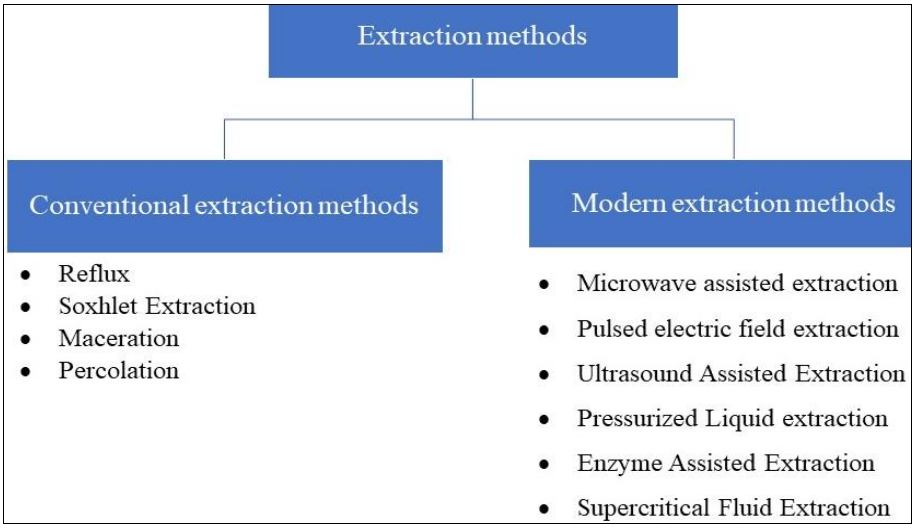

Fig 4: Extraction techniques involved in extraction of bioactive compound

3.1 Conventional extraction methods

3.1.1. Reflux

The principle of the method is heating the solvent for a while and then allow to condense. In reflux extraction, a solution can heat in a controlled manner at a constant temperature. This method helps maintain the stability of the compound. The disadvantage of this method includes the need for more amount of solvent and a long time. The use of organic solvents causes environmental damage and increases the cost of operation. Because of the high equipment availability, these methods are still in use [45].

3.1.2. Soxhlet Extraction or continuous hot extraction

Soxhlet extraction is also known as continuous hot extraction. The apparatus used for this extraction is a soxhlet extractor [46]. This extractor setup consists of a round bottom flask, siphon tube, distillation path, expansion adapter, condenser, cooling water inlet, cooling water outlet, heat source, and thimble. Drying plant materials is essential to avoid enzymatic reactions. Grinding and size reduction of sample material are necessary to improve contact between sample and solvent [47]. The sample is filled in a porous thimble located in the chamber. The bottom flask contains extraction solvents heated, vaporized into a sample thimble, condensed in the condenser, and dripped back. The advantage of this method is that compared to maceration, these methods require a shorter processing time. Disadvantages include using toxic and flammable liquid organic solvent, which is not eco-friendly; extracting compound should be thermostable [48].

3.1.3. Maceration

The meaning of maceration is 'softening'. This method is suitable for extracting bioactive compounds from drugs [49]. It is a solid-liquid extraction. The plant material is soaking in a suitable solvent for a long time with shaking. No need for complicated utensils, and it is a cheap method and require a long time for extraction and is not an eco-friendly method [50].

3.1.4. Percolation

It is the most frequently used procedure for the extraction of active compounds. The apparatus is a percolator, and it consists of a narrow, cone-shaped vessel open at both ends [51]. The dried and finely powdered material is mixed with solvent in a clean container and allowed to macerate for 24 hrs [46].

3.2 Modern extraction methods:

3.2.1. Microwave-assisted extraction (MAE)

This is a novel method for the extraction of bioactive compounds by using microwave energy [52]. In this process, microwave energy is used to heat solvents and samples, promoting the partition of a compound of interest. Microwaves are electromagnetic waves and non-ionizing radiations. Their frequency range is from 300 MHz to 300 GHz. They consist of two perpendicular oscillating fields, such as electrical and magnetic fields. Ionic conduction and dipole rotation of the microwave converts microwave energy into thermal energy. Solvent with a high dielectric constant is desirable because it will absorb more microwave energy [53]. This extraction process can be performed in the "closed-vessel system" or "open-vessel system". For shorter extraction time and higher yield, the MAE system increases the temperature rapidly. Analysing several samples simultaneously is possible in MAE [54]. In an open vessel system, the extraction occurs at atmospheric pressure, and the maximum temperature depends on the boiling point of the solvent. An open system has a cooling system on top of the vessel for the condensation of vapours, this will prevent the loss of vapour [55]. Factors influence MAE includes temperature, pressure, extraction time, power, and nature of the solvent. Because of the use of less solvent, it is an environment-friendly method. In MAE, extraction is possible without solvent or water [56].

3.2.2. Pulsed electric field (PEF) extraction

It is a non-thermal extraction method [57]. The principle of this

method is the disruption of the plant cell membrane by using a high electric field. This electric charge breaks interactions of membrane molecules leading to creating of pores and increasing permeability. This is called electroporation or electro- permeabilization [58]. This is a desirable method for extracting thermolabile compounds [59]. Factors influencing PEF extraction include electric field strength, pulse shape, pulse duration, frequency, and extraction [60]. This method will cause reversible or irreversible changes in the cell membrane [61]. Among this irreversible change is more efficient than reversible change [62].

3.2.3. Ultrasound-Assisted Extraction (UAE)

Ultrasound extraction is one of the modern methods and the extraction of bioactive compounds. The frequency of ultrasound waves is higher than 20 kHz. This high energy creates cavitation bubbles which leads to rupture of cell membrane and release of bioactive compounds. The solvent will interact with the bioactive compounds and leads to extraction [63]. The efficiency of this method is high due to some mechanisms like material fragmentation, erosion, sonicapillary effects, sonoporation, etc. [64].

Table 1: Difference between ultrasonic assisted extraction (UAE) system: a. Bath system, b. Probe system [65]

Bath system	Probe system
Ultrasonic wave supplying by a bath.	Wave supplying by the probe.
Bath is a stainless-steel tank.	It consists of a probe and sample vessel (Stainless steel or glass).
The operating frequency is 40KHz.	The operating frequency is 20KHz.
A large and more amount of sample treatment is possible.	Small and only less amount of sample treatment is possible.
Compared to the probe system power is lower.	Higher power delivery.

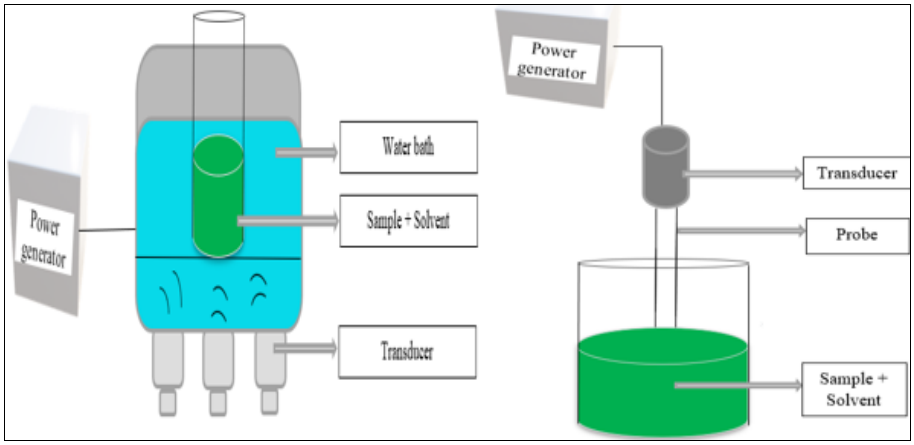

Fig 5: Ultrasonic assisted extraction (UAE) system

3.2.4. Pressurized Liquid extraction (PLE)

High pressure is applied to prevent the vaporization of solvents at a higher temperature. Because of this high pressure and temperature, the solvent will effectively penetrate through the sample. This will lead to the extraction of the compound of interest. The benefits of PLE are less solvent consumption, more efficiency than classic methods, and shorter extraction time. Automation is possible in this extraction [66]. This method is unsuitable for thermolabile compounds and the selectivity of extraction is less [67].

3.2.5. Enzyme Assisted Extraction (EAE)

It is one of the eco-friendly methods which extract bioactive compounds by the action of enzymes on cell walls under optimum conditions. In most cases, water is used as a solvent. It requires low temperature and energy. This method is suitable for extracting phytochemicals inside the cell, like polyphenols [68]. Examples of enzymes are cellulase, pectinase, tannase, and polyphenols [69]. This method is useful for the extraction of insoluble bound phenolic compounds [70].

3.2.6. Supercritical Fluid Extraction (SFE)

Extraction by using supercritical fluids as solvent (e.g., supercritical CO_2) [71]. Supercritical fluids have temperature and pressure above the critical point. They are in mesophase because of their fluid-like and gas-like properties [72]. Supercritical CO_2 (SC CO_2) is less expensive, nontoxic, preserves extracts from oxidation, and its critical temperature is 31.3ºC. Because of these reasons, supercritical CO_2 is used commonly. Due to its non-polar nature, co-solvents are also required to extract polar compounds. For the extraction of anthocyanins, ethanol and methanol are co-solvents [71]. SC CO_2 is used for the extraction of thermolabile compounds due to its user-friendly critical temperature [73]. The other properties of supercritical fluid for fast extraction are their high diffusivity and low density, viscosity, and surface tension compared to other solvents [74].

Table 2: Qualitative tests involved in primary detection of plant secondary metabolites

Sr. No	Test	Procedure	Inference (Indicating Positive Test)	Reference
1	Test for alkaloid:	Extracts are treated with dilute Hydrochloric (HCL) acid and filtered.		
	Mayer's test	Filtrates + few drops of Mayer's reagent (Potassium Mercuric Iodide).	Presence of brownish / reddish ppt indicate presence of alkaloid.	[51]
	Dragendroff's test	Filtrates + Dragendroff's reagent (solution of Potassium	Presence of red colour ppt indicate presence	[51]

		Bismuth Iodide)	of alkaloid	
	Wagner's test	Filtrates + few drops of Wagner's reagent	Presence of brownish / reddish ppt indicate positive results for alkaloid.	[75]
	Hager's test	Filtrates + Hager's reagent (saturated picric acid solution).	Formation of yellow colour ppt	[51]
2	Test for glycosides	Extracts are hydrolysed with dil. HCl, and then used for detection of glycosides		[51]
	Modified Borntrager's Test	Extract + ferric chloride solution boil for 5min, cooled and add equal volume of benzene + benzene layer is separated which is further treated with ammonia solution.	Presence of rose-pink colour in ammoniacal layer indicate positive test.	[76]
	Legal's Test	Extracts + sodium nitroprusside in pyridine + 10% sodium hydroxide.	Initial pink colour converted in to blood red colour indicates the presence of cardiac glycosides.	[77]
3.	**Test for saponins**			
	Foam test	a) Extracts + distilled water up to 20ml and shake thoroughly in a graduated cylinder for 15 minutes. b) 1ml extract was treated with 1% lead acetate solution	Presence of firm layer of foam indicates the saponins. b) Presence of white ppt indicate positive test.	[78]
	Froth test	0.5 gm of extract + 2 ml of distilled water.	foam produced and persists for at least ten minutes it indicates the positive result for saponins	[51]
4	**Test for phytosterols**			
	Salkowski's Test	Extracts + chloroform and filtered. Filtrates + few drops of conc. sulphuric acid, shaken and allowed to stand.	Presence of golden yellow colour indicates the positive result for triterpenes.	[51]
	Liebermann Burchard test	Extracts + chloroform and filtered it. The filtrates + few drops of acetic anhydride solution, boiled and cooled. Conc. sulphuric acid was added in solution.	Presence of brown ring at the junction indicates the positive test of phytosterols	[51] [79]
5	**Test for phenols**			
	Ferric chloride test	Extracts + 2ml of distilled water was added followed by a two to three drops of 10% ferric chloride solution.	Formation of bluish/ black colour indicate positive test.	[75]
6.	Test for coumarin	Extract + 3 mL of 10% NaOH.	Presence of yellow colour indicate positive test	[80]
7.	**Test for tannins:**			
	Gelatin Test	Extract + 1% gelatin solution containing sodium chloride.	Presence of white precipitate indicates the positive test for tannins	[51]
8.	**Test for flavenoids**			
	Alkaline reagent test	Extracts + 1 ml of 2N NAOH solution	Initial intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.	[51]
	Lead acetate test	Extracts are treated with few drops of lead acetate solution.	Presence of yellow colour precipitate indicates the positive test for flavenoids.	[51]
9.	Test for terpenoids	Extract (5 ml) + 2.0 ml of chloroform evaporated to dryness on the heating plate and then boiled with 3 ml of H_2SO_4 concentrated.	Presence of grey colour indicate positive test.	[81]
10.	**Test for carbohydrate**			
	Molisch's Test:	Extract (2 ml) + few drops of Molisch's solution + few drop of concentrated sulphuric acid were added through the side of the test tube to form a layer.	Formation of the purple – colour ring at the junction indicates the presence of Carbohydrates.	[82]
	Benedict's test	Filtrates + Benedict's reagent heated gently on boiling water bath	Presence of orange red precipitate indicates the positive test for reducing sugars.	[51]
	Fehling's Test	Filtrates treated with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions.	Presence of red precipitate indicates the positive test for reducing sugars	[51]
11.	**Test for proteins**			
	Xanthoproteic Test	Extract + few drops of conc Nitric acid. forms a yellow-coloured product known as Xanthoprotein. This is due to reaction with aromatic amino acids.	Presence of yellow colour indicates the presence of proteins	[83]
	Ninhydrin Test	Extract + 2 ml 0.25% w/v Ninhydrin reagent was added and boiled for few minutes.	Presence of blue colour indicates the positive test for amino acid	[51]

4. Quantitative methods involved in detection of plant secondary metabolite

4.1. Estimation of total phenolic content (TPC) by Folin–Ciocalteu Assay

The estimation of total phenolic content is essential in the detection of secondary metabolite functions [84]. The spectrophotometric technique is the most commonly used method for measuring total polyphenols. The Folin–Ciocalteu assay is one of the most widely used assays for detecting and quantifying phenolics. The Folin–Ciocalteu assay works on the principle of reducing the Folin–Ciocalteu reagent in the presence of phenolics, which results in the formation of molybdenum–tungsten blue colour that is measured through a spectrophotometer at wavelength 760 nm [85]. The Folin–Ciocalteu reagent is a mixture of phosphotungstic acid and phosphomolybdic acid, which are reduced to blue oxides of tungsten and molybdenum after phenol oxidation, the blue colour is then measured with a spectrophotometer. The quantity is usually expressed in gallic acid or catechin equivalents. This reaction occurs under alkaline conditions, which are maintained by the addition of sodium carbonate. Under these conditions, the electron is easily transferred from the phenol molecule, which is a mixture of phosphotungstic acid and phosphomolybdic acid, which, after phenol

oxidation, is reduced to blue tungsten and molybdene oxides [86]. The main disadvantage of this method is its low specificity because the folic ciocalteu reagent is quantified total phenols as well as any reducing agent. The colour reaction can occur with any oxidizable phenolic hydroxy group, such as aromatic amines, ascorbic acid, Cu (I), and Fe (II) [87]. This Folin–Ciocalteu assay was initially designed to measure tryptophan, it is an aromatic amino acid which do not have any phenolic group (Folin and Ciocalteu, 1927), and it employs a reagent containing complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids [88].

4.2. Estimation of total flavonoids content (TFC) by aluminium chloride Assay

The flavonoids are class of secondary metabolites that are similar to polyphenols but have a different structure and can be presence in many fruits and vegetables as aglycones or glycosides [32]. The quantitative measurement of flavonoids is done through the spectrophotometric technique [87]. The principal behind the aluminium chloride colorimetric method for determining total flavonoid content is that keto- and hydroxyl groups of flavones and flavanols are formed an acid-stable complex with aluminium. Furthermore, acid-labile complexes can be formed with flavonoids specific dihydroxyl groups [89]. It is a colorimetric method that uses $NaNO_2$ followed by $AlCl_3$*$6H_2O$ and NaOH to produce a bright orange colour. A UV-visible spectrophotometer is used to measure the absorbance immediately at 510 nm. The findings are calculated and expressed in micrograms of (+)-catechin equivalents [88].

4.3. Estimation of anthocyanin by pH differential method

The pH differential method determines the total anthocyanin content based on structural changes of anthocyanin molecules at pH 1.0 and 4.5, and absorbance is measured at 520 and 700 nm. Two buffer systems, namely 0.025 M potassium chloride (KCL) (pH 1.0 adjusted by hydrochloric acid) and 0.4M sodium acetate buffer (pH = 4.5), keep the desired pH. The sample is diluted with buffer to obtain an absorbance ranging from 0.2 to 1.4. A UV-Vis spectrophotometer is used to measure the absorbance of the mixture at 520 and 700 nm. The total anthocyanin content was calculated in cyanidin-3-glucoside equivalents [90, 91]. The method is suitable for determining the total monomeric anthocyanin content based on structural changes in the anthocyanin chromophore between pH 1.0 and 4.5 [92]. Anthocyanin absorbs and transmits light at specific wavelengths, giving them colour. The positive charge on the molecule's C ring is mainly responsible for anthocyanin pigmentation. However, this is pH-dependent; at pH 1.0, the molecules carry a positive charge and are pigmented; at pH 4.5 and higher, the charge is neutralized. The anthocyanin loses its pigmentation and becomes colourless [93]. As a result, at pH 1.0, the molecule strongly absorbs light with wavelengths ranging from 460 to 550 nm; however, at pH 4.5, it is colourless. Thus, the difference in absorbance at 520 nm and 700 nm is proportional to pigment concentration.

Absorbance = [Ab (520 nm) – Ab (700 nm)] pH 1.00 – [Ab (520 nm) – Ab (700 nm) pH 4.5]

$$\text{Anthocyanin (mg/L)} = \frac{A \times MW \times DF \times V \times 1000}{a \times l \times m}$$

Where A = absorbance, MW = molecular weight of cyanidin-3-glucosode (449.2 g/mol), DF = dilution factor, V = solvent volume (mL), a = molar absorptivity (26,900 $L.mol^{-1}. cm^{-1}$), and l = path length (1 cm) [91]

5. Methods involved in the estimation of antioxidant activity of plant secondary metabolite.

Reactive oxygen species (ROS) generally form due to oxidative phosphorylation, during which attack biomolecules such as cellular DNA and proteins. Increased ROS levels can cause breaks in single and double-stranded DNA, which can lead to cell ageing, cardiac disease, mutagenic changes, and cancerous tumour progression. Consumption of naturally antioxidant-rich foods is the most effective way of tackling such unfavourable transformations and health risks [94].

5.1 Estimation of total antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) Assay

The DPPH assay is commonly used to determine antioxidant activities, dependent on the mechanism by which antioxidants act to inhibit lipid oxidation, thereby determining free radical scavenging capacity. The method is commonly used to determine the antioxidant activity of extracts. DPPH (1,1-diphenyl-2-picrylhydrazyl) is a very stable free radical that reacts with compounds that can donate hydrogen atoms and has a UV–VIS absorption at 518 nm. The method is based on antioxidants scavenging DPPH, which after a reduction reaction, decolorizes the DPPH methanol solution. The assay assesses antioxidant ability to reduce the DPPH radical [95]. The radical scavenging activity can be measured using the DPPH assay. When neutralized by radical scavengers, the deep purple colour solution turns colourless or pale yellow. As a result, it is recognized as a quick and easy method to measure radical scavenging activity [96]. The result is expressed in terms of IC_{50}, which represents the concentration required to inhibit a radical 50% of the time [97].

$$\text{DPPH scavenging effect} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

5.2 Ferric reduction antioxidant power assay (FRAP)

The method is associated with two different mechanisms, hydrogen atom transfer (HAT) or single electron transfer (SET). Examples of single electron transfer assay (SET) are ferric reducing antioxidant power assay (FRAP) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) [87]. Benzie and Strain (1996) originally developed the FRAP assay to measure the reducing power in plasma, but it has since been adapted and used as a botanical approach to assessing antioxidant capacity [98]. It is a typical single electron transfer (SET)-based assay that measures the reduction of ferric ion (Fe^{3+})- ligand complex by antioxidants in an acidic medium to the intensely blue-coloured ferrous (Fe^{2+}) complex. To maintain iron solubility and electron transfer, the reaction is carried out in an acidic environment (pH 3.6). Although the original FRAP assay uses tripyridyltriazine (TPTZ) as the iron-binding ligand, other than TPTZ, ferrozine is used as a ligand for ascorbic acid –reducing power evaluation [99]. The FRAP reagent is a solution of ferric chloride and TPTZ in acetate buffer (pH 3.6) used in the FRAP assay [100]. Antioxidant activity is measured using a UV VIS spectrophotometer at 593 nm, and the results are expressed in terms of micromolar Fe^{2+} equivalents or relative to an antioxidant standard [101]. Trolox is primarily used as a positive control in this assay, and results can be expressed as mol/ml.

5.3 2, 2′-Azinobis- 3-Ethylbenzthiazolin-6-Sulfonic Acid Assay (ABTS)

The ABTS assay was described by Miller *et al*., 1997. The method for determining the total antioxidant capacity of body fluids and drug solutions was developed based on the absorbance of the $ABTS^+$ radical cation [102]. Metmyoglobin was first reacted with H_2O_2 in the original assay to form the ferrylmyoglobin radical, then reacted with ABTS to form the $ABTS^{\bullet+}$ radical [103]. The chemical compound ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) is used to study the reaction kinetics of specific enzymes. 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$), a green-blue stable radical cationic chromophore, is produced by oxidation shows absorbance maxima at 414, 645, 734, and 815 nm. In the ABTS assay, also known as the Trolox equivalent antioxidant capacity (TEAC) assay. The ABTS assay measures free radical scavenging ability of antioxidant molecule to scavenge ABTS in the aqueous phase. Trolox (water-soluble vitamin E analogue) is used as a standard [104]. The ABTS cation radical can be measured spectrophotometrically at 734 nm [105]. The Trolox equivalent antioxidant capacity (TEAC) assay (reported in Trolox equivalent units) determines the strength of an antioxidant when compared to Trolox. The ABTS assay results are typically reported in a dose-dependent manner (moles ABTS radical cation consumed/moles antioxidant consumed over a given time interval) [106].

5.4 Oxygen Radical Scavenging Capacity (ORAC) assay

The Oxygen Radical Scavenging Capacity (ORAC) assay was developed by Cao *et al*. (1993). It is an example of a hydrogen atom transfer (HAT) assay to measure the total antioxidant capacity of secondary antioxidants. The assay is based on De Lange and Glaze's 1989 fluorescence method for peroxy radicals. It is recognized as one of the most widely used antioxidant activity assays in the research and food industries. However, it has the disadvantage of requiring fluorometers, which are not always readily available in analytical laboratories [85]. The ORAC assay makes use of AAPH [2, 2′-azobis(2-amidinopropane) dihydrochlorideability] to form peroxyl radicals when heated in the presence of sufficient oxygen. These radicals reduce the fluorescence of a probe. The amount of reduction is determined by the antioxidant, which acts by quenching the produced radicals [107]. This assay plots fluorescence emission versus time to determine the kinetics of the decrease in fluorescence for each sample compared to a blank. The ORAC assay has several limitations, including lengthy and labour-intensive sample preparation, which is especially problematic when analysing large numbers of samples. This limitation, however, has been overcome by the development of a high-throughput ORAC assay that employs fluorescein as the fluorescent probe [108]. ORAC assay describe both the thermodynamic and kinetic characteristic of a reaction and provide results based on the net area under the curve (AUC) fluorescence decay/ time curve of the probe in the presence and absence of antioxidants [94].

5.5 Cupric reducing antioxidant capacity (CUPRAC)

Antioxidant assays are classified into two types: hydrogen atom transfer (HAT) assay and electron transfer (ET) assay. CUPRAC assay is a type of electron transfer assay. CUPRAC is a newly discovered (2004) electron transfer ET-based TAC assay for total antioxidant quantification. ET-based spectrophotometric assays evaluate an antioxidant's capacity by reducing a chromogenic oxidant (probe), which changes colour once reduced [109]. The redox system of copper (II)-neocuproine complex (copper-reducing antioxidant capacity method or CUPRAC) is a very trusted and accurate method for determining total antioxidant capacity [110]. The CUPRAC method can detect all antioxidant compounds, including thiols, in complex sample mixtures [111]. The assay is generally based on measuring the absorbance of Cu (I)-neocuproine (Nc) chelate, which is formed by a redox reaction between antioxidant molecules and CUPRAC reagent copper (II)-neocuproine and absorbance is recorded at a wavelength of 450 nm [109]. The CUPRAC regent is a chromogenic reagent which forms copper (II) chelate. This reagent is required pH 7. The reactive Ar-OH groups of polyphenolic antioxidants are oxidised to the corresponding quinones (ascorbic acid is oxidised to dehydroascorbic acid). The coloured complex is formed due to the reduction of Cu (II)-Nc is to the highly coloured Cu (I)-Nc chelate with maximum absorption at 450 nm [94].

5.6 Phosphomolybdenum assay

The antioxidant such as flavonoids and polyphenols detect by DPPH and ABTS scavenging assay, whereas the antioxidant such as ascorbic acid, some phenolics, a-tocopherol, and carotenoids are generally detected by phosphomolybdenum method [112]. The phosphomolybdenum assay is based on the principal of reduction of Mo (VI) to Mo (V) presence of an antioxidant compound [113] that results in the formation of a green phosphate Mo (V) complex at acidic pH. The absorbance is recorded by UV VIS spectrophotometer at a wavelength of 695 nm [114].

5.7 Superoxide radical scavenging activity

The anion radical, which is associated with the phenazine methosulfate-nicotinamide adenine dinucleotide (PMS NADH) system, is generally responsible for superoxide radical scavenging activity [115]. The nitro blue tetrazolium (NBT) reduce into a purple formazan by superoxide radicals generated by non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system [116]. The method which is described by Robak and Gryglewski (1988) are used to determine superoxide anion scavenging activity. 3.0 mL of Tris–HCl buffer containing nitroblue tetrazolium (NBT), NADH solution, 1.0 mL sample, and Tris–HCl buffer, is used to generate superoxide anion radicals. The reaction is carried out by adding phenazine methosulfate (PMS) solution to the mixture. The reaction mixture in incubated at 25 °C for 5 min and absorbance is taken on 560 nm by UV VIS spectrophotometer against blank [114]. Quercetin was used as positive control [116].

5.8 Catalase assay

The UV spectrophotometric method, which relies on monitoring the change in 240 nm absorbance at high levels of hydrogen peroxide solution (30 mM), is the most commonly used method for measuring catalase activity. High levels of hydrogen peroxide (H_2O_2) immediately inhibit the catalase enzyme by altering its active site structure, though the extent to which this occurs varies [117]. The catalase activity was calculated using the molar extinction coefficient of H2O2, 43.6 M cm^{-1} where one unit of activity equals one mmol of H_2O_2 degraded per minute and is expressed in milligrams of protein [118].

5.9 Hydrogen peroxide (H_2O_2) scavenging activity

The hydrogen peroxide (H_2O_2) scavenging activity is widely use method generally used to determine the natural antioxidants present in plant extracts it is an UV -VIS spectrophotometric method which measure the decrement of H_2O_2 in an incubation system containing H_2O_2 and the scavenger [119]. In the hydrogen peroxide scavenging activity assay, the solution of concentration of hydrogen peroxide is prepared in phosphate buffer (pH 7.4). The ascorbic acid, gallic acid and tannic acid is used as standard which is added to a hydrogen peroxide solution, the absorbance is recorded after 10 min of incubation period at wavelength 230 nm against blank. The blank solution contains only phosphate buffer (pH 7.4) without hydrogen peroxide [120].

6. Bioactivity property of plants secondary metabolites

Bioactivity is an alternative term to the biological activity, which is govern by the biological active compounds. The compound is categorized as biological active when it has direct beneficial effect on living organisms by interacting with components of living tissue [121].

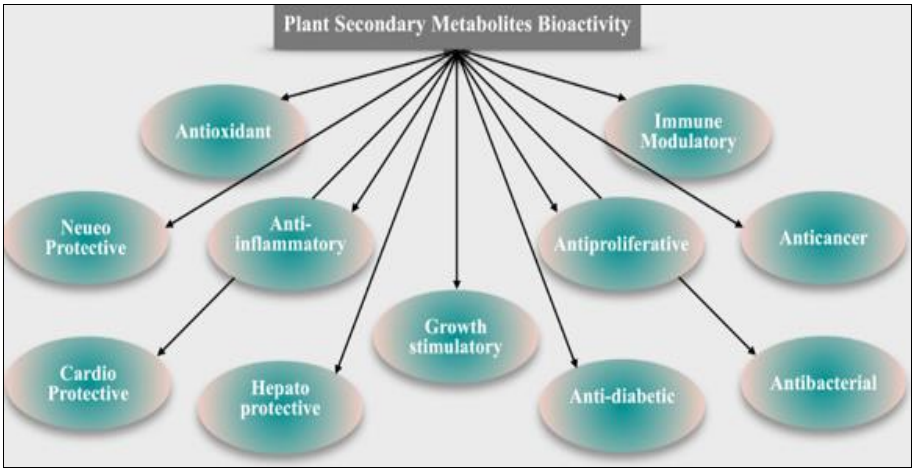

Fig 6: Schematic representation of bioactivity showed by plant secondary metabolites

6.1. Antioxidant activity

Antioxidants play a crucial role in maintaining the homeostasis of free radicals/reactive oxygen species/oxidants in the human body by destabilizing or neutralizing the free radicals [122]. Antioxidant action depends on its bond dissociation enthalpy (BDE). The BDE is inversely proportional to the antioxidant activity of the antioxidant [123]. The natural sources of antioxidants are mainly dietary sources such as beans. Phenolic acids, stilbenes, flavonoids and tannins are potent antioxidants and are also involved in stimulating the endogenous antioxidant molecules [124]. Rutin and gallic acid confer the concentration-dependent antioxidant activity [125]. The polyphenols and flavonoid concentrations directly correlate with antioxidant activity [126]. The ratio of flavonoids: to polyphenols also determines the antioxidant activity when there is no direct correlation with its concentration [127]. The antioxidant properties of phenolic and flavonoid content helps to prevent ROS mediated liver damage [122]. Coumarins (Daphnetin, esculin and scoparone) also exhibit the antioxidant properties and promising agents to treat intestinal inflammatory disease.

6.2. Anti-inflammatory

Inflammation is a protective adaptive response of the innate immune system against tissue injury or damage or other stimulants such as pathogens and allergens. The inflammations are also triggered by an unhealthy lifestyle and diet and result in inflammatory stress, which may contribute to development of a number of chronic diseases. To treat inflammation, mankind is using plant-based materials from several generations. The dietary supplements of polyphenols have an excellent effect on combating inflammation and reversing the induced inflammation [128]. Polyphenols, more specifically flavonoids, have strong anti-inflammatory activity. This may be because of its antioxidant property [129]. Alkaloids such as chuanbeinone, verticine, and verticinone exhibit anti-inflammatory activity. When administrated in rats, these alkaloids significantly inhibited ammonia induced cough [130]. Chabamide, a dimeric alkaloid isolated from *Piper nigrum* was shown to confer anti-inflammatory activity by induction of the Nrf2/heme-oxygenase-1 pathway [131]. Tannins also inhibit the inflammatory events in cyclophosphamide-induced haemorrhagic cystitis [132]. Coumarin and hydrocoumarin also have the potential to exhibit anti-inflammatory activity [133].

6.3. Growth stimulatory activity

Polyphenols help maintain human health because of their antioxidant and growth stimulatory effect on beneficial bacteria of the gut. Polyphenols significantly stimulate the growth of *Bifidobacterium*. The polyphenol extract of *Sesbania grandiflora*, rich in rutin, significantly increases the probiotic bacteria *Lactobacillus acidophilus* [134].

6.4. Antibacterial activity

Alkaloids have shown the potential to function like antibiotic, and exhibit broad-spectrum antibacterial activity through various approaches such as chelating the ions, enzyme inhibition, transcription factor inhibition, and metabolite substrate deactivation [135]. Tea polyphenols are also antibacterial against *Escherichia coli* and *Staphylococcus aureus* by increasing oxidative stress and disrupting cell membranes. The growth of methicillin-resistant *Staphylococcus aureus* (MRSA) could be inhibited by persimmon tannin (PT) through multifaceted effects [136]. Coumarin, (±)-euryacoumarin A isolated from *Eurya* stem exhibit potent antibacterial activity against *B. subtilis*, *B. cereus*, *S. aureus*, and MRSA.

6.5. Antiproliferative activity

Inhibition of cancer cell growth is an ultimate solution to prevent cancer. The antiproliferative compounds

systematically and selectively arrest/inhibit cell growth. Such compounds isolated from *Lespedeza bicolor* exhibit strong antiproliferative activity against blood cancer cells by arresting the cells in the G1 phase and reducing BCL3 levels [137]. Resveratrol, a phytoalexin compound found in grapes, inhibits the growth of estrogen receptor (ER)- positive MCF-7 cells in a concentration-dependent manner. In MCF-7 cells, resveratrol inhibits the growth-promoting effect of 17-β-estradiol in a dose-dependent way [138].

6.6. Immune modulatory activity

Host immune modulation is a vital phenomenon played by plant secondary metabolites. The tannins from *Terminalia chebula* show significant immune modulation activity and inhibitory activity against Hepatitis C virus (HCA NS3/4A) [139]. Tannins also provide a leishmanicidal effect along with immune-modulatory training by stimulating macrophages for nitric oxide (NO), tumour necrosis factor (TNF) and interferon (IFN) production [140]. Resveratrol and 6 gingerol showed the immune modulation against lipopolysaccharide (LPS) and pathogen-associated molecular pattern (PAMP) stimulation in Atlantic salmon macrophages [141]. Curcumin from turmeric inhibits immature dendritic cells and stops inflammation that activate naive CD4+ T-cells. Curcumin inhibits interleukins IL-6 and IL-12 production, which ultimately inhibits the differentiation of naive CD4+ T-cells into TH17 and Th1 [142, 143].

6.7. Anticancer

India is the production factory of medicinal plants; appreciable work is done on plants to treat cancer and many phytoproducts have been recognized as anticancer drugs. Compound conjugated with different chemicals are in trials which exhibits chemoprotective activities. Inhibition of angiogenesis is the sole factor of cancer treatment. Some of the plants which originated anticancer agents are studied in cancer cell lines [144]. Bioactive xanthomicrol, 5, 4'-dihydroxy-6,7,8,3'- tetramethoxyflavone, and ursolic acid present in *Adenosma bracteosum Bonati* has shown anticancer activity against human large cell lung carcinoma (NCI-H460) and hepatocellular carcinoma (HepG2) with the highest radical scavenging activity IC_{50} values of 4.57 ± 0.32 and 5.67 ± 0.09 µg/mL respectively followed by ursolic acid with percent inhibition 13.05 ± 0.55 and 10.00 ± 0.16 µg/mL for NCI-H460 and HepG2 respectively [137]. Recently nanoparticle-based tools are emerging to develop nanomedicines from plant-derived drugs for better anticancer activity with respect to enhance controlled release of the compound at targeted site and in finding new out for their administration. Generally, phyto originated drugs are categorized under four classes based on their actions; inhibitors of methyltransferases, preventives of DNA damage or antioxidants, inhibitors of histone deacetylases (HDAC) and disruptors of mitotic division. sulforaphane, isothiocyanates, isoflavones and pomiferin are some prominent examples of HDAC inhibitors. Mainly plant secondary metabolites which are recognized for their anticancer properties are polyphenols, brassinosteroids and taxols [145]. Ethanolic extracts of medicinal herbs *C. halicacabum*, *G. celosioides*, *C. halicacabum* and *S. dulcis* were studied for the anticancer activity against cholangiocarcinoma (CCA) cells. In comparison to others, *S. dulcis* extract showed the most inhibitory effect on cell lines KKU-100 AND KKU-213 56.06 and 74.76, respectively at a 250 µg/mL concentration of extracts for 72 h. treatment time. While at higher concentrations of 400 and 500 µg/mL, there was a significant increase in BAX/Bcl-2 ratio and cell membrane permeability, showing the induction of apoptosis specifically in the KKU-213 cell line [146]. *Andrographis paniculate* has shown cytotoxicity against breast cancer cells (MCF-7), P388 lymphocytic cells and colon cancer cells (HCT-116). In addition, andrographolide present in *A. paniculate* showed an inhibitory growth effect on cell line HT 29 and enhanced growth and division of human peripheral blood lymphocytes in myeloid leukemia M1 cell line from mouse [147].

6.8. Anti-diabetic

Diabetes mellitus (DM), also known as diabetes, has affected 25% of the world's total population together in developed and developing countries. In diabetes patients, cells cannot metabolize sugar either because of a lack of sufficient insulin secretion by pancreatic cells or lack of effective utilization of insulin. In response to that, body breaks down its fats, proteins and glycogen for energy purposes, resulting in elevated sugar levels and excess ketone body generation. Herbal extract contribute to antidiabetic activity because of their potential to boost the performance of pancreatic cells to increase insulin secretion or trigger the reduction in glucose absorption by the intestine [148]. Coumarin and quercetin inhibit the dipeptidyl peptidase-IV (DPP-IV) enzyme which is essential in lowering blood glucose levels in type-II diabetes myelitis [149]. Phenolic acids help to maintain blood glucose homeostasis by inhibiting the activity of enzymes such as α-amylase and α- glucosidase, which are responsible for elevating the blood glucose level. Their mode of action is directly on islet β- cells which induce glucose-stimulated insulin secretion and maintain blood glucose levels [125]. Many plants, *Santalum spicatum* (5.43 ug/ml), *Aloe vera* (80 ug/ml), *Ocimum tenuiflorum* (8.9 ug/ml), *Moringa stenopetala* (1470 ug/ml) and methanolic extract of *Eugena cumini* (632 ug/ml) secondary metabolites have shown impressive inhibitory activity in terms of IC_{50} values (ug/ml) against α- amylase. In addition, there are many reports available for inhibitory activity against α -glucosidase such as *Acacia ligulate*, methanol extract of *Marrubium radiatum*, hydroalcoholic extracts of *Camellia sinensis*, *Mucuna pruriens and Fagonia cretica* with IC_{50} values 1.01 ug/ml, 68.8 ug/ml, 299 ug/ml, 0.8 ug/ml and 4.62 ug/ml respectively [148]. To check the effect on DPP-IV enzyme activity both studies were done on 22 medicinal plants which are known for antidiabetic activity by *in vitro* fluorometric assay and in *in vivo* high-fat-fed (HFF) obese diabetic rats. Four most active extracts containing DPP-IV inhibitors- sitagliptin, vildagliptin and diprotin A showed maximum (95-99%) inhibitory activity and were selected and fed (250 mg/5 ml/kg, body weight) to HFF obese diabetic rats. Maximum inhibitory effects were exhibited by *Anogeissus Latifolia* (96%) with IC_{50} 754 ug/ml while other extracts of *A. marmelos*, *M. indica*, *C. cochinchinesis*, *T. foenum-graecum* and *A. indica* exhibited 44%, 38%, 31%, 28% and 27%, respectively [150].

6.9. Hepatoprotection

The liver is one of the most crucial body organs and plays multi-biological roles such as in metabolism of carbohydrates, proteins and lipids. Nowadays, acute and chronic liver dysfunction is becoming a global concern, as achieving medical treatments for this is a very tedious and less efficient process. Hepatoprotective activity can be measured in terms of liver markers such as AST, ALT, ALP, total protein,

albumin and bilirubin and by histopathological investigation [151]. Methanolic extract of *Euphorbia retusa forssk* was evaluated against in CCL4 induced liver damage in rats by accessing MDA, GSH, NO and ALT levels in serum. It was found that administration of extract (200 mg/ kg body weight) significantly decreased AST and ALT while inhibited the NO and MDA, and also there was an increase in GSH. This hepato- protective activity was attributed because of phenolic compounds present in the extract namely gallic acid, ellagic acid, kaempferol, quercetin, kaempferol-3-O-β-D-glucopyranoside, quercetin-3-O- β-D-glucopyranoside and 3,3-dimethoxy ellagic acid [152]. The shade dried fruits of *Arrtocarpus lakoocha* were extracted in methanol by maceration and hepato-protective activity of the extract was investigated against paracetamol (500 mg/kg) induced hepatotoxicity in mice. In HPLC analysis, chromatotropic acid, quercetin, gallic acid, cinnamic acid, ferulic acid, and kaempferol were found to be significant compounds in the extract [133]. Some liver treatment formulations have used flavonoids, dihydromyricetin (DMY), hyperoside and silybin. To use as a liver cell injury model, cell line L02 was treated with 200 μM emodin for 48 h and the hepatoprotective effect was studied by giving different concentrations of DMY/hyperoside/silybin for the same time duration. CCK-8 assay was performed for detection of cell viability, mRNA and protein expression was studied by RT-qPCR and western blotting of the CYP7A1 (bile acid synthetic pathway gene), the bile acid efflux transporter, bile salt export pump, the nuclear factor erythroid -2-related factor 2 (Nrf2) and drug processing gene CYP1A2, respectively. The study revealed that DMY, hyperoside, and silybin protected cells against emodin at a very low concentration (10 μM) dose-dependent in order of hyperoside > DMY > silybin. There was a decrease in expression of CYP7A1 at both mRNA and protein levels with a decrease in expression of the CYP1A2 gene with enhanced expression of Nrf2 nuclear protein expression [153]. Many phytochemicals belonging to classes flavonoids, polyphenols, saponins, terpenes and alkaloids have been shown to have a protective effect against AFB1 caused liver diseases by improving tissue's ability to sustain oxidative and inflammatory stress and in addition, promoting AFB1 detoxification and metabolism inhibition. Such exploration of phytochemical induced effects and mechanisms to prevent and treat various human diseases can provide new insights into developing new plant-based drugs and functional foods [154].

6.10. Cardiovascular diseases

Cardiovascular diseases represent a worldwide socioeconomic problem; solving it also depends on lifestyle changes such as dietary habits. Several foods and dietary supplements have been studied for anti-inflammatory, antioxidant properties and modulating mitochondrial function. Resveratrol is most studied phyto-compound in addition to that *Brassica oleracea*, curcumin, berberine are studied for cardioprotective potential [155]. Nature is important source for finding new drugs, some of the famous examples are aspirin from the *Salix alba* L., digoxin from *Digitalis purpurea*, ephedrine from *Ephedra sinica* and lovastatin sourced from *Monascus purpureas* L., taxol from *Taxus brevifolia*, reserpine from *Rauvolfia serpentia* and many more [156]. *In vivo* anti-inflammatory and antioxidant effects of ethanolic extract of *Mahonia aquifolium* was studied in turpentine oil- induced acute inflammation in rat model in comparison to diclofenac, all extracts of flower and green fruits resulted in decreased in serum nitric oxide (NOX), total oxidative stress (TOS), 3-nitrotyrosine (3NT), malondialdehyde (MDA) and increase in total thiols (SH) [157]. Another way to use natural plant metabolites in cardiovascular disease treatment is activation of cardiac sarcoplasmic reticulum calcium pumping ATPase which is mainly responsible for instant removal of calcium ions from cytosol for allowing faster relaxation of cardiac muscle cells. Such activity is evident in simple phenol-gingerol from rhizome part of *Zingiber officinale* [158].

6.11. Neuroprotection

Several classes of polyphenols and there *in vivo* resultant metabolites can reach several tissues, including the brain. Many evidences available suggest that oxidative cascades of events lead to neurodegeneration linked diseases such as Parkinson's, Alzheimer's disease (AD), Huntington's disease, and amyotrophic lateral sclerosis. Plenty of *in vivo* models have been studied for diseases, and also several biomedical and epidemiological reports are available providing evidence that polyphenols are natural antioxidants which are responsible for neuroprotective activity [143].

Amyloid peptides, also known as Aβ fibrils, are markers of AD. Aβ fibrils are mainly derived after the action of γ-secretase and β-secretase on amyloid precursor protein (APP) which is present as an integral protein of the plasma membrane. These peptides cause neurotoxicity induced by oxidative stress, and cellular prion protein causes lowering in glutathione levels, leading to neuronal death [159]. Insoluble Aβ fibrils oligomerize with each other, reach synaptic clefts, and activate kinases, resulting in over phosphorylation of microtubule-associated T protein. Excess polymerization of insoluble amyloid fibres aggregates into plaques, and this plaque formation is always followed by recruitment of nearby microglia; such activation of microglia causes local inflammation. Two forms of Aβ play a role in plaque formation, Aβ40 and Aβ42. Aβ40 is present in higher amounts, and it is less soluble and neurotoxic compared to Aβ42. While Aβ42 is the opposite in properties, it is more aggregation-prone and the primary toxic component of amyloid fibers assembly. The ratio value of Aβ40 to Aβ42 aggregation interferes with the function of ion channels, alterations in calcium balance, increment in mitochondrial oxidative pressure and also causes disturbances in glucose-related metabolisms [160]. Green tea contains many bioactive compounds, mainly rich in flavonoids (30% on a dry basis leaf) representing catechins and their derivatives, flavonols, phenolic acids, caffeine and theanine. These bioactive are strong warriors against singlet oxygen, superoxide ions, hydroxyl and peroxyl radicals. Perhaps a decade ago, green tea is well studied for its potential with respect to the prevention and treatment of cancer, cardiovascular and inflammatory diseases [161].

7. Conclusion

Plants can produce a diverse range of organic compounds, which are further classified as two different types of primary and secondary metabolites. The secondary metabolite is a metabolic intermediate or product that is required for plant growth, but it also plays an essential role in plant survival and abiotic and biotic stress conditions. The secondary metabolites are potent source of bioactive compounds, and current research proves that these bioactive compounds have many health beneficial properties and so an in- depth understanding of plant bioactive compound is necessary. Therefore, the bioactive compounds which have a key role in

pharmaceutical and nutraceutical industries need to be studied for their appropriate extraction, identification and characterization in plants. In extraction, selection of appropriate solvent system is essential to prevent decomposition of metabolites as well as loss of their bioactivity. This review article has presented the classification, biosynthesis pathways, extraction methods and analytical techniques involved in the identification and characterization of plant bioactive compounds as well as their bioactivity in a single platform.

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