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A review: Extraction, isolation and determination of secondary metabolite (*Phenols*) from medicinal plants and its benefits

Divya Raj, Mrudula Sanjay Shah and Deoraj Sharma

Abstract
A significant class of extensively present secondary metabolites in the plant kingdom with a wide range of structural variations are phenolic compounds. These substances can be found as free or matrix-bound substances, aglycones or glycosides, monomers, or as parts of highly polymerized structures. They are also not evenly distributed throughout the plant, and their stability varies greatly. This significantly complicates their extraction and isolation processes, making it impossible to recommend a single standardized procedure for all phenolics and/or plant materials. Instead, procedures must be optimized based on the nature of the sample, the target analytes, and the study's overall goal. We have attempted to summarize the main steps in the extraction, isolation, and determination of phenols from medicinal plants.

Keywords: Medicinal pants, secondary metabolites, phenols, extraction, isolation, determination

Introduction
Numerous chemical substances, commonly referred to as secondary metabolites, are produced by vascular plants. The fundamental life activities of plants are controlled by these low-molecular-weight substances. Phenols are thought to make up roughly 8000 different naturally occurring plant chemicals. They have an aromatic ring with at least one hydroxyl substituent, which is their defining structural property. Numerous phenolic compounds are produced constitutively, however under stress conditions, such as those caused by infection, plant tissue damage, UV radiation, and high temperatures, phenolic levels can rise or new phenolic compounds can be synthesized in recent years, medicinal plants have become much more well-liked and consumed. This phenomenon has made it possible to assess the quality of plant products more quickly and accurately. Estimating the pharmacological activity of medicinal plants can be greatly aided by determining the presence of phenolic chemicals. Phenolic acids, aromatic secondary metabolites that are common in the plant kingdom, play a vital role within this enormous category of chemicals. Both hydroxyl and carboxyl groups can be found in these molecules. The hydroxyl derivatives of benzoic and cinnamic acids are examples of phenolic acids. Gallic, vanillic, salicylic, caffeic, and p-coumaric acids, which are active components of many plants, have received a lot of attention. As examples, consider the phenolic acids found in thyme, which include Gallic, caffeic, and rosmarinic acids. Other examples include sage, which contains ferulic, Gallic, rosmarinic, vanillic, and caffeic acids, and rosemary, which is known for its high content of vanillic, caffeic, and rosmarinic acids. (Agnieszka Arceusz *et al*., 2013) [1].

Nutritionists have just recently become aware of the health benefits of dietary polyphenols, despite their widespread distribution. Due to their powerful antioxidant qualities, abundance in the diet, and verifiable effects in the prevention of many oxidative stress-related disorders, polyphenols have attracted the attention of researchers and food makers. Epidemiologic data, as well as results from experiments conducted *in vitro* and *in vivo,* are used to infer the preventative effects of these second plant metabolites in terms of cardiovascular, neurological illnesses, and cancer, and the resulting dietary advice. Additionally, it was shown that polyphenols can modify the function of a variety of enzymes and cell receptors. In this approach, polyphenols have various different distinct biological functions in the prevention and/or treatment of diseases in addition to their antioxidant characteristics.

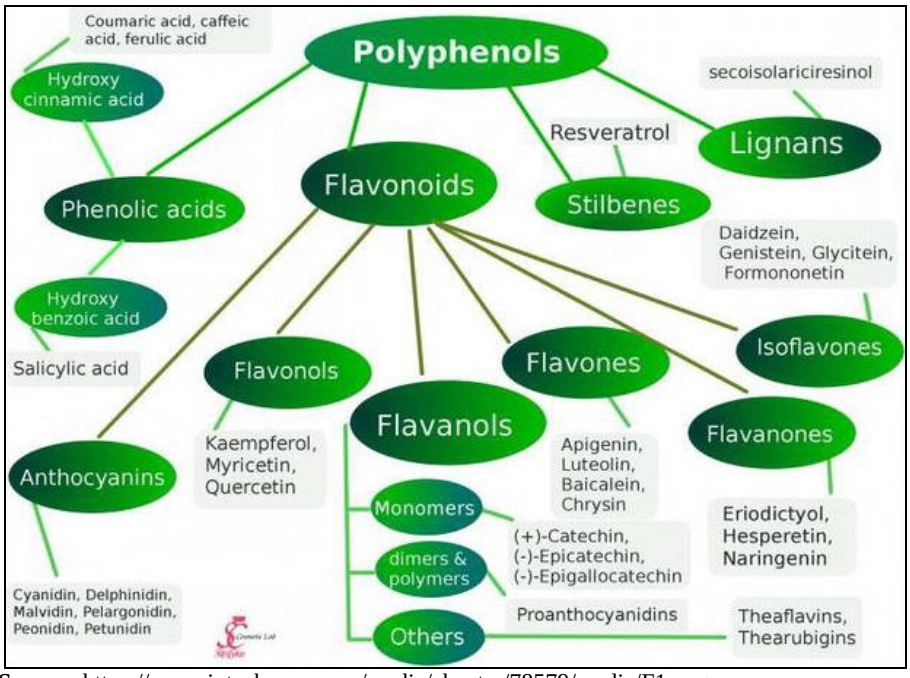

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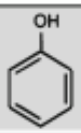
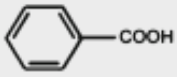
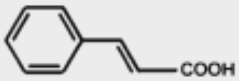
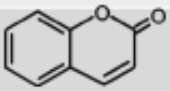
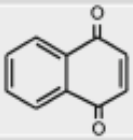
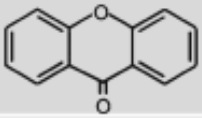
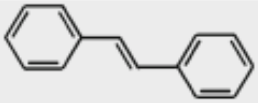
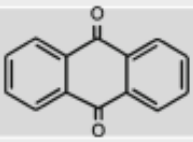
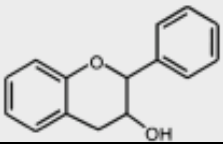
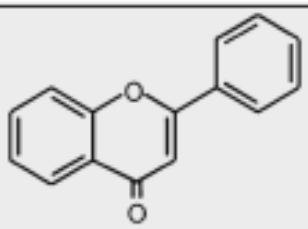
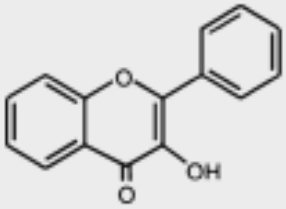
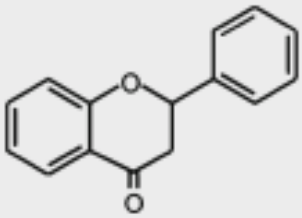
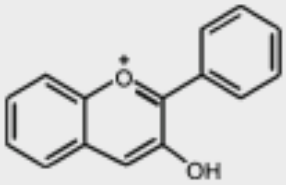
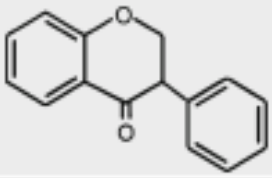
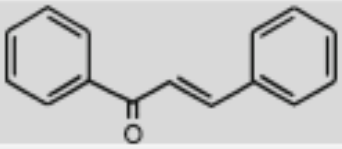
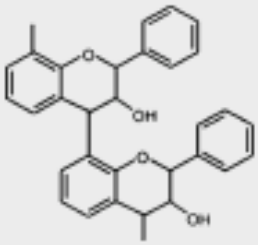
Fig 1: Phenolic Groups

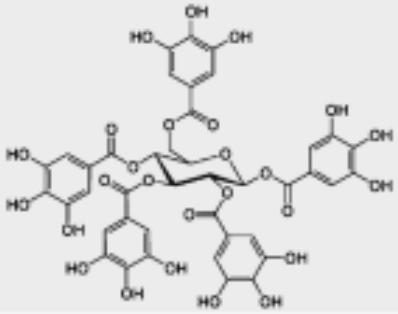
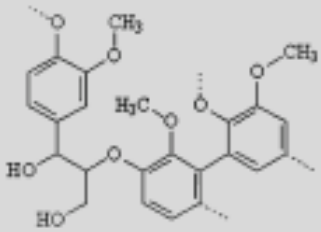
Classification

Class	Basic skeleton	Examples
Simple phenols (C6)		Phloroglucinol, catechol, resorcinol, vanillin, syringaldehyde
Phenolic acids Hydroxybenzoic acids (C6-C1)		*p*-hydroxybenzoic acid, protoctechuic acid, vanillic acid, syringic acid, gallic acid, gentisic acid, salicylic acid
Hydroxycinnamic acids C6-C3) and derivatives		Caffeic acid, coumaric acid, ferulic acid, sinapic acid, chlorogenic acid (5-caffeoylquinic acid)
Coumarins (C6-C3)		Scopoletin, umbelliferone, aesculetin
Naphtoquinones (C6-C4)		Juglone, pumblagin
Xanthones (C6-C1-C6)		Mangostin, mangiferin
Stilbenes (C6-C2-C6)		Resveratrol, piceid, e-viniferins
Anthraquinones (C6-C2-C6)		Emodin, physcion,
Flavonoids (C6-C3-C6) Flavan-3-ols		(Epi)catechin, (epi)gallocatecin

(continued)

Class	Basic skeleton	Examples
Flavones		Apigenin, luteolin, chrysin, scutellarein, diosmetin, chrysoeriol
Flavonols		Quercetin, kaempferol, myricetin, galangin, fisetin, morin
Flavanones		Hesperidin, naringenin, taxifolin, eriodictyol, isosakuranetin
Anthocyanins		Cyanidin, delphinidin, malvidin, pelargonidin, petunidin, peonidin
Isoflavones		Genistein, daidzein, glycitein, formononetin, biochanin A, puerarin
Chalcones		Phloretin, arbutin, butein, naringenin chalcone
Condensed tannins (proanthocyanidins) $(C6\text{-}C3\text{-}C6)_n$		Procyanidins, prodelphidinins

(continued)

Class	Basic skeleton	Examples
Hydrolysable tannins (gallotannins, ellagitannins)		Pentagalloylglucose, vescalagin, castalagin
Lignans $(C6\text{-}C2)_2$		Secoisolariciresinol, mataires-inol, sesamin, pinoresinol, syringaresinol
Lignins (C6-C3)n		

Source: Extraction and Isolation of Phenolic Compounds Celestino Santos-Buelga, Susana Gonzalez-Manzano, Montserrat Dueñas, and Ana M. Gonzalez-Paramas

Secondary Metabolites

Plants also use secondary metabolites (such as colorful flavonoids, volatile essential oils, and tetraterpenes), which function as signaling chemicals, to entice insects for pollination or other animals for seed dispersion. Terpenoids, alkaloids, and flavonoids are being employed in pharmaceuticals and nutritional supplements to treat or prevent a wide range of diseases, and several of these compounds appear to be particularly helpful at resisting and preventing cancer. (Watson, A.A. *et al*., 2001, Raskin, I. *et al*., 2002, Reddy, L. *et al*., 2003) [38, 24, 25].

Between 14 to 28 percent of higher plant species are thought to be utilized medicinally, and 74 percent of pharmacologically active plant-derived components have been discovered as a result of study into ethnomedical plant use (Ncube, N.S. *et al*., 2008) [17]. Secondary metabolites are metabolic intermediates or products that are found as differentiation products in particular taxonomic groups, are produced through a wider variety of biosynthetic pathways than are available in general metabolism, and are not required for the growth and survival of the producing organism. Plants have a powerful defense system thanks to volatile monoterpenes or essential oils, notably against pathogenic fungus and herbivorous insect pests. These combustible terpenoids draw pollinators and are crucial for plant-plant interactions (Tholl, D. 2006) [35].

Table 1: Secondary metabolites from plant cell, Tissue and organs cultures

Plant Name	Active Ingredient	Culture Type
Adhatoda vasica	Vasine	Shoot culture(24)
Agastache rugosa	Rosmarinic acid	Hairy root(25)
Ammi majus	Umbelliferone	Shootlet(26)
	Triterpenoid	Suspension(27)
Angelica gigas	Deoursin	Hairy root(28)
Arachis hypogaea	Resveratol	Hairy root(29)
Artemisia annua	Artemisinin	Callus(30)
Aspidosperma ramiflorum	Ramiflorin	Callus(31)
Azadirachta indica	Azadirachtin	Suspension(32)
Brucea javanica	Cathin	Suspension(33)
Bupleurum falcatum	Saikosaponins	Root(34)
Camellia chinensis	Flavones	Callus(35)
Capsicum annum	Capsiacin	Callus(36)
Cassia acutifolia	Anthraquinones	Suspension(37)
C. senna	Anthraquinone	Hairy root(38)
Catharanthus roseus	Indole alkaloids	Suspension(39)
	Vincristine	Suspension(40)
	Catharathine	Suspension(41)
Cayratia trifoliata	Stilbenes	Suspension(42)
Centella asiatica	Asiaticoside	Hairy root(43)
		Callus(44)
Drosera rotundifolia	7-Methyljuglone	Shoot culture(45)
Eleutherococcus senticosus	Eleuthrosides	Suspension(46)
Eriobotrya japonica	Triterpenes	Callus(46)
Fabiana imbricata	Rutin	Callus and Suspenson(47)
Fagopyrum esculentum	Rutin	Hairy root(48)
Fritillaria unibracteata	Alkaloids	Multiple shoot(49)
Gentiana macrophylla	Glucoside	Hairy root(50)
Gentianella austriaca	Xanthone	Multiple shoot(51)
Glycyrrhiza glabra	Glycyrrhizin	Hairy root(52)
Gymnema sylvestre	Gymnemic acid	Callus(53)
Hemidesmus indicus	Lupeol, Rutin	Shoot culture(54)
Hypericum perforatum	Hypericin	Multiple shoot(55)
Mentha arvensis	Terpenoid	Shoot(56)
Momordica charantia	Flavonoid	Callus(57)

Source: Saurabh Pagare *et al*., 2015) [31]

Phenolic Compound

The biggest class of secondary metabolites, phenols include polymers like tannins and lignins as well as simple molecules with a single aromatic ring. They include the flavonoids coumarins, quinones, napthoquinones, and anthraquinones that give plants their smell, color, and flavor. Animals are affected physiologically by some of the chemicals. Foods are frequently flavored with vanillin. It is only a basic phenol. Aspirin is a precursor to salicylic acid, which is widely used. First discovered in *Ephermerantha lonchophylla* (*Flickingeria xantholeuca*), denbinobin is a 1,4-phenanthrenequinone that has been shown to be present in *Dendrobium nobile* and *D. candidum*. It has been discovered to inhibit HIV-1 replication through an NF-kappaB-dependent pathway (Sanchez-Duffhunes *et al*. 2008) [30].

Additionally, it could prevent the invasion or spread of stomach and breast malignancies by preventing angiogenesis and inhibiting the formation of tumours (Tsai *et al*. 2011, Song *et al*. 2012) [36, 33]. Denbinobin exhibits an antifibrotic effect on the liver by eliciting selective apoptosis in hepatic stellate cells but not in normal hepatic cells, and may thus be a helpful starting point for creating chemicals to safeguard the liver against cirrhosis (Yang *et al*. 2011) [39]. Since denbinobin has been synthesized, additional research and clinical testing ought to be conducted. This is most likely the orchid-isolated phenanthrene or phenol that shows the most promise. (Source: Secondary Metabolites of Plants, Springer International Publishing Switzerland 2016) [32].

Extraction of Phenols

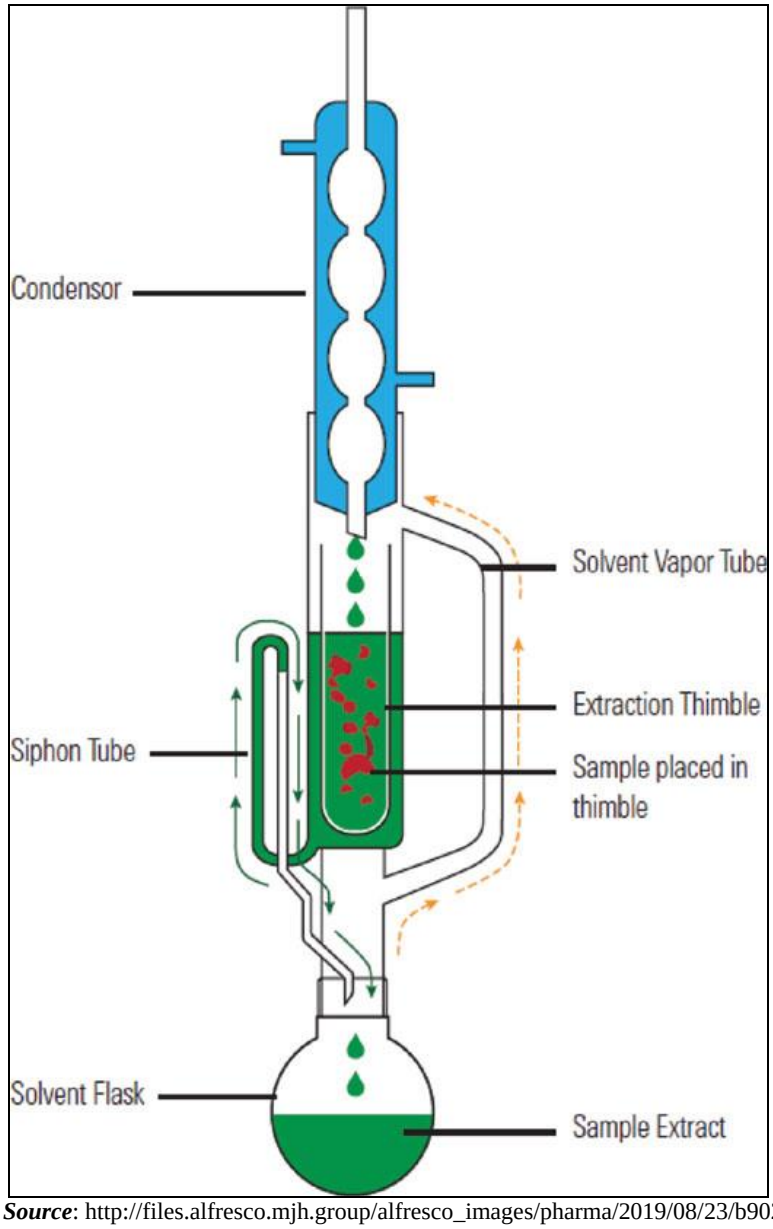
The solvents with low boiling points and evaporation temperatures are most frequently utilized to extract analytes from solid samples. The solvents must also be chemically neutral, non-toxic, and non-flammable. They must also not negatively affect the stability of the chemicals being analyzed or the instruments. Common solvents include methanol, ethanol, acetone, and ethyl acetate, as well as water-based combinations of these substances (Naczk M *et al*., 2006, Sahin S *et al*., 2011) [16, 29].

Insoluble bound complexes of phenols and phenolic acids also exist; these complexes are linked to cell wall polymers by ester and glycosidic linkages and cannot be extracted by organic solvents (Mattila P *et al*., 2007) [15], Plazonić A *et al*., 2009) [22]. Prior to extraction, bound phenolic acids are commonly released via base hydrolysis, acid hydrolysis, or both (Agnieszka Arceusz *et al*., 2013) [1].

Soxhlet extraction

The initial step in the procedure leading to the isolation of secondary metabolites from plant sources is extraction. Soxhlet extraction is a traditional technique used for this purpose. This process ensures that a plant material is digested several times and that extraction is ongoing while a fresh batch of solvent is being provided. This results in the complete extraction of a plant material by maintaining the biggest differential between the concentrations of analyte in the cell solution and in the solvent throughout. To extract phenolic acids from plant material, several extractions using a Soxhlet equipment were used (*Sambucus nigra* L., *Polygonum aviculare*). The digesting procedure took 15 hours, and the solvent used was methanol. Application of this modification of extraction in the case of wild common lilac allowed a high extraction yield of phydroxybenzoic, vanillic and ferulic acids (Waksmundzka-Hajnos M *et al*., 2007) [37]. Soxhlet extraction was also used for qualitative and quantitative analysis of phenolic compounds occurring in *Salvia halophila* and *S. virgata* (Akkol EK *et al*., 2008) [2].

The main disadvantages of Soxhlet extraction are that it is a time-consuming process and uses costly solvents, which must be of appropriate quality. For these reasons, in recent years other methods have been preferred that are less time-consuming and require smaller volumes of solvents (Rajaei A *et al*., 2010) [23].



Source: http://files.alfresco.mjh.group/alfresco_images/pharma/2019/08/23/b902cfb8-6438-41bb-b42e-38c70ab4b071/LCGC2_i3.jpg

Fig 2: Soxhlet extraction

Ultrasound-assisted extraction (UAE)

The principle behind ultrasound-assisted extraction (UAE) is that ultrasonic vibrations applied to an extracted material increase the effectiveness of a sample's ability to be penetrated by a solvent. This process is known for its high speed and simplicity, and it often takes several minutes. The effectiveness of the extraction process is influenced by elements such as particle size, sonification duration, and amplitude in addition to the type of solvent, sample size, extract pH, temperature, and pressure (Khan MK *et al*., 2010, Kagan IA. 2011) [12, 11]. Despite these issues, the approach is regarded as the easiest one that can be used in a lab (Casazza AA *et al*., 2010, Garcia-Salas P *et al*., 2010) [5, 9].

One of its benefits is the ability to extract multiple samples at once in a reasonably short period of time. However, the extract must be decanted or put through the proper paper filters. On the other hand, after extraction, an opaque solution could become stuck on an HPLC column. In actuality, plant cell walls are disrupted during the UAE process, allowing for the efficient extraction of metabolites present in the cells. In some experiment it has examined various ginger species, used this technique. Methanol was used as the solvent for the 30-minute UAE procedure. The method of extraction is not limited to ultrasound-assisted extraction (Pawar N *et al*., 2011) [19].

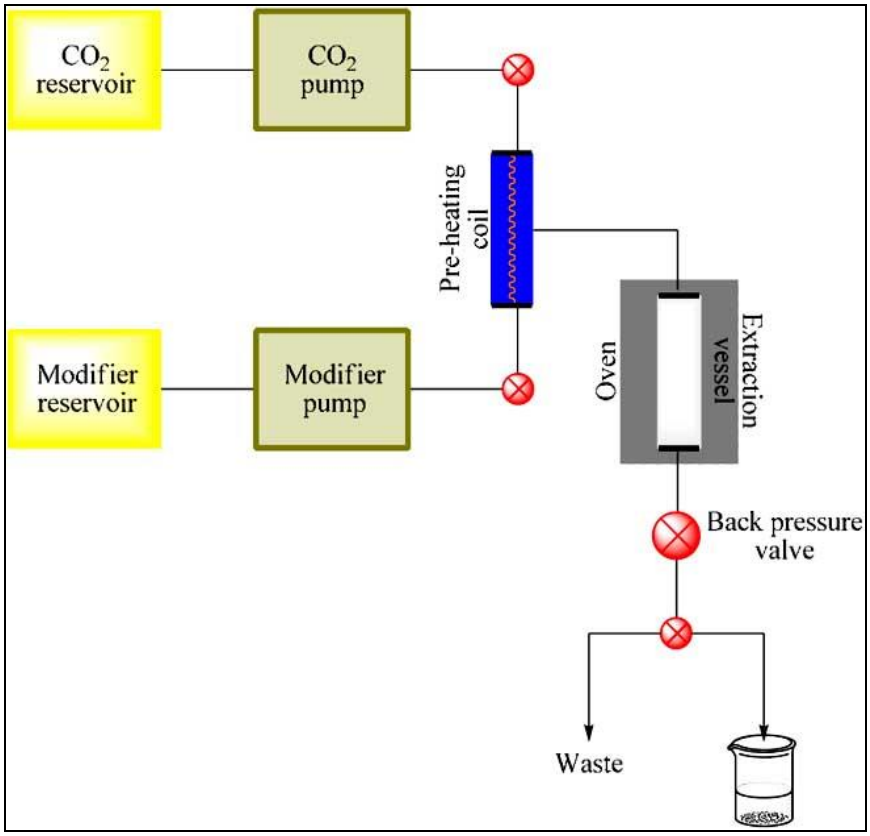
The yield increased with longer extraction times, but after 60 minutes, a decline in yield was seen, according to an extraction profile related to ultrasonication time. Aromatic plants, including Rosmarinus officinalis and Origanum majorana, were the subject of the analysis. Ultrasonication was carried out for 1 hour at 25° and 60 °C while traditional digestion took place in a water bath for 2 hours at 90 °C. The 60 °C temperature and the methanolic solvent seemed to be the most effective. In these circumstances, the greatest extraction yield was attained (Benetis *et al*., 2008) [4].

Supercritical fluid extraction (SFE)

This kind of extraction frequently comes before HPLC analysis. Modern techniques like supercritical fluid extraction have several benefits over traditional extraction techniques. Low temperatures are one of them, which is advantageous when analyzing substances that are thermally labile. High selectivity, large solvent volume reductions, low sample mass (a few mg), quick extraction times, automation potential, and off-line and on-line coupling with the majority of chromatographic techniques are further benefits (GC, HPLC). Due to its low cost and low toxicity, the solvent employed in SFE is often a fluid in a supercritical state, such as carbon dioxide. However, it is not the optimum solvent for phenolic compounds because of their polarity is low in comparison with that of phenolics (Pinelo M *et al*., 2007) [20].

Despite this, researchers isolated phenolic components from the seeds of Psidium guajava L., family Myrtaceae, using carbon dioxide. The effectiveness of supercritical fluid extraction is influenced by a variety of factors (Castro-Vargas *et al*. 2010) [6]. They fall into two categories. The first includes factors that affect how the extraction is carried out, such as pressure, temperature, time, sample weight, and flow rate. The second group includes variables related to a sample's matrix, including its shape, homogeneity, solubility, and analyte desorption capacity. In addition, the pH affects the SFE process. The analyte's extraction yield and speed from an aqueous phase might be affected by its fluctuations.

SFE is frequently utilised in the food, cosmetic, and pharmaceutical industries. Supercritical fluid extraction is used to remove physiologically active chemicals from plant matter, primarily those that can't be separated by a straightforward solvent extraction method (Agnieszka Arceusz *et al*., 2013) [1].



Source: https://www.researchgate.net/profile/Waseem-Gul-3/publication/233604150/figure/fig1/AS:341220330098690@1458364674640/Fig-1-Supercritical-fluid-extraction-SFE-diagram.png

Fig 3: Supercritical fluid extraction

Accelerated solvent extraction (ASE)

The pressurized fluid extraction (PFE) and pressurized liquid extraction are other names for the relatively recent technique known as the accelerated solvent extraction method, which was invented and is primarily supplied by Dionex (Dionex Corporation, Sunnyvale, CA) (PLE).

The same solvents used in conventional procedures are employed in the ASE approach, but a greater pressure (about 3.3-20.3 MPa) and higher temperature (around 40-200 °C) are used. A sample obtained using this method is put into a stainless-steel extraction vessel. The analysis takes only a few minutes-between five and fifteen. The ability to extract samples with high levels of humidity, a quick extraction time, improved sample penetration by the solvent, superior extraction kinetics, automation of the process (Agnieszka Arceusz *et al*., 2013) [1].

Shake extraction

In addition to the methods of sample extraction outlined above, extraction using various shaking devices has been done in order to analyse plant material. Shaking is used to increase the surface area where the solvent interacts with the plant material, hence speeding up and improving the efficiency of the entire process. For the examination of phenolics in *Phalaris canariensis* L., a sample is suspended in a specific volume of solvent and shaken at a predetermined stirrer speed (Li *et al*. 2011) [14].

Shaking extraction was also utilised in the analysis of the rosmarinic acid found in lemon balm leaves. Aqueous methanol solutions were used as solvents with methanol/water ratios of 40, 60, and 80 percent (v/v). Additionally, the rosmarinic acid in the leaves of lemon balm was also examined using the extraction by shaking method. With methanol/water ratios of 40, 60, and 80 percent (v/v), aqueous methanol solutions were employed as solvents. Additionally, the effects of temperature (25, 40, and 55 °C) and extraction time (30, 60, and 90 min) on the process were investigated Kim S *et al*., 2010) [13].

The procedure' ideal extraction conditions were achieved when it was run for 60 minutes using a 60:40 (v/v) methanol/water solution. One of the shaking methods is solid-liquid extraction carried out in a separation funnel, however extraction yields are less predictable since shaking speed and strength cannot be precisely controlled. The three extraction techniques that are most frequently used in gas chromatography (GC) analysis are solid-phase micro-extraction (SPME), headspace single-drop micro-extraction (HSDME), and microwave-assisted extraction (MAE). The last method is frequently employed to separate active ingredients from plant matter. The type and amount of the solvent, radiation potential, extraction duration, and process temperature are the factors that have the biggest effects on the efficacy of the method (Agnieszka Arceusz *et al*., 2013) [1].

Both the dry roots of Eucommia ulmodies, in which chlorogenic acid, among other things, was determined by using a methanol and water mixture and conducting the extraction at 40 °C, and the root of Salvia miltiorrhizae were extracted using the MAE technique in order to determine selected phenolic acids. To identify the phenolic components in the herbs Hypericum perforatum and Thymus vulgaris, a somewhat lower temperature of 30 °C was utilized. The extractant was HCl solution (Agnieszka Arceusz *et al*., 2013) [1].

Isolation of Phenols

Column Chromatography

To isolate chemicals or create simpler mixes, extracts obtained from plants and food can be further fractionated. Column chromatography has traditionally been used to perform fractionation. Commercially available chromatographic resins with a wide range of diverse separation methods, such as adsorption, size exclusion, ion exchange, etc., have all been employed to some degree to separate phenolic chemicals from plant extracts. Adsorption, which is primarily controlled by the formation of hydrogen bonds between phenolic proton donors and acceptor groups in the resin as well as by hydrophobic interactions with the aromatic rings of the phenolic compounds, is the basis for the majority of common column chromatography separations of phenolic compounds. As a result, it would be reasonable to assume that the affinity for the stationary phase would rise as the amount of hydroxyl phenolic groups and aromatic rings increased. However, it is difficult to anticipate the adsorption capacity since other elements, such as the resin's physical attributes (material, surface area, particle size, porosity), are also important (solvent, pH, temperature). The hydro/lipophilicity of the compounds is further altered by the inclusion of different substituents (such as methoxyl and carboxyl groups, sugars, or acylating moieties), which alters their retention properties. The features of the extracts and the kind of target chemicals must be considered when selecting the resin and separation parameters (eluents, flow, loading capacity, etc.). The extraction of phenolic acids and flavonoids from plant and food extracts has been primarily accomplished using Amberlite ®XAD-2, a nonionic hydrophobic polymeric adsorbent made of a cross-linked polystyrene copolymer resin. Polyamide adsorbents are also advised for the polarity-based separation of phenolic chemicals and have been the subject of numerous applications.

For the separation of flavan-3-ols (*i.e*., catechins and proanthocyanidins) on the basis of both size exclusion and adsorption mechanisms using elution by aqueous alcohol and/or aqueous acetone solvents, Sephadex LH-20 (a hydroxypropylated cross-linked dextran) and Fractogel (Toyopearl) HW-40 (a hydroxylated methacryl Although other structural factors, such as the kind of inter-flavonoid connections and the types of elementary units, also have an impact, proanthocyanidins elute in a pattern that is closely correlated to their molecular size. Additionally, these resins have been used to fractionate anthocyanin pigments.

Cyclodextrins have been used to separate phenolic chemicals more recently. Cyclodextrins are a kind of cyclic oligosaccharides having hydrophilic outer faces and a hydrophobic interior made of a-D-glucose linked by a-(1,4)-linkages. These structures have been employed for the selective separation of various phenolic groups, such as isoflavones, tea catechins or flavanones, and flavanone glycosides, by forming inclusion complexes with analytes of the right size and shape. Reversed-phase (RP) columns, which are typically C 8 or C 18 alkyl bonded phases, are the stationary phases that are most frequently utilised today for the separation of phenolic compounds, whether at analytical or preparative scales. When high pressures are used, chemicals separate more effectively, as seen in the following by HPLC equipment's.

The size of the particles in the stationary phase is the primary distinction between analytical HPLC, lower pressure column chromatography, and (semi)preparative HPLC. Resolution often increases with decreasing particle size, which is crucial for analytical HPLC. Smaller diameters, on the other hand, significantly raise pressure and prevent high flow rates. In (semi)preparative HPLC, it is typically necessary to use

particle sizes over 3 mm (typically 5-10 mm), although the particle size of lower pressure chromatographic columns is larger than 10 mm. When using preparative HPLC, column internal diameters typically range from 5 to 20 mm while the column length and sample concentration are constant.

Phenolic compounds include ionizable hydroxyl groups, thus it's crucial to utilize an acid modifier to inhibit ionization and avoid these groups' potentially damaging interactions with lingering metal ions in the stationary phase. As a result, maintaining a low pH level enhances the precision and repeatability of the retention properties while also preventing peak tailing. The pH range of 2-4 is advised for elution. Citrate, ammonium acetate, ammonium formate, and phosphate buffers at low pH have all been used in addition to aqueous acidified solvents such as acetic, formic, TFA, phosphoric, and very infrequently perchloric acid. The most popular additions used in flavonoid separation are acetic acid, formic acid, ammonium acetate, and ammonium formate.

Counter-Current Chromatography

As a potent instrument for the isolation of chemicals at the preparative and semi-preparative scale, CCC has emerged as a separation technology that competes favorably with column chromatography. The separation of chemicals in CCC, unlike other chromatographic methods, is based on their partitioning between two immiscible solvents, one of which is utilized as the "stationary phase" and another as the "mobile phase." This is an all-liquid chromatography method. The partitioning of the analytes between the two immiscible phases may also be aided by the addition of an auxiliary solvent that is miscible in both phases.

The two immiscible phases are vigorously mixed together by this force. According to definition, the less dense or lighter phase travels to the "head" end of the coil or column and the denser or heavier phase-often the aqueous phase-to the opposite end, or "tail." Simply flipping the head/tail designation around will shift the motion's direction. Whether a normal phase separation is necessary (*i.e.*, mobile phase: organic upper phase; stationary phase: aqueous lower phase) or reverse phase separation, which will depend on the distribution ratios of the target compound and whether they are required in an organic solvent for ease of drying down, will determine which phase is used as the mobile phase (Sutherland IA *et al*., 2009) [34].

The most recent hydrodynamic systems, like high-speed counter-current chromatography (HSCCC), first introduced by Ito, where a double-axis gyratory movement causes a variable gravity field to act on the column, are different CCC devices from the oldest hydrostatic systems, like DCCCs, that only operated by gravity to move the mobile phase through the stationary phase. While there are universal separation principles, there are important variations in the effectiveness and speed of separation. In a recent study, comprehensive information on the various pieces of equipment and their features may be obtained. The absence of solid supports prevents the analytes from adhering to the column, removing tailing of solute peaks and preventing sample loss owing to irreversible degradation with a solid stationary phase (Pauli GF *et al*., 2008, C. Santos-Buelga *et al*., 2012) [18].

In contrast to traditional chromatographic methods, where certain polyphenols have a propensity to become irreversibly adsorbed in the solid support matrix, this represents a distinct advantage. The ability to load and fractionate crude samples, the ability to easily scale up to larger fractionation systems by simply changing the tubing coil (column) to larger sizes, the capability of complete sample recovery by pumping out the liquid stationary phase from the coil system, and its low cost because it does not use pricey absorbents and columns are all additional benefits of this technique (Sutherland IA *et al*., 2009) [34].

Over the past two decades, a significant number of articles focusing on the fractionation and/or separation of phenolic chemicals, particularly flavonoids, using CCC have been published (Sutherland IA *et al*., 2009) [34]. In contrast to traditional chromatographic methods, where certain polyphenols have a propensity to become irreversibly adsorbed in the solid support matrix, this represents a distinct advantage. The ability to load and fractionate crude samples, the ability to easily scale up to larger fractionation systems by simply changing the tubing coil (column) to larger sizes, the capability of complete sample recovery by pumping out the liquid stationary phase from the coil system, and its low cost because it does not use pricey absorbents and columns are all additional benefits of this technique. Over the past two decades, a significant number of articles focusing on the fractionation and/or separation of phenolic chemicals, particularly flavonoids, using CCC have been published (Sutherland IA *et al*., 2009) [34].

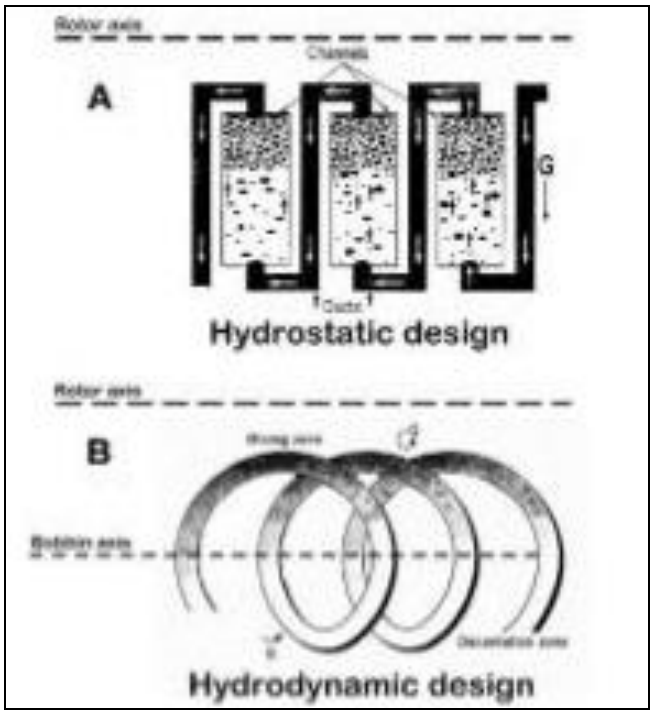


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Fig 4: Counter-Current Chromatography

Determination of phenols

Numerous chromatographic techniques have been used to separate, purify, and identify phenolic chemicals in plant materials [10]. Furthermore, chromatographic methods are often employed to investigate how phenolic chemicals interact with other food components.

High-performance liquid chromatography (HPLC)

The chemical make-up of the constituents, the extraction method, particle size, time, and storage conditions of the plant material before analysis, as well as the determination method and the presence of interfering agents, such as fats, terpenes, and chlorophyll, all affect the quantitative analysis of

phenolic compounds in plant material. High-performance liquid chromatography is the most frequently used chromatographic technique. This approach has been tried with various column types, mobile phases, column temperatures, and, to a lesser extent, mobile phase flow rates. The most frequent components of the mobile phase are water, methanol, and acetonitrile. Modifiers are occasionally required in order to make it easier to resolve the components. Formic acid, ammonium acetate, and acetic acid are the most widely used modifiers because they stop chromatogram tailing. As far as the length of the analysis is concerned, it is not a constant parameter because it can be altered depending on how fast the mobile phase is flowing. It is possible to use detection devices for the HPLC process, including chemiluminescence detectors (CL), coulometric electrode array systems (CEAD), UV-Vis spectrometers with either single wavelength or diode-array capacity, and mass spectrometers (MS) Robards K. *et al*., 2003, (Agnieszka Arceusz *et al*., 2013) [28, 1].

Waksmundzka-Hajnos *et al*. used reversed-phase, high-performance liquid chromatography with UVV is detection to analyze the *Sambucus nigra* L. inflorescence and the *Polygonum aviculare* L. foliage. Two mobile phases were employed in the application of isocratic elution. For S. nigra, the first was a 22:78 (v/v) mixture of methanol and orthophosphoric acid, while for *P. aviculare* L., a 25:75 (v/v) mixture of methanol and water with a 1-percent addition of acetic acid was utilised. At 520 nm, phenolic compounds were discovered (Agnieszka Arceusz *et al*., 2013) [1].

For the investigation of phenolics in numerous Lamiaceae plant species, Santos-Gomes *et al*. used HPLC. Acetonitrile, water, acetic acid, and methanol were used as solvents A and B, respectively, in the mobile phase. A flow rate of 0.8 mL/min was used with gradient elution. Based on a comparison of the compounds' retention periods with the standards, specific phenolic compounds were identified using an analytical wavelength of 280 nm (Agnieszka Arceusz *et al*., 2013) [1].

HPLC with UV-Vis detection was employed by Benetis *et al*. to analyse the phenolic components in *Achillea millefolium* L. To avoid ionisation of phenolic groups, gradient elution was used with an acetonitrile/water mobile phase and trifluoroacetic acid (TFA) as a modifier. Two different types of columns-Xterra RP18 (Waters) and Ascentis RP-Amide Supelco-were tested prior to the analysis, and the latter was picked. A 10 L sample was put into the column with a flow rate of 1.5 mL/min and detection at 360 nm to analyse the phenolic chemicals at 25 °C (Agnieszka Arceusz *et al*., 2013) [1].

The determination of phenolic acids in Echinacea purpurea, lemon balm leaves, aqueous extracts of Hypericum perforatum, and 32 medicinal plants growing in Poland also used liquid chromatography with UV detection. At 330 nm, the caffeic acid derivatives in the first instance were identified. Gallic, vanillic, and syringic acids were measured at 280 nm in the second case, caffeic, ferulic, and p-coumaric acids at 325 nm in the third example, and all phenolic acids at 210 nm (Agnieszka Arceusz *et al*., 2013) [1].

Additionally, HPLC combined with mass spectrometry (MS) is employed in some labs. In order to simultaneously determine six phenolic acids in rat plasma following intravenous administration of Guanxinning, a traditional Chinese medicine preparation made of lyophilized powder containing Salvia miltiorrhiza Bge, Guo *et al*. used the LC-MS approach. The caffeic acid in products from Echinacea sp., chlorogenic acid in lemon balm leaves, and caffeic and protocatechuic acids in sage roots were all detected using the same method. Additionally, 18 phenolic acids and mono- and diglycosidic flavonoids were identified and their structures established using LC-MS and NMR methods (Agnieszka Arceusz *et al*., 2013) [1].

Gas chromatography (GC)

One of the less used methods for determining phenolic acids is gas chromatography. Only a few small molecule phenolic acids (below 600 D) may be analysed using this method. Furthermore, the high temperatures needed for GC can cause sample degradation. Mass spectrometry coupled with GC (GC-MS) is frequently used to analyse phenolic acids in plant materials.

Fiamegos *et al*. used GC-MS to ascertain the amount of phenolic acids present in medicinal plant raw materials and their infusions. They employed GC-MS equipment with SIM (selective ion monitoring). Due to the method's modification, currents were only recorded for a small number of ions that had masses that were typical of the analysed analyte. The SIM technique involves at low concentrations in complex mixtures (Agnieszka Arceusz *et al*., 2013) [1].

In order to determine the phenolic acids in aromatic plants like nettle and common rue, Proestos *et al*. also used GC-MS. They employed N,O-bis(trimethylsilyl)-trifluoroacetamide and trimethylchlorosilane as silylating chemicals, capillary gas chromatography, silylation as a derivatization method, and silylation as a silylation process. In addition to HPLC with UV detection at 280 nm, they also used capillary gas chromatography (CGC-MS). By comparing the spectra of the compounds with those in the mass spectra library, the compounds in GC-MS are identified (Agnieszka Arceusz *et al*., 2013) [1].

Capillary electrophoresis (CE)

In the investigation of phenolic chemicals, capillary electrophoresis (CE) has recently grown in importance. This technique enables the isolation and identification of non-polar, non-ionic chemicals as well as polar substances with ionic and non-ionic character. Small sample weights, quick analysis times, high resolution potential, and less electrolyte volume are advantages of CE. The kind of buffer, its pH and concentration, the type of capillary and its volume, the temperature of electrophoresis, the voltage, and the manner of sample injection must all be optimized for this approach to produce the desired resolution. Each of these factors will be influenced by the CE approach, the chemical characteristics of the phenolic acid under study, and its matrix.

Extracts made from chamomile flowers were analysed using capillary electrochromatography (CEC). Fluorimetric, phosphorimetric, chemiluminescent, UV-Vis, IR, and Raman spectrometry's, as well as refractometry and electrochemical methods (conductometric, potentiometric, amperometric, and voltamperometric), as well as other techniques, like mass spectrometry and radiometry, are used as detection methods in capillary electrophoresis and its modifications. The detectors based on UV, MS, and amperometric techniques are the most widely utilized. High sensitivity and selectivity are guaranteed. For the study of phenolic acids in plant materials, CE with UV detection is frequently utilized, and a borate buffer with a pH of 9.2 is the most frequently used electrolyte (Agnieszka Arceusz *et al*., 2013) [1].

The phenolic acids in Strobili lupuli, Cortex fraxini, and the fruits of sea buckthorn berries were measured using this buffer. A MOPSO buffer solution, which is used less

commonly, was employed to measure the chlorogenic acid in Hypericum perforatum along with Tris solution and boric acid, pH 8.3. The caffeic and chlorogenic acids in Matricaria chamomilla L. were also determined using a phosphate buffer with acetonitrile at a pH of 2.8 (Agnieszka Arceusz *et al*., 2013) [1].

Capillary zone electrophoresis (CZE)

Apart from CE, another popular technique applied for the determination of phenolic compounds, is capillary zone electrophoresis (CZE). This method was used, among others, for the determination of rosmarinic acid in commercial sage tea-bags. Of the tested solvents, such as methanol, acetone and acetonitrile, the best was methanol. As the extraction technique, ultrasonication was used, and for detection, UV spectrometry at 210 nm. Resolution of the compounds was performed in a quartz capillary, 50 µm in diameter, and a solution of borate as a separating buffer was used. Beside CE and CZE, sometimes micellar electrokinetic chromatography (MEKC) is also used. By using this technique, phenolic acids were determined in the roots and pods of Echinacea purpurea [9], as well as in the herb of Artemisia capillaris (Agnieszka Arceusz *et al*., 2013) [1].

Capillary zone electrophoresis (CZE), in addition to CE, is a widely used method for determining phenolic chemicals. Rosmarinic acid in commercial sage tea bags was measured using this approach, among others. Methanol performed the best of the tested solvents, including acetone, acetonitrile, and acetone. Ultrasonication was employed as an extraction method, while UV spectroscopy at 210 nm was used as a detection method. A solution of borate was utilized as a separating buffer during the resolution of the compounds in a quartz capillary with a diameter of 50 m. Micellar electrokinetic chromatography (MEKC), in addition to CE and CZE, is occasionally utilized. Using this method, phenolic acids in Echinacea purpurea's roots and pods as well as in the plant Artemisia capillaries were identified (Agnieszka Arceusz *et al*., 2013) [1].

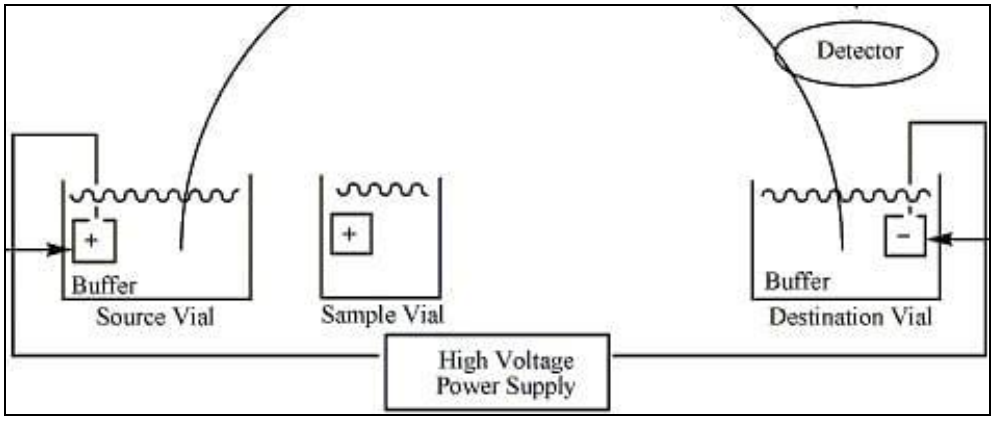

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Fig 5: Capillary zone electrophoresis

Benefits

Plant Phenolics and Cancer

Environmental, chemical, physical, metabolic, and genetic factors all play a direct or indirect impact in the development and progression of tumours. Cancer is a multi-stage disease. Strong and reliable epidemiological data show that eating a diet high in antioxidant-rich fruits and vegetables considerably lowers the chance of developing various cancers, suggesting that certain dietary antioxidants may be useful tools for reducing the incidence and mortality of cancer. Due to their safety, low toxicity, and widespread acceptability, these agents found in the food represent a very promising class of substances (Fresco, P *et al*., 2006) [8]. As a result, the discovery and development of such drugs has grown significantly in importance during the past few years in experimental cancer research. One of the most common and widespread groups of plant metabolites, phenolic chemicals are an essential component of the human diet. It was discovered that this group of chemicals exhibits a wide range of biological actions, many of which are primarily connected to modulation of carcinogenesis, in addition to their core antioxidant activity. These naturally occurring phenolic compounds or extracts have had their anticarcinogenic and anticancer properties evaluated using a variety of *in vitro* and *in vivo* techniques (Jin Dai *et al*., 2010).

Antioxidant Properties

Antioxidants are substances that, by scavenging free radicals and reducing oxidative stress, can delay, inhibit, or prevent the oxidation of oxidizable materials. When endogenous antioxidant capacity is outstripped by excessive levels of reactive oxygen and/or nitrogen species (ROS/RNS, such as superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxynitrite), oxidative stress results. This causes a variety of biomacromolecules, including enzymes, proteins, DNA, and lipids, to oxidise. Chronic degenerative disorders like cancer, coronary heart disease, and ageing are all influenced by oxidative stress (Ames *et al*., 1993) [3]. Recently, phenolics have been recognised as effective antioxidants *in vitro*, outperforming vitamins C and E and carotenoids. (Rice-Evans *et al*., 1995, Rice-Evans *et al*., 1996) [26, 27].

Phenolics have been largely blamed for the negative correlation between fruit and vegetable consumption and the risk of oxidative stress-related disorders including cancer, cardiovascular disease, or osteoporosis. Following pathways have been postulated as potential mediators of the antioxidant effects of phenolic compounds:

1) Neutralising reactive oxygen and nitrogen species (ROS/RNS);
2) preventing the development of ROS/RNS by blocking certain enzymes or chelating trace metals involved in free radical production; and
3) Enhancing or protecting antioxidant defence. (Jin Dai *et al*., 2010).

Conclusion

Summary: In addition to the subjects we have discussed, this review has focused on a small number of plant secondary

metabolites and their potential roles in defense mechanisms and ecological adaptability. The plant kingdom contains a huge variety of additional chemicals with a very variable distribution. Products from plant secondary metabolism help plants grow and develop, but they are not necessary for the plant to survive. Secondary metabolites play crucial ecological roles in plants by defending them against herbivore predation and microbial pathogen infection. They operate as odor, color, and taste attractants for pollinators and seed-dispersing animals. They serve as facilitators of plant-plant rivalry and plant-microbe symbioses. The ecological roles of plants' secondary metabolites, then, have a significant impact on their capacity to compete and survive. The generation of secondary metabolites by genetic engineering also uses biotechnological methods. A significant part for the same may also be played by plant tissue culture. Additionally, natural phenolics have been shown to interfere with cancer formation at all stages. The prevention of cancer growth by phenolic compounds depends on a variety of fundamental cellular mechanisms, involving a range of cellular basic machinery, in addition to their antioxidant action. Additionally, in-depth research on this class of chemicals will shed light on potential therapeutic applications in the fields of medicine and the life sciences.

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