Year	Sem.	Subject Code	Title of the paper	Hours/ Week
2018 -2019 onwards	II	18MBO23C	PAPER –VI- PHYTOCHEMISTRY	7

Unit V

Plant Secondary metabolites: Brief outline of shikimate, acetate and mevolonate pathway of secondary metabolites; Structure, classification and biological significance of alkaloids, terpenoides and polyphenolic compounds; Extraction, isolation and identification of alkaloids and terpenoids

Prepared by
Dr. M M Sudheer Mohammed
Associate Professor of otany
Mobile-9443274469

2 Secondary metabolism in plants

2.1 Metabolism and its significance

Metabolism in living matter may be described as a large group of enzyme-controlled and regulated chemical reactions that produce energy in the form of ATP, produce substances needed for growth and the development of tissues, and help the organism to survive in different circumstances. Depending on the biosynthetic origin, general occurrence and biochemical role of the compounds produced in metabolism, they are called either primary or secondary metabolites.

Primary metabolic routes produce primary metabolites, which are present almost everywhere in nature and are essential for all life forms. These compounds include the common carbohydrates, fats, proteins and nucleic acids that are needed to create and maintain life. Apart from fats, the compounds are polymeric and usually chemically large molecules. Typically they are involved in the energy regulation of organisms and with growth and development of tissues; in short, they are the building blocks of organisms.

The secondary metabolites are of more limited occurrence. While some do not have a proven function in the organism, others apparently play a vital role. Some secondary metabolites of plants are found only in certain species of families, showing the individuality of species. Their production may also vary with conditions: the environment of the organism or the available nutrition. They may have a role in providing defense against pest and pathogens, providing protection against UV radiation and stress, or acting as attractive volatile odor compounds or pigments [1-3]. Reactions involving secondary metabolites produce an enormous selection of compounds, some of them substances that may be pharmacologically active in humans and useful as medicines or food additives. Some famous examples (Fig 1) of secondary plant metabolites with pharmacological activities are the analgesic and antipyretic compound salicin (1) isolated from willow (*Salix* species) and used as a template for the synthesis of acetylsalisylic acid (aspirin), the anticancer drug taxol (paclitaxel, 2) isolated from Pacific yew (*Taxus brevifolia*) and the strongly analgesic, narcotic, and addictive compound morphine (3) isolated from opium (*Papaver somniferum*).

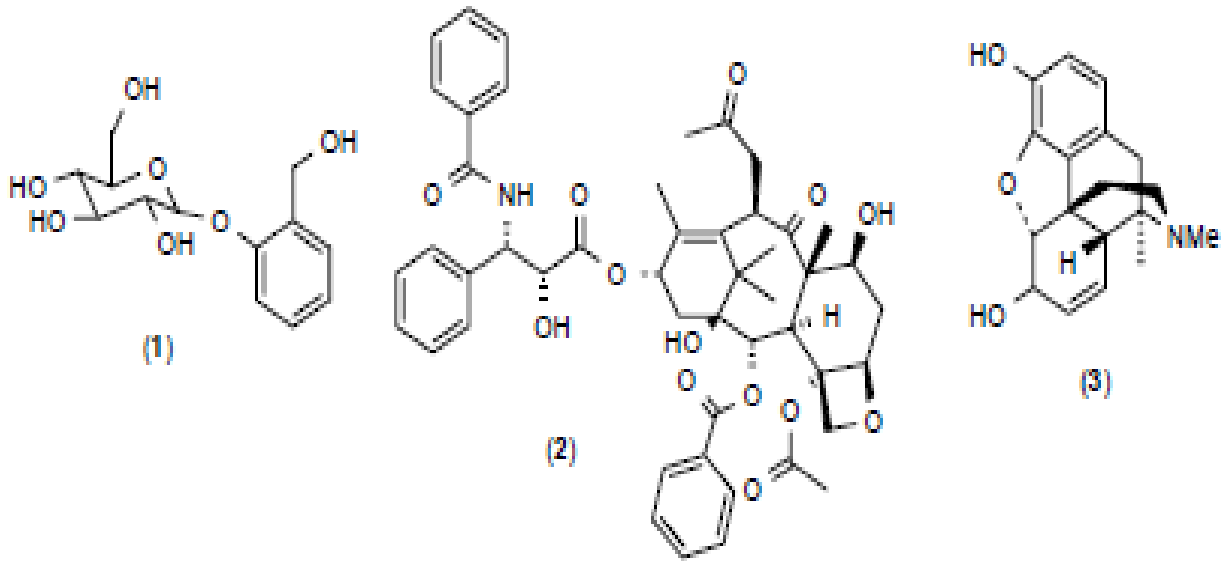


Fig. 1. Famous examples of secondary metabolites with pharmacological activity: (1) salicin, (2) taxol (paclitaxel), (3) morphine.

2.2 Major metabolic pathways

The pathways for modifying and synthesizing carbohydrates, proteins, fats and nucleic acids are the same in all organisms, apart from minor variations. Also, the biosynthesis of most secondary metabolites begins from a relatively small group of compounds, which are modified into an unlimited number of compounds through various synthesis pathways. The most important of these metabolic pathways are those with acetyl coenzyme A (acetyl-CoA), shikimic acid, or mevalonic acid intermediates, and known as the acetate, shikimate, and mevalonate pathways, respectively. These key intermediates are formed from products of the glycolytic pathway or their intermediates (Fig 2). These major metabolic pathways are briefly described below [1,2,7]. In addition to these key intermediates, a wide variety of antibiotics, peptides, proteins, and alkaloids are formed from amino acids derived from acetyl-CoA via citric acid (Krebs) cycle [1, 8], or via the shikimate pathway.

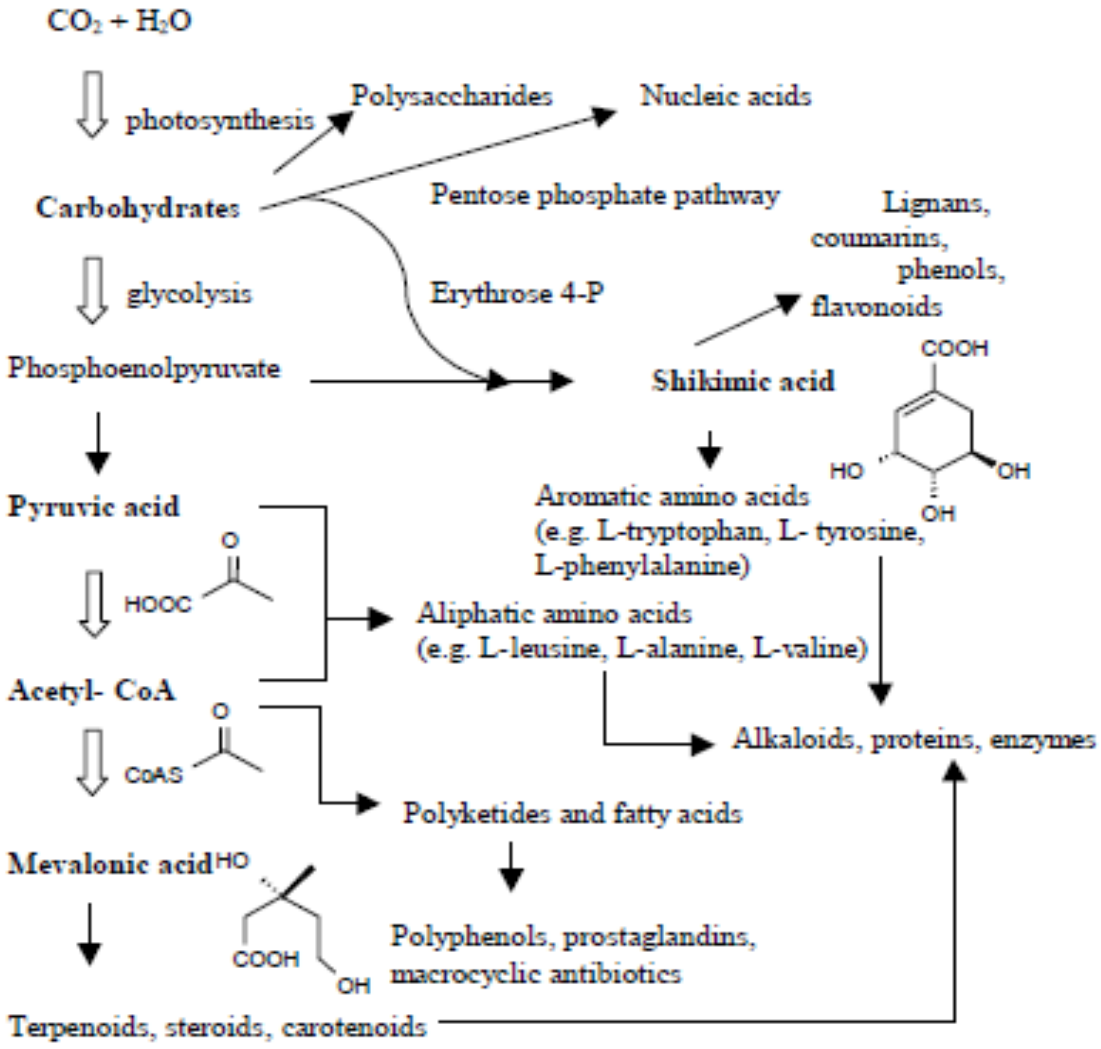


Fig. 2. Common biosynthetic pathways to secondary metabolites via acetyl-CoA, shikimic acid, mevalonic acid and amino acids.

ACETATE PATHWAY

The acetate pathway produces a very large number of acetyl-CoA based compounds, sometimes classified as polyketides. Included as phenols such as phloroglucinols and anthraquinones, fatty acids, prostaglandins, thromboxanes, leukotrienes, and macrolide antibiotics. Polyketide structures are derived from poly-β-ketoester chains that are formed from acetyl-CoA units via malonyl-CoA in condensation reactions, as shown in Figure 4. The polymerization of polyketide chains then either continues further to produce long and highly reactive poly-β-keto chains that form a wide variety of aromatic compounds, or alternatively the carbonyl groups are reduced before reaction with the next malonyl group, and reaction leads to fatty acids and macrolide antibiotics. This

biosynthesis pathway via polyketide formation leads to the usually easily recognizable meta-substitution pattern in phenols and aromatic compounds.

2.2.3 Shikimate pathway

The shikimate pathway begins with a combination of the glycolytic pathway intermediate phosphoenolpyruvate (PEP) and the pentose phosphate pathway product erythrose-4-phosphate, which leads, after a few reaction steps, to 3-dehydroquinic acid and then through various reaction routes to shikimic acid and eventually to a number of different metabolites, including phenols, cinnamic acid derivatives, flavonoids, coumarins, lignans, and alkaloids (Fig 5). In contrast to products of the acetate pathway, the substitution pattern found in phenols of the shikimate pathway is characteristically ortho or para. The C_6C_3 phenylpropane units present in many metabolites originate from phenylalanine and tyrosine structures. Flavonoids are produced through a combination of products of the shikimate and the acetate pathways, where three malonyl-CoA units are added to a shikimate pathway intermediate such as cinnamoyl-CoA to form a polyketide (as in the acetate pathway). The polyketide is then folded to form a chalcone structure that is transformed further to flavonoid.

2.2.4 Mevalonate pathway

Mevalonic acid is formed from three molecules of acetyl Co-A, but it leads the metabolism to different compounds from those of the acetate pathway. The mevalonic acid is transformed into five-carbon-containing C_5 isoprene units, which in the form of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) combine to form a large number of terpenoid and steroid structures (Fig 6).

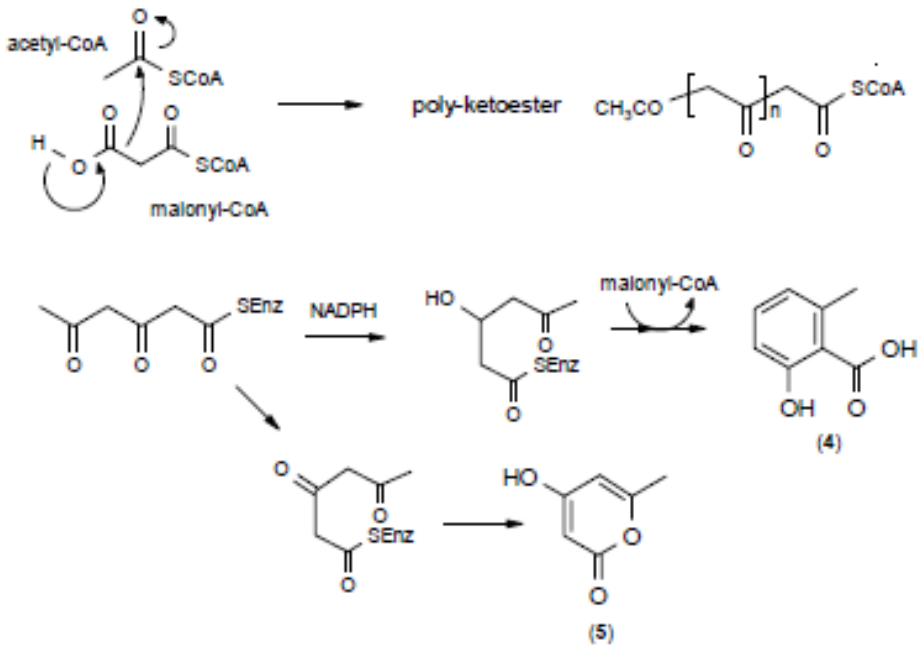


Fig. 4. Formation of polyketides from acetyl-CoA. (4) 6-methylsalicylic acid, (5) triacetic acid lactone.

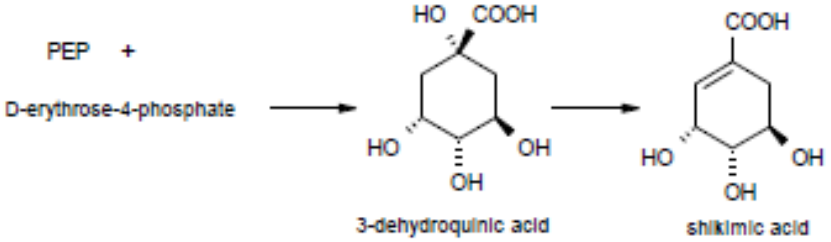


Fig. 5. Origin of the shikimate pathway.

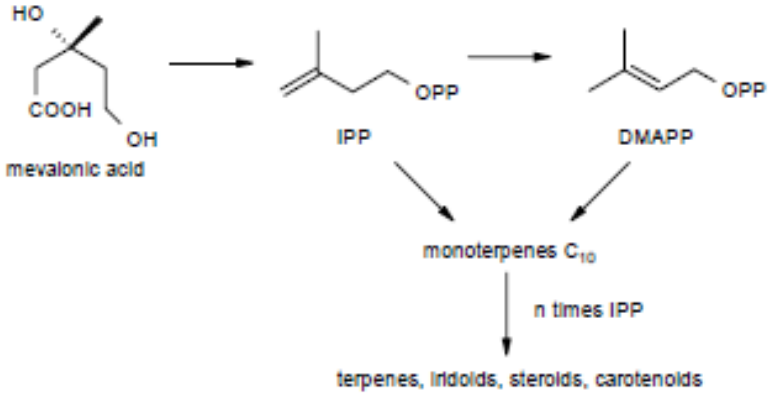
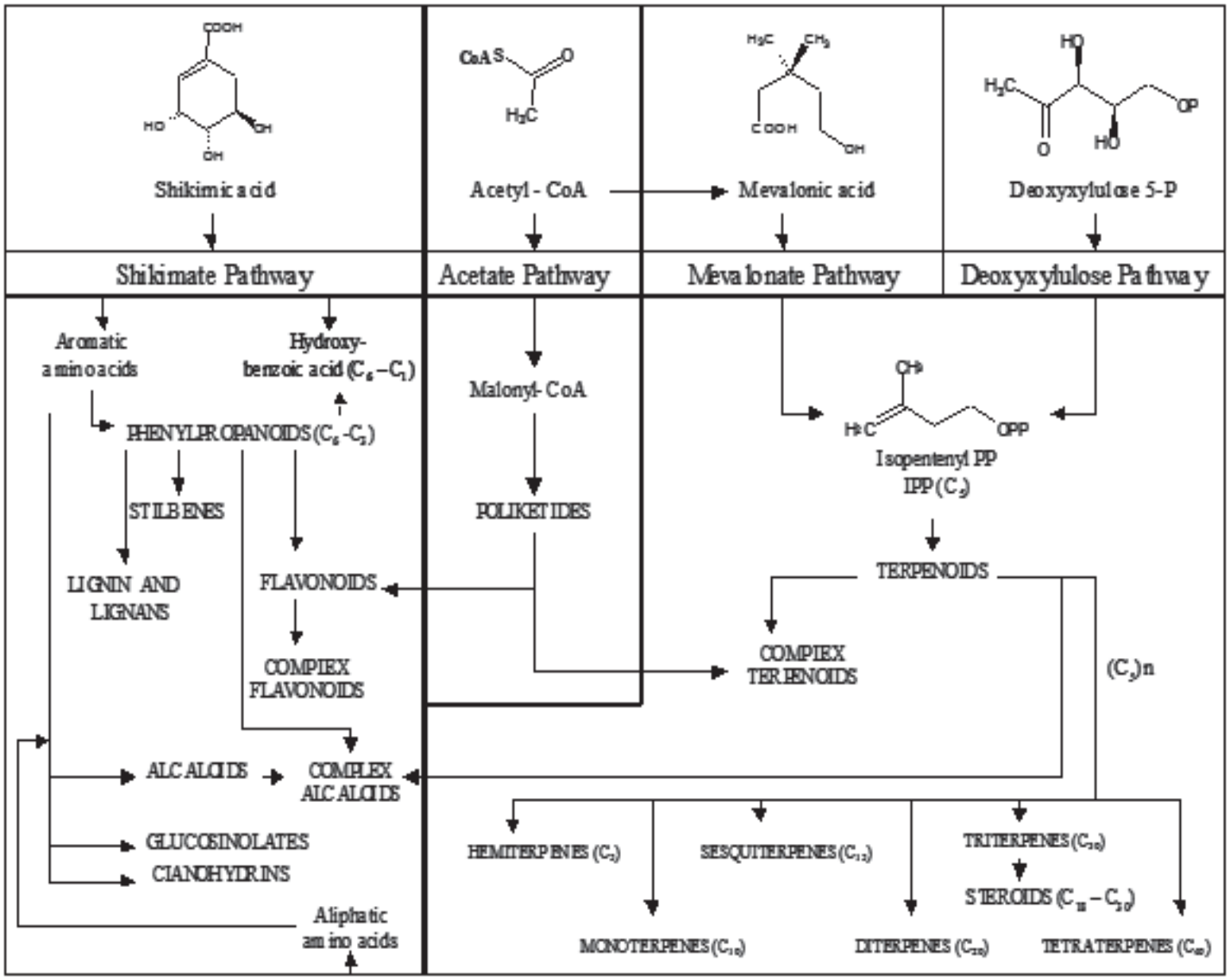


Fig. 6. Formation of terpenoids from mevalonic acid.


Shikimic acid
Acetyl - CoA
Mevalonic acid
Deoxyxylulose 5-P
Shikimate Pathway
Acetate Pathway
Mevalonate Pathway
Deoxyxylulose Pathway
Aromatic amino acids
Hydroxy-benzoic acid (C6 –C1)
PHENYLPROPANOIDS (C6 -C3)
STILBENES
LIGNIN AND LIGNANS
FLAVONOIDS
COMPLEX FLAVONOIDS
Malonyl- CoA
POLIKETIDES
Isopentenyl PP IPP (C5)
TERPENOIDS
COMPLEX TERPENOIDS
(C5)n
ALCALOIDS
COMPLEX ALCALOIDS
GLUCOSINOLATES
CIANOHYDRINS
Aliphatic amino acids
HEMITERPENES (C5)
SESQUITERPENES (C15)
TRITERPENES (C30)
STEROIDS (C28 – C29)
MONOTERPENES (C10)
DITERPENES (C20)
TETRATERPENES (C40)

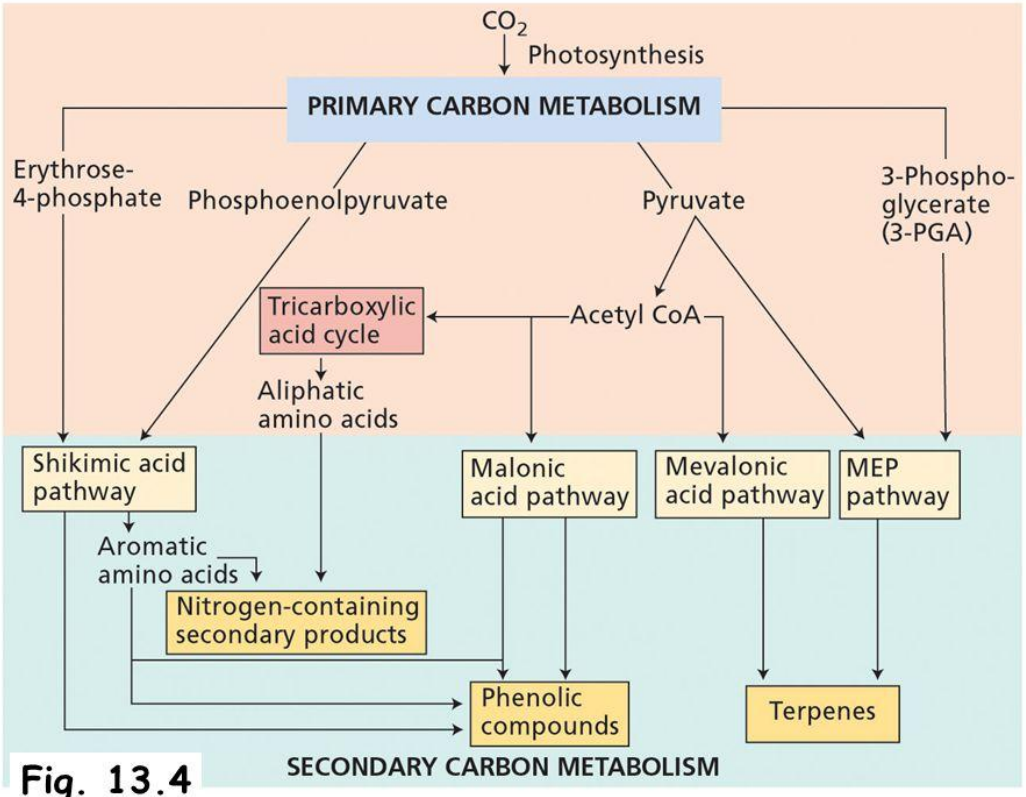
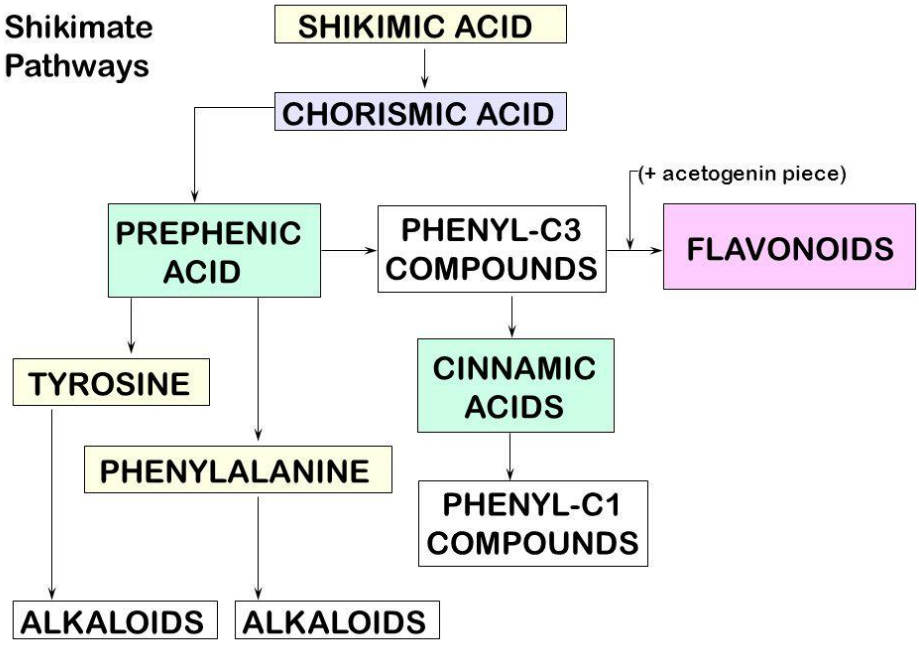


Fig. 13.4

PLANT PHYSIOLOGY, Third Edition, Figure 13.4 © 2002 Sinauer Associates, Inc.



Shikimic Acid Pathway in Biosynthesis of Phenolic Compounds in Higher Plants

The shikimate pathway consists of seven reaction steps, beginning with an aldol-type condensation of phosphoenolpyruvic acid (PEP) from the glycolytic pathway, and D-erythrose-4-phosphate, from the pentose phosphate cycle, to produce 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP). A key branch-point compound is chorismic acid, the final product of the shikimate pathway. The shikimate pathway is described in this chapter, as well as factors that induce the synthesis of phenolic compounds in plants. Some representative examples that show the effect of biotic and abiotic stress on the production of phenolic com-pounds in plants are discussed.

The secondary metabolism is a biosynthetic source of several interesting compounds useful to chemical, food, agronomic, cosmetics, and pharmaceutical industries. The secondary pathways are not necessary for the survival of individual cells but benefit the plant as a whole [1]. Another general characteristic of second-ary metabolism is that found in a specific organism, or groups of organisms, and is an expression of the individuality of species [2]. The secondary metabolism provides chemical diversity to organic molecules with low molecular weight that are related by the respective pathways; such organic molecules are called secondary metabolites. The secondary metabolites are often less than 1% of the total carbon in plant molecules [3]. These organic molecules isolated from terrestrial plants are the most studied, and their syntheses have an important role in the protection against pathogens, unfavorable temperature and pH, saline stress,

heavy metal stress, and UVB and UVA radiation [3]. Secondary metabolism reflects plant environments more closely than primary metabolism [4]. There are three principal kinds of secondary metabolites biosynthesized by plants: phenolic compounds, terpenoids/isoprenoids, and alkaloids and glucosinolates (nitrogen- or sulfur-containing molecules, respectively) [5]. Phenolic compounds are biosynthesized by the shikimate pathway and are abundant in plants. The shikimate pathway, in plants, is localized in the chloroplast. These aromatic molecules have important roles, as pigments, antioxidants, signaling agents, electron transport, communication, the structural element lignan, and as a defense mechanism [6], **Figure 1**. The seven steps of the shikimate pathway and the metabolites for branch point are described in this chapter, as factors that induce the synthesis of phenolic compounds in plants. Some representative examples that show the effect of biotic and abiotic stress on the production of phenolic compounds in plants are discussed.

The shikimate pathway

The shikimate biosynthesis pathway provides precursors for aromatic molecules in bacteria, fungi, apicomplexan, and plants, but not in animals [2, 7]. Shikimic acid is named after the highly toxic Japanese *shikimi* (*Illicium anisatum*) flower from which it was first isolated [8]. This biochemical pathway is a major link between primary and secondary metabolism in higher plants [6]. In microorganisms, the shi-kimate pathway produces aromatic amino acids L-phenylalanine (L-Phe), L-tyrosine (L-Tyr), and L-tryptophan (L-Trp), molecular building blocks for protein biosyn-thesis [9]. But in plants, these aromatic amino acids are not only crucial components of protein biosynthesis; they also serve as precursors for diverse secondary metabo-lites that are important for plant growth [10]. These secondary metabolites are called

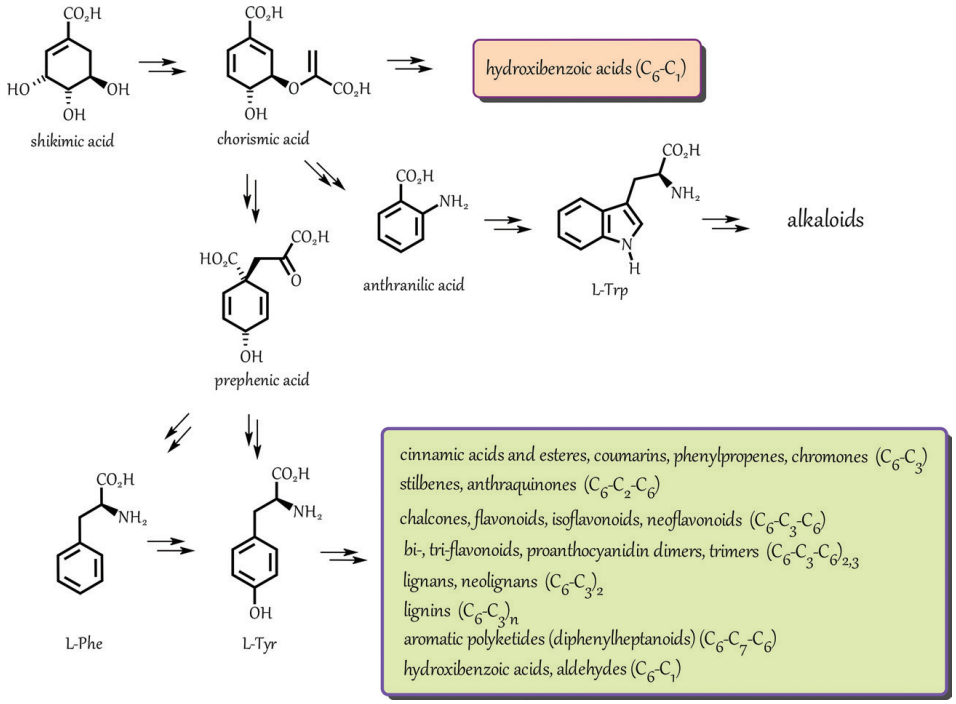


Figure 2.
The shikimic and chorismic acids are the common precursors for the synthesis of L-Phe, L-Tyr, and L-Trp and diverse phenolic compounds.

phenolic compounds and are synthesized when needed by the plant [11]. These molecules play an important role in the adaptation of plants to their ecosystem, and their study advances biochemical techniques and molecular biology [3, Bourgaud]. The principal aromatic phenolic compounds synthesized from L-Phe and L-Tyr are cinnamic acids and esters, coumarins, phenylpropenes, chromones (C_6-C_3), stilbenes, anthraquinones (C_6-C_2-C_6), chalcones, flavonoids, isoflavonoids, neoflavonoids (C_6-C_3-C_6), and their dimers and trimers, respectively (C_6-C_3-C_6)$_{2,3}$, lignans, neolignans (C_6-C_3)$_2$, lignans (C_6-C_3)$_n$, aromatic polyketides, or diphenylheptanoids (C_6-C_7-C_6) [12]. L-Trp is a precursor of alkaloids in the secondary metabolism [2]. Additionally, diverse hydroxybenzoic acids and aromatic aldehydes (C_6-C_1) are biosynthesized via branch points in the shikimate pathway, **Figure 2**. Phenolic compounds biosynthesized from the shikimate pathway have structural versatility.

The shikimate pathway consists of seven sequential enzymatic steps and begins with an aldol-type condensation of two phosphorylated active compounds, the phosphoenolpyruvic acid (PEP), from the glycolytic pathway, and the carbohydrate D-erythrose-4-phosphate, from the pentose phosphate cycle, to give 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP), **Figure 3**. The seven enzymes that catalyze the pathway are known: 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHPS; EC 4.1.2.15, now EC 2.5.1.54), 3-dehydroquinatesynthase (DHQS; EC 4.2.3.4), 3-dehydroquinate dehydratase/shikimate dehydrogenase (DHQ/SDH; EC 4.2.1.10/EC 1.1.1.25), shikimate kinase (SK; EC 2.7.1.71), 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS; EC 2.5.1.19), and chorismate synthase (CS; EC 4.2.3.5) [13], **Table 1**.

The shikimate pathway has special characteristics that are present only in bacteria, fungi, and plants. The absence of the pathway in all other organisms provides the enzymes catalyzing these reactions with potentially useful targets for the development of antibacterial agents and herbicides. For example, 5-*enol*pyruvylshikimate 3-phosphate synthase (EPSP-synthase) catalyzes the transfer of the enolpyruvyl (carboxyvinyl) moiety from PEP to shikimic acid 3-phosphate (S3P) [6].

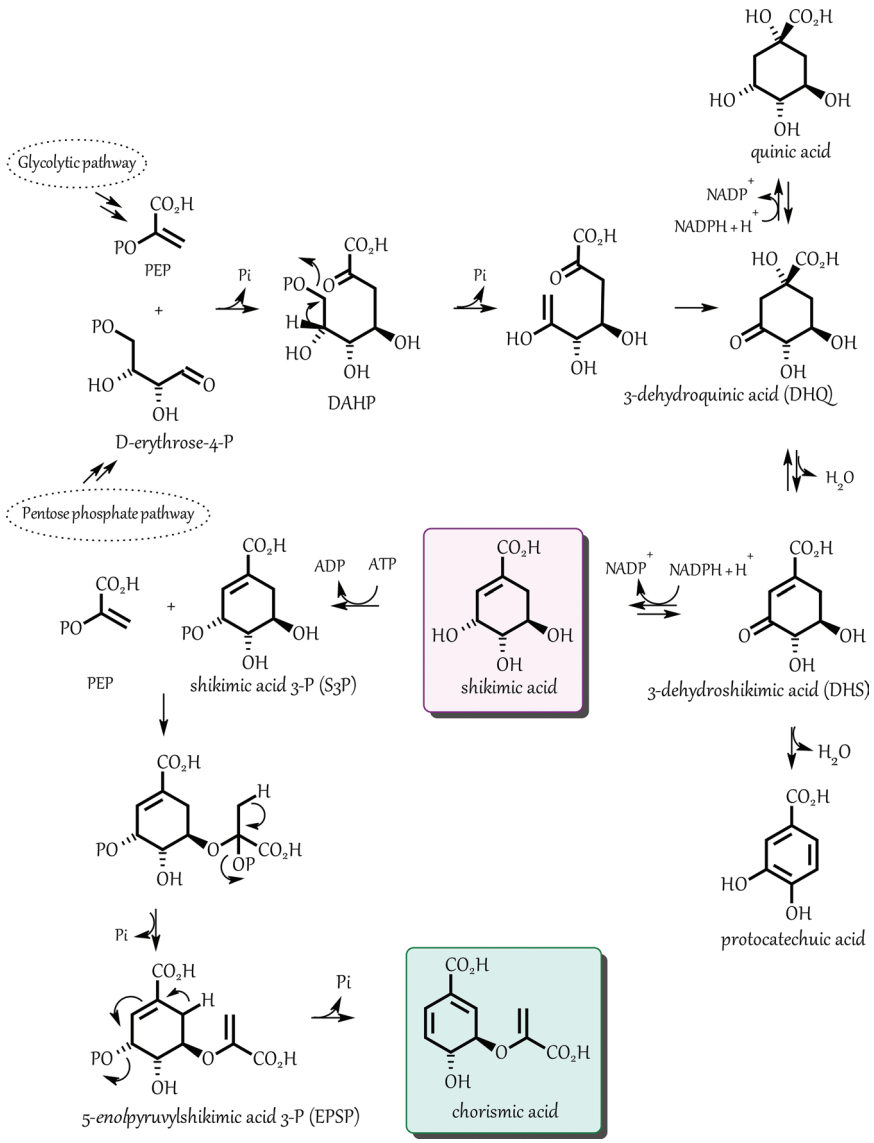


Figure 3.
Shikimate pathway.

In the second reaction step, DAHP loses phosphate (Pi); the enolic-type product is cyclized through a second aldol-type reaction to produce 3-dehydroquinic acid (DHQ). The 3-dehydroquinate synthase (DHQS) catalyzes this cyclization in the shikimate pathway. The DHQ dehydrates to produce 3-dehydroshikimic acid (DHS) (3-dehydroquinate dehydratase); this compound has a conjugated double carbon-carbon, **Figure 3**. The protocatechuic and the gallic acids (C_6-C_1) are produced by branch-point reactions from DHS [2]. The fourth step in the pathway is a reduction reaction of DHS with reduced nicotinamide adenine dinucleotide phosphate (NADPH), **Figure 3**. The fifth section of the pathway is the activation of shikimic acid with adenosine triphosphate (ATP) (shikimate kinase, SK) to make shikimic acid 3-phosphate (S3P). The sixth chemical reaction is the addition of PEP to S3P to generate 5-*enol*pyruvylshikimic acid 3-phosphate; the enzyme that catalyzes this reaction step, 5-*enol*pyruvylshikimate 3-phosphate synthase (EPSPS), has been extensively studied. The reason for this interest is because glyphosate [*N*-(phosphonomethyl)

Reaction step	Substrate	Enzyme/cofactor	Product
1	Phosphoenolpyruvate (PEP), erythrose-4-phosphate	3-Deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (DAHPS; EC 4.1.2.15, now EC 2.5.1.54)/ Co^{2+}, Mg^{2+} or Mn^{2+} [15]	3-Deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP), Pi
2	3-Deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP)	3-Dehydroquinate synthase DHQS (EC. 4.2.3.4)/Co^{2+}, NAD^+ [15, 16]	3-Dehydroquinic acid (DHQ), Pi
3	3-Dehydroquinic acid (DHQ)	3-Dehydroquinate dehydratase (DHQ dehydratase EC 4.2.1.10) [15]	3-Dehydroshikimic acid (DHS), H_2O
4	3-Dehydroshikimic acid (DHS), NADPH + H^+	Shikimate dehydrogenase (SDH; EC 1.1.1.25) [18–21]	Shikimic acid, $NADP^+$
5	Shikimic acid, ATP	Shikimate kinase enzyme (SK; EC 2.7.1.71)	Shikimic acid 3-phosphate (S3P), ADP
6	Shikimic acid 3-phosphate (S3P), PEP	5-*Enol*pyruvylshikimate 3-phosphate synthase, also called aroA enzyme (EPSPS; EC 2.5.1.19) [25]	5-*Enol*pyruvylshikimate 3-phosphate (EPSP), Pi
7	5-*Enol*pyruvylshikimate 3-phosphate (EPSP)	Chorismate synthase (CS; EC 4.2.3.5)/$FMNH_2$ [2, 19, 30, 31]	Chorismic acid, Pi

Pi, phosphate; NAD^+, oxidized nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; $FMNH_2$, reduced flavin mononucleotide.

Table 1.
Substrates, enzymes, and products of the shikimate pathway.

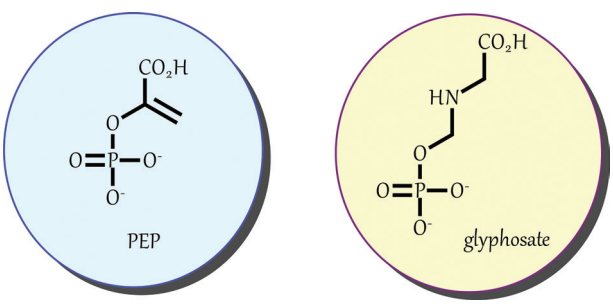


Figure 4.
PEP and glyphosate (powerful inhibitor of the 5-enolpyruvylshikimate 3-phosphate synthase, EPSPS).

glycine] is a powerful inhibitor of EPSPS [2], so glyphosate has been used as a broad-spectrum systemic herbicide. It is an organophosphorus molecule, phosphonic acid, and glycine derivative that has a similar molecular structure to PEP, **Figure 4**.

The last reaction step of the shikimate pathway is the production of chorismic acid from catalytic action on the chorismate synthase (CS). This reaction is a 1, 4-*trans* elimination of Pi, to yield the conjugated molecule, chorismic acid, **Figure 3**.

2.1 Synthesis of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP)

The first reaction of the shikimate pathway is an aldol-type condensation of PEP and carbohydrate erythrose-4-P, to give 3-deoxy-D-*arabino*-heptulosonic acid

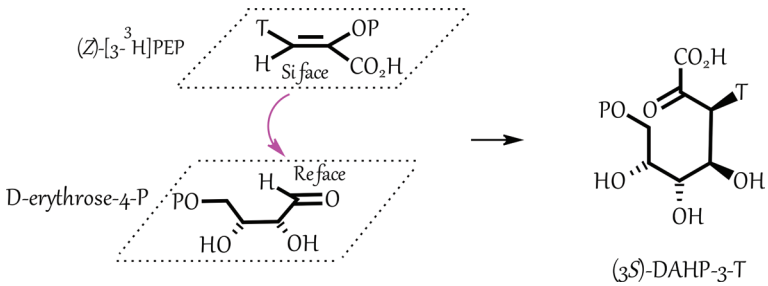


Figure 5.
Stereochemistry of the condensation reaction of (Z)-[3-^{3}H]PEP and D-erythrose-4-phosphate by DAHP synthase [14].

7-phosphate (DAHP), **Figures 3** and **5**. A new stereogenic center is generated in the condensation product DAHP catalyzed by the 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase enzyme (DAHPS; EC 4.1.2.15, now EC 2.5.1.54). Results of enzymatic kinetic and labeled PEP with tritium (*Z*)-[3-^{3}H] PEP suggest that the nucleophilic attack of PEP is from the *Si* face of PEP to the *Re* face of the carbonyl group of D-erythrose-4-P, **Figure 5** [14]. Two isoenzymes of DAHPS have been found for the catalysis of this first reaction step. One isozyme needs only Mn^{2+}, and the other, either Co^{2+}, Mg^{2+}, or Mn^{2+} for the catalysis [15].

2.2 Synthesis of 3-dehydroquinic acid (DHQ)

The second reaction of the shikimate pathway is an intramolecular aldol-type reaction cyclization, where the enol (C6-C7) of DAHP nucleophilically attacks the carbonyl group (C2), to produce a six-member cycle, the 3-dehydroquinic acid (DHQ), **Figures 3** and 6. The enzyme that catalyzes this reaction, 3-dehydroquinate synthase DHQS (EC. 4.2.3.4), is a carbon-oxygen lyase enzyme that requires Co^{2+} and bound oxidized nicotinamide adenine dinucleotide (NAD^+) as cofactors [15, 16]. The Co^{2+} is essential for the catalytic activity of DHQS. Bender et al. [16] found that DHQS, from *Escherichia coli*, is a monomeric metalloenzyme that contains tightly bound Co^{2+}, and DHQS is deactivated with ethylenediaminetetraacetic acid (EDTA). The presence of the substrate (DAHP) blocks the inactivation by EDTA. The NAD^+ cofactor dissociates form the DHQS enzyme rapidly in the presence of DAHP [16]. The reaction mechanism of the enzyme-catalyzed conversion of DAHP to DHQ involves five transformations from the DAHP hemiketal form, a pyranose: (1) oxidation of the hydroxyl at C5 adjacent to the lost proton that requires NAD^+ (NAD^+ need never dissociate from the active site), (2) the elimination of Pi of C7 to make the α,β-unsaturated ketone, (3) the reduction of C5 with NADH + H^+, (4) the ring opening of the enol to yield an enolate, and (5) the intramolecular aldol-like reaction to produce DHQ. All five-reaction steps occur through the function of DHQS, **Figure 6**.

The reduction reaction of DHQ leads to quinic acid at this branch point in the shikimate pathway. Quinic acid is a secondary metabolite that is free, forming esters or as part of alkaloids such as quinine. Quinic acid is found in high quantities in mature kiwi fruit (*Actinidia chinensis* and other species of *Actinidia*) and is a distinguishing characteristic of fresh kiwi fruit [7]. Also, the quinic acid is abundant in roasted coffee [17].

2.3 Synthesis of 3-dehydroshikimic acid (DHS) and shikimic acid

The third and fourth reaction steps of the shikimate pathway are catalyzed by a bifunctional enzyme: 3-dehydroquinate dehydratase/shikimate dehydrogenase

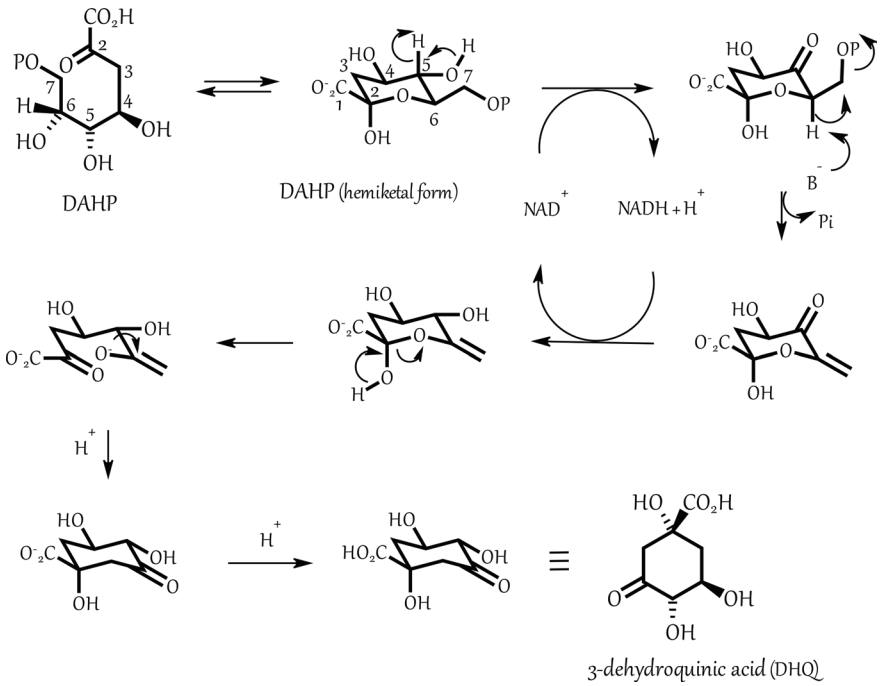


Figure 6.
Reaction mechanism of DAHP (hemiketal form) to 3-dehydroquinic acid (DHQ) by 3-dehydroquinate synthase DHQS (EC. 4.2.3.4) [16].

(DHQ dehydratase/SDH; EC 4.2.1.10/EC 1.1.1.25). The DHQ dehydratase enzyme is a hydro-lyase kind, and the SDH is an oxidoreductase enzyme. The DHQ dehydratase, in the third reaction step, converts DHQ into 3-dehydroshikimic acid (DHS) by eliminating water, and this reaction is reversible, **Figure 7**. The DHS is converted to shikimic acid in the fourth reaction step, by the reduction of the carbonyl group at C-5 by the catalytic action of SDH with NADPH, **Figure 3**. The biosynthesis of DHS is a branch point to shikimic acid and to the catabolic quinate pathway. If the DHS dehydrates, it produces protocatechuic acid (C_6-C_1) or gallic acid, **Figure 3**. Gallic acid (C_6-C_1) is a hydroxybenzoic acid that is a component of tannins [2].

Two structurally different kinds of 3-dehydroquinate dehydratase are known: type I (not heat-stable) and type II (heat-stable). Type I enzyme is present in bacteria and higher plants, and type II is found in fungi, which have both types of enzymes [18, 19]. The catalytic mechanism of the type I DHQ dehydratase has been detected by electrospray MS [20]. This catalytic mechanism involves the amino acid residue Lys-241 that forms a Schiff base with the substrate and product, **Figure 7** [21]. The fourth step is the reduction of DHS with NADPH that enantioselectively reduces the carbonyl of the ketone group of DHS to produce shikimic acid (shikimate dehydrogenase, SDH), **Figure 3**.

Sigh and Christendat [22] reported the crystal structure of DHQ dehydratase/SDH from the plant genus *Arabidopsis*. The crystal structure has the shikimate bound at the SDH and the tartrate molecule at the DHQ dehydratase. The studies show that Asp 423 and Lys 385 are key catalytic amino acids and Ser 336 is a key-binding group.

2.4 Synthesis of shikimic acid 3-phosphate (S3P)

The shikimate kinase enzyme (SK; EC 2.7.1.71) catalyzes the phosphorylation of the shikimic acid, the fifth chemical reaction of the shikimate pathway, and the products are shikimic acid 3-phosphate (S3P) and ADP, **Figures 3** and **8**.

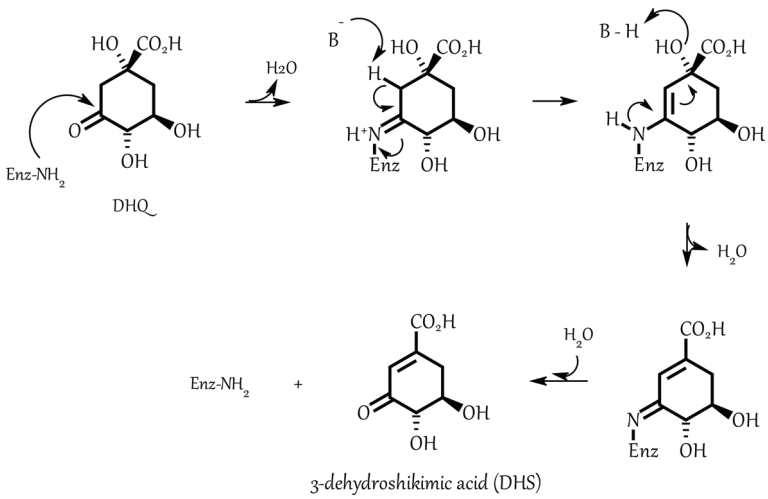


Figure 7.
Reaction mechanism to produce 3-dehydroshikimic acid (DHS) by type I DHQ dehydratase enzyme [21].

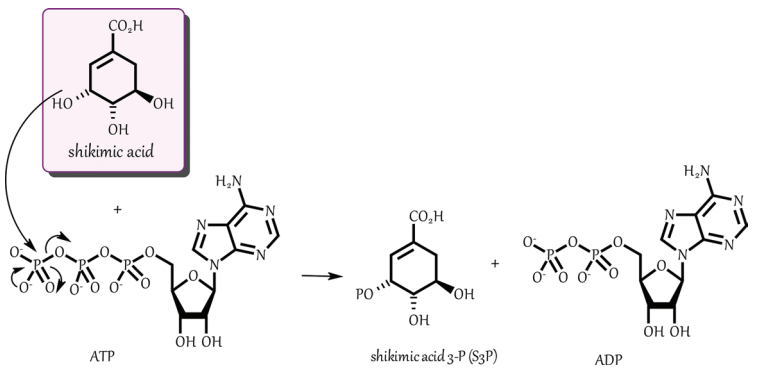


Figure 8.
Phosphorylation of shikimic acid with ATP.

Shikimic acid is phosphorylated with ATP in the 5-hydroxyl group of shikimic acid. SK is an essential enzyme in several bacterial pathogens and is not present in the human cell; therefore the SK enzyme has been classified as a protein target for drug design, especially for chemotherapeutic development of antitubercular drugs [23, 24].

2.5 Synthesis of 5-*enol*pyruvylshikimate 3-phosphate (EPSP)

The 5-*enol*pyruvylshikimate 3-phosphate synthase, also called aroA enzyme (EPSPS; EC 2.5.1.19), catalyzes the condensation of PEP to the 5-hydroxyl group of S3P in the sixth reaction of the shikimate pathway to form 5-*enol*pyruvylshikimate 3-phosphate (EPSP). The reaction mechanism involves the protonation of PEP to subsequent nucleophilic attack of the hydroxyl at C-5 of S3P to form an intermediate that loses Pi to form EPSP, **Figure 9** [25].

EPSPS is the most studied enzyme of the shikimate pathway because it plays a crucial role in the penultimate step. If this enzyme is inhibited, there is an

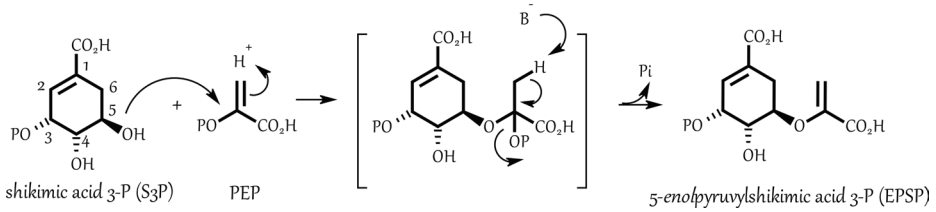


Figure 9.
Reaction mechanism of the condensation of S3P with PEP by EPSPS (EC 2.5.1.19) to form EPSP [25].

accumulation of shikimic acid [26], and the synthesis of aromatic amino acid is disabled, leading to the death of the plant [27]. Therefore, EPSPS is used as a target for pesticides, like glyphosate, **Figure 4**, the active ingredient in the herbicides RoundUp™, Monsanto Chemical Co., and Touchdown™, Syngenta. Glyphosate (*N*-(phosphonomethyl)glycine) inhibits EPSPS and is a potent nonselective herbicide that mimics the carbocation of PEP and binds EPEPS competitively [28]. Because the glyphosate is nonselective and kills food crops, there is interest in finding glyphosate-tolerant genes for genetically modified crops [29]. Two types of EPSPS enzymes have been identified: type I EPSPS (sensitive to glyphosate) identified mostly in plants and bacteria and type II EPSPS (nonsensitive to glyphosate and has a high affinity for PEP), found in some bacteria [27].

2.6 Synthesis of chorismic acid

The seventh and last reaction step of the shikimate pathway is the 1,4-*trans* elimination of the Pi group at C-3 from EPSPS to synthetize chorismic acid. This last step is catalyzed by chorismate synthase (CS; EC 4.2.3.5) that needs reduced flavin mononucleotide ($FMNH_2$) as a cofactor that is not consumed [2, 19]. The $FMNH_2$ transfers an electron to the substrate reversibly [30]. Spectroscopic

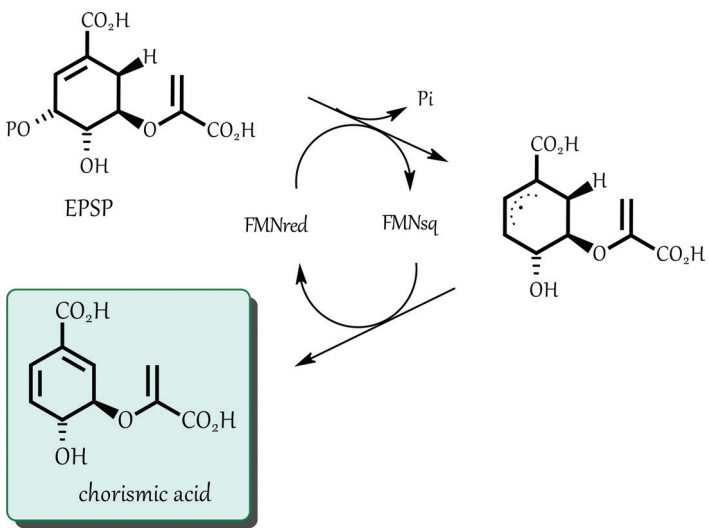


Figure 10.
Reaction of mechanism to yield chorismic acid by chorismate synthase [30].

techniques and kinetic isotope effect studies suggest that a radical intermediate in a non-concerted mechanism is developed [30, 31], **Figure 10**. Chorismic acid, the final molecule of the shikimate pathway, is a key branch point to post-chorismic acid pathways, to obtain L-Phe, L-Tyr, and L-Trp, **Figure 2**. L-Phe is the substrate to phenylpropanoid and flavonoid pathways [13].

3. Factors that induce the synthesis of phenolic compounds in plants

The expression of phenolic compounds is promoted by biotic and abiotic stresses (e.g., herbivores, pathogens, unfavorable temperature and pH, saline stress, heavy metal stress, and UVB and UVA radiation). UV radiation is divided into UVC (≤280 nm), UVB (280–320 nm), and UVA (300–400 nm). UVA and UVB radiation are transmitted through the atmosphere; all UVC and some UVB radiation (highly energetic) are absorbed by the Earth's ozone layer. This accumulation is explained by the increase in enzymatic activity of the phenylalanine ammonia-lyase and chalcone synthase enzymes, among others [12]. Studies have been done about the increase of phenolic compounds, such as anthocyanins, in plants when they are exposed to UVB radiation [13]. Another study demonstrates that UVB exposure enhances anthocyanin biosynthesis in "Cripps pink" apples (*Malus* x *domestica* Borkh.) but not in "Forelle" pears (*Pyrus communis* L.) [32]. This effect may be due to UV radiation exposure and the cultivar of the plants studied. It is known that if plants are under stress, they accumulate phenolic compounds.

The increase in phenolic compounds in blueberry (*Vaccinium corymbosum*) plantlets cultivated in vitro exposed to aluminum (Al) and cadmium (Cd) has also been studied. These heavy metals cause high toxicity in plants, because they increase the oxidative stress by the production of reactive oxygen species (ROS). The authors of the study suggest that the phenolic compounds, specifically chlorogenic and ellagic acids, **Figure 11**, reduce the ROS in blueberry plants [33].

An interesting study was carried out in 2011 by Mody et al., where they studied the effect of the resistance response of apple tree seedlings (*Malus* x *domestica*) to a leaf-chewing insect (*Spodoptera littoralis*) [34]. The authors found a significant herbivore preference for undamaged plants (induced resistance) was first observed 3 days after herbivore damage in the most apical leaf. Also, the results showed higher concentrations of the flavonoid phlorizin, **Figure 12**, in damaged plants than undamaged plants. This indicates that insect preference for undamaged apple plants may be linked to phlorizin, which is the main secondary metabolite of the phenolic type in apple leaves.

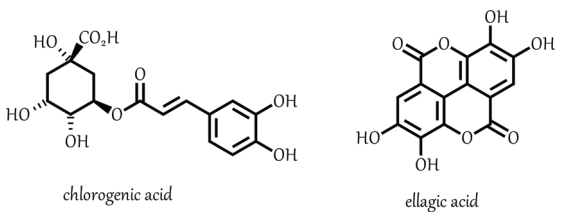


Figure 11.
Chemical structure of chlorogenic (C_6-C_3) and ellagic (C_6-C_1) acids.

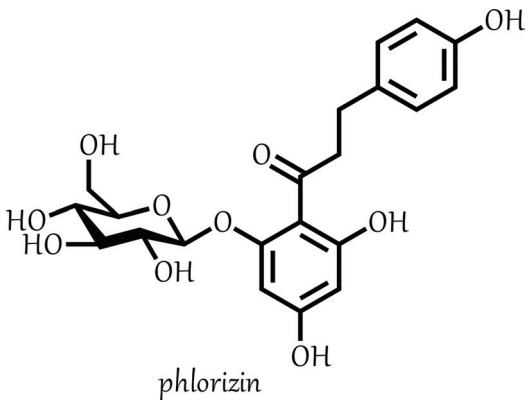


Figure 12.
Chemical structure of phlorizin (C_6-C_3).

4. Conclusions

Knowledge of the biosynthetic pathway of shikimic acid leads to understanding the reaction mechanisms of enzymes and thus discovering antimicrobials, pesticides, and antifungals. Studies with isotopic labeling of substrates, the use of X-ray diffraction, nuclear magnetic resonance (NMR), mass spectrometry (ES), biotechnology, as well as organic synthesis have contributed to explaining the shikimate pathway. Although the seven steps of the biosynthetic pathway are elucidated, these metabolites are the precursors of phenolic compounds, more complex molecules that are necessary for the adaptation of plants to the environment. So, the shikimate pathway is the basis for the subsequent biosynthesis of phenolic compounds. There is scientific interest in continuing to investigate the biosynthesis of phenolic compounds from several points of view: pharmaceuticals, agronomy, chemical and food industries, genetics, and health.

Secondary metabolites are organic molecules that are not involved in the normal growth and development of an organism. Secondary metabolites are frequently produced at highest levels during a transition from active growth to stationary phase. The producer organism can grow in the absence of their synthesis, suggesting that secondary metabolism is not essential, at least for short term survival.

Although the initial isolation and identification of PSMs was driven by knowledge about their pharmacological functionality, they were, until the late 1950s, largely considered as detoxification or waste products of primary metabolism with only accidental biological function. In reflections on the history of the study of plant chemistry, Hartmann (2007) pointed out that biologists studying plant-herbivore-interactions had eventually suggested important biological functions of PSMs and so laid the basis for the field of chemical ecology. At present, an estimated 200,000 PSMs have been isolated and identified. Although this number seems large, it is actually small relative to the approximately 391,000 described plant species, of which nearly 369,000 (94%) are vascular plants. In a more specific example, 990 plant species have been analyzed for their floral emissions, and only approximately 1,720 compounds belonging to seven major compound classes have been isolated and identified from the floral headspace).

The relatively low number of described PSMs is partly because the isolation, structural description and categorization of PSMs started much later than did the categorization of plants, and only a relatively small proportion of plants have been profiled. However, most compounds and compound classes are widely expressed among members of different plant phyla. For example, terpenes (isoprenes) form one of the most diverse compound classes, are the major components of floral and vegetative volatile compound emissions, and have various ecological functions. Some of those compounds such as β-caryophyllene, limonene, and linalool, together with the phenylpropanoids benzaldehyde and salicylic acid, are present in 50–70% of floral emissions of all plants studied. This strong overlap in plant metabolism among species as well as the often cited apparent paradox of PSM multifunctionality, on the one hand, and PSM functional redundancy, on the other, raises one of the long ststanding questions in the study of plant chemistry:

Biosynthesis and storage of plant secondary Metabolites

Biosynthesis of PSM is organ-, cell- or developmentspecific in almost all higher plant species. In most cases the pathways, and indeed the genes involved in their synthesis, are tightly regulated and may be linked to environmental, seasonal or external triggers. Cellular sites of synthesis are compartmentalised in the plant cell, with the majority of pathways being at least partially active inthe cytoplasm. However, there is some evidence that compounds such as alkaloids, quinolizidines, caffeine and some terpenes are synthesised in the chloroplast . The biosynthesis of protoberberine occurs in cell vesicles and coniine and some amines are synthesised in mitochondria The synthesis of lipophilic compounds is usually associated with the endoplasmic reticulum, as are many of the postsynthetic modifications such as hydroxylation.

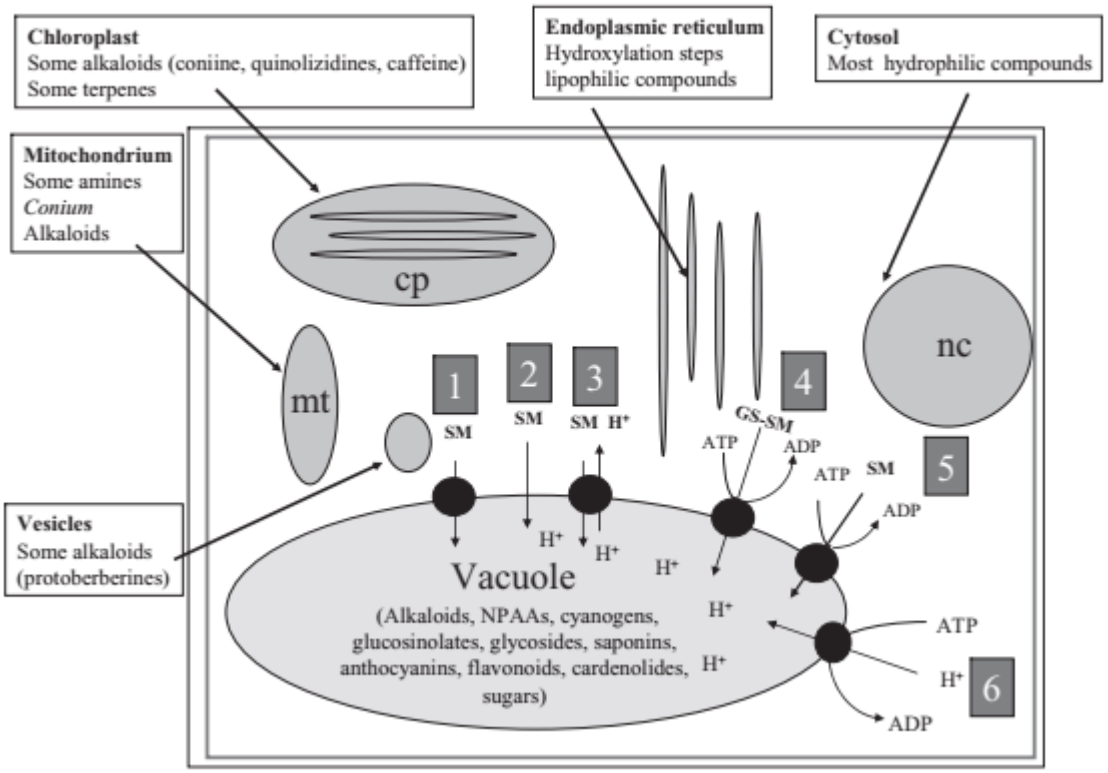


Figure:- *Compartmentation of biosynthesis and sequestration. Abbreviations: SM, secondary metabolites; GS-SM, conjugate of SM with glutathione; NPAAs, non-protein amino acids; ATP, adenosine triphosphate; ADP, adenosine diphosphate; mt, mitochondrion; cp, chloroplast; nc, nucleus; 1, passive transport; 2, free diffusion; 3,H+/SM antiporter; 4, ABC transporter for SM conjugated with glutathione; 5 ABC transporter for free SM; 6, H+-ATPase.*

Although PSM are often detected throughout the plant, their initial site of synthesis is often restricted to a single organ such as roots, fruits or leaves. Thereafter, they can be transported around the plant via the phloem or xylem or by symplastic or apoplastic transport and stored in a number of different tissues. The site of storage often depends on the polarity of the compounds, with hydrophilic compounds such as alkaloids, glucosinolates and tannins being stored in vacuoles or idioblasts, whilst lipophilic compounds such as the terpene-based essential oils are stored in trichomes, glandular hairs, resin ducts,thylakoid membranes or on the cuticle. For some compounds that are present in the plant as defence barriers, e.g. alkaloids, flavonoids, cyanogenic glycosides, coumarins, storage may be in the epidermis itself. Storage may be tissue- or cellspecific, with flowers, fruits and seeds being rich sources of many PSM, especially in annual plants. In perennial species PSM are present in high levels in bulbs, roots, rhizomes and bark of the roots and stems. PSM may not be the end products of metabolism, but may have a regular rate of turn over). N-containing PSM such as alkaloids, cyanogenic glycosides, non-protein amino acids, NSP and protease inhibitors are stored by the plant and are metabolised at germination to serve as N or C sources for the developing seedlings. There is also a turnover of carbohydrates (e.g. oligosaccharides) and lipids during germination. The concentration of some PSM, such as quinolizidine alkaloids and some

monoterpenes, have also been shown to vary in a diurnal fashion, suggesting an interplay between synthesis and turnover, and active transcription of the genes involved.

Biological significance of plant secondary metabolites (PSM),

Although PSM have been used for thousands of years in human medicine, for flavouring, as stimulants and hallucinogens, as fragrances in cosmetics and household fresheners and as therapeutic agents, their native function in plants remains contentious. The primary use of many PSM is probably for plant defense. Whereas animals and birds can evade predation by relatively rapid movement, plants do not have this capability, and so have evolved elaborate mechanisms of protection. PSM are generally thought to be present in plants primarily for defence purposes and this view has been extended on the basis of some convincing evidence. The proposed functions include: defence against grazing herbivores and insects; defence against micro-organisms, including bacteria, fungi and viruses; defence against other plants competing for nutrients and light; protection against the damaging effect of UV light.

However, PSM perform other roles including: acting as volatile attractants to promote pollination by birds and insects; colouring for the purpose of either camouflage or attraction; signals to promote colonisation by beneficial symbiotic micro-organisms such as mycorrhizal fungi and N-fixing rhizobia. They may also have a possible nutritional role, particularly the N-containing PSM, during germination of seeds. Interestingly, plants also regulate the synthesis and storage of PSM so that the more vulnerable tissues such as fruits and young leaves contain higher concentrations of PSM than senescing tissues. The physical location of structures such as trichomes, which serve as the sites of synthesis and storage of essential oils, also provides support for a defence role for these compounds. Trichomes are located on the surfaces of leaves, and are usually the first point of contact for browsers or insect predators. Tannins are usually located in leaf vacuoles beneath the epidermal surface. The volatile nature of the essential oils or the astringent and bitter taste of tannins and alkaloids respectively can be a clear deterrent to predators.

(a) Defence against herbivores (insects, vertebrates)
(b) Defence against fungi and bacteria
(c) Defence against viruses
(d) Defence against other plants competing for light, water and nutrients
(e) Signal compounds to attract pollinating and seed-dispersing animals
(f) Signals for communication between plants and symbiotic microorganisms (N-fixing Rhizobia or mycorrhizal fungi)
(g) Protection against UV light or other physical stress
(h) Selected physiological functions.

Extraction

The initial step during extraction is the preparation of plant tissues. The extraction can be done on clean and ground leaves, barks, roots, fruits, and flowers, from fresh or dried plant

material. In order to maintain the freshness of the samples and avoid possible chemical damage, it is recommended that the interval between harvest and the initiation of extraction does not exceed 3 hours since the plant tissue is fragile and tends to deteriorate faster than dry tissue. Otherwise it is preferable to dry the plant by air-drying, microwave-drying, oven-drying or lyophilization. Each of these methods has advantages and disadvantages. Another critical point to view during pre-treatment of the plant is the particle size of plant material. The smaller the particle size, the higher the area of contacts between the plant material and the solvent, and consequently the more effective the extraction of the chemicals.

Extraction is the process that allows separating SM from the plant by using solvents of different polarity. As a result of the extraction remains two phases: a liquid phase containing solubilized metabolites and a solid containing the insoluble cell debris. Conditions as temperature and time are important factors to achieve high-quality extracts67. The most common extraction methods are maceration, infusion, percolation, decoction, Soxhlet or continuous extraction, microwave assisted extraction (MAE), ultrasound-assisted extraction (UAE), accelerated solvent extraction (ASE), and supercritical fluid extraction (SFE).

Maceration is a solid-liquid extraction technique. The method consists of using a solvent or a mixture of solvents having different polarities and a particular affinity with compounds that are going to be extracted. The mixture (plant solvent) is placed in a container with lid and let it rest for two or three days until the compounds could be transferred from vegetal tissues to the solvent. This method is widely used with soft vegetal material.. The infusion is a maceration process too but uses shorter extraction times and the solvent usually is cold or boiling water. This method is used to obtain a diluted solution of compounds that are easily extracted.

The decoction is a more convenient method for extracting water-soluble compounds from roots and barks that are stable at high temperatures and usually results in oil-soluble compounds compared to maceration and infusion. The decoction method is carried out boiling the vegetal material in water by 15 minutes, then cooling, filtering and adding water until it reaches the desired volume. Finally, percolation is an extraction method that shares similar fundamentals. The method uses a conical filtration camera open on both sides where the material is placed with the solvent. The camera is connected to a flask and once the material is inside the camera, the system is opened to let it strain. The solvent can be used several times to rinse the material until the saturation point.

Another way to conduct the extraction of SM is using a Soxhlet apparatus. In this method, a Soxhlet extractor, a condenser, and a round bottom flask are used. The finely ground vegetal material is loaded into the thimble of a strong paper of cellulose and then placed in the Soxhlet extractor. The solvent goes in the round bottom flask, and it needs to be heated. The solvent vapors go into the thimble and then return to the flask after being condensed. The system is left, at least for sixteen hours. The main advantage of Soxhlet extractor is the use of smaller quantity of solvent compared to maceration.

However, the exposure to hazardous and flammable organic solvents, with potential toxic emissions is high.

The microwave-assisted extraction (MAE) is another popular and easy technique in which the sample is heated using electromagnetic radiation. This method improves the extraction of intracellular compounds due to the rupture of the cellular wall. Increasing temperature, the humidity inside the cell is transformed into vapor; as a result the intracellular pressure increases and the lysis is provoked. This factor comes together with other effects in the solution that benefit the interaction of the compounds to be extracted with the solvent. The main disadvantage is the possible thermal degradation.

The ultrasound-assisted extraction (UAE) facilitates partition of analytes with the occurrence the fragmentation of cell wall provoked by the collisions between the electromagnetic waves and the particles. There are two forms of applying it: in direct contact with the sample or using an ultrasound bath, where the contact is given through the walls of the bottle. In the first case the efficacy is 100 times higher than the second one. The procedure is simple, low cost and can be used in both small and larger-scale extraction. In the method called accelerated solvent extraction (ASE), high temperatures and high pressures are applied to the samples. The time required to achieve the extraction is reduced to one hour, which is an advantage in comparison with other methods (48h or 72h). This is a method that separates efficiently analytes from the matrix. Since the nature of the solvent is an important fact in each method of extraction, the solvents used in this method determine the efficiency of the results. The solvents system, temperature, and time of action are determinants in accelerated solvent extraction. In the case of extraction of bixin the most efficient mixture of solvent was cyclohexane: acetone (6:4) at 50°C for 5 minutes.

The supercritical fluid extraction (SFE) involve a supercritical fluid. It is a substance that has both physical properties of gas and liquid in its critical point. Pressure and temperature are determinant factors to reach this critical point. The utility of the supercritical fluid is their gas behavior and solvating capacity of liquids. The most used solvent is CO_2. due to its capacity to dissolve nonpolar analytes, it has low cost and low toxicity. This method is very selective, very fast, has a high yield percentage and the resultant product has high quality. It has been used in the coffee industry to decaffeinate coffee, or in other industries to extract essential oils.

Detection of Alkaloids

Extraction of Alkaloids

Extraction of alkaloids are based on the facts that they occur in plants as salts and depending on their basicity. In other words, extraction depends on the differential solubility of the bases and salts in H_2O and organic solvents.

Solvent exraction for alkaloids may be studied under the two medium, alkaline and acidic media.

Tests for Alkaloids - General Reagents.

A. Precipitation Reactions

.a. Mayer’s reagent:

Potassium Mercuric Iodide

It is called also Valser's reagent. The reaction gives white or yellow color mostly amorphous precipitates with alkaloids, except the purine bases, ephedrine Colchicine and ricinine.

Mercuric Chloride 1.36 g

Potassium Iodide 5.00 g

Water qs 100.0 mL

b. Dragendorff's reagent:

Potassium Iodide + Bithmus Nitrate

It is called also Krauts Reagent. It produces orange-red precipitate which is usually amorphous.

Bismuth Nitrate 8.00 g

Nitric Acid 20.5 g

Potassium Iodide 27.2 g

Water qs 100.0 mL

c. Wagner's reagent:

Iodine-Potassium Iodide

It produces brown or reddish-brown precipitates with all alkaloids.

Iodine 1.3 g

Potassium Iodide 2.00 g

Water qs 100.0 mL

d. Marme's reagent:

[KI — 20 g in 20 ml dist. Water

CdCl2 — 10 g in 50 ml dist. water]

To about 3 ml of extract, a few drops of Marme's reagent are added. Then dil. H_2SO_4 is added to the mixture.

White or yellow precipitate is formed. Precipitate may be dissolved on excess addition of Marme's reagent or ethanol.

e. Bouchardat's Reagent:

[KI + I2

Brownish precipitate is formed.

f. Hager's Reagent

Picric Acid

Gives yellow crystalline precipitate.

g. Tannic Acid Solution(%5 w/v)

It gives buff colored precipitation which is soluble in diluted acid or ammonia.

h. Ammonium Reineckate Solution(2%)

The solution produces yelow-orange precipitates with heterocyclic nithrogen alkaloids, with quaternary and some tertiary amines.

As a warning, the specificity of the general reagents is not absolute, and peptides, proteins, and some coumarins can give incorrect positive results. Therefore, there are some specific reagents used for specific alkaloids so as to make certain.

Qualitative test for terpenoids (Salkowski test):

Dried extract (50mg) was taken and soaked in 5 mL of ethanol. Extract was mixed in 2 mL of chloroform. It was slightly warmed then cooled. 3 ml of concentrated H_2SO_4 was added slowly along the sides of test tubes. A radish brown colored precipitation was formed at the interface indicating the presence of terpenoids

CLASSIFICATION OF ALKALOIDS

1. Taxonomical classification: This classification is based on the distribution of alkaloids in various plant families, like solanaceous or papilionaceous alkaloids. Sometimes they are grouped as per the name of grouped genus in which they occur, e.g. ephedra, cinchona, etc.

2. Biosynthetic classification: This method gives significance to the precursor from which the alkaloids are biosynthesized in the plant. Hence the variety of alkaloids with different taxonomic distribution and physiological activities can be brought under same group, if they are derived from same precursor, e.g. all indole alkaloids from tryptophan are grouped together. Alkaloids derived from amino acid precursor are grouped in same class such as ornithine, lysine, tyrosine, phenylalanine, tryptophan, etc.

3. Pharmacological classification: This classification is based on the physiological action or biological activity of alkaloids on animals like CNS stimulants or depressants, sympathomimetics, analgesics, purgatives, etc. This method does not take account of chemical nature of alkaloids. Within the same chemical structure the alkaloids can exhibits more than one physiological action e.g. morphine is narcotic-analgesic, while quinidine is cardiac depressant.

4. Chemical classification: this classification is most accepted way to specify the alkaloids. The alkaloids are categorised into three divisions.

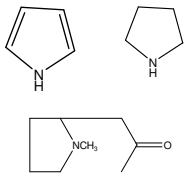
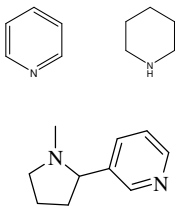
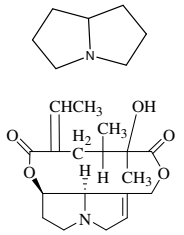
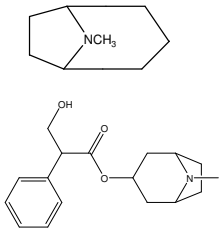
a. True alkaloids: These have heterocyclic ring with nitrogen and derived from amino acids.

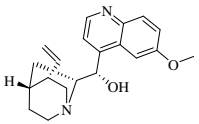
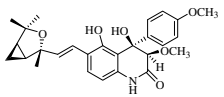
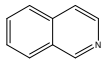
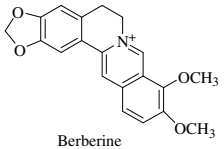
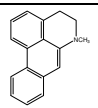
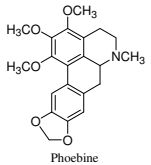
b. Proto alkaloids: These does not have heterocyclic ring with nitrogen and derive from aminoacids, e.g. colchicine.

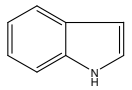
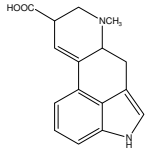
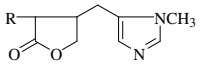
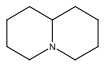
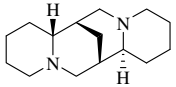
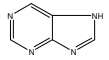
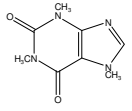
c. Pseudo alkaloids: These have heterocyclic ring with nitrogen and derived from terpenoids or purines but not derived from amino acids.

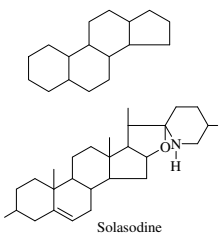
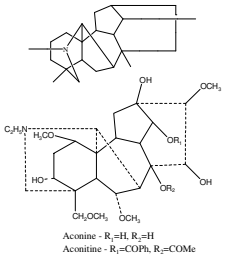
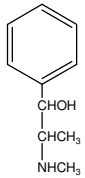
The chemical classification of alkaloids is summarized in below table and a general scheme for classification of alkaloids is shown below.

Table 1. Classification of alkaloids along with some common examples and sources

S.No.	Class	Basic ring	Example	Biological sources	References
1.	Pyrrole and Pyrrolidine	 Hygrine	Hygrine, nicotine, cuscohygrine, coca alkaloids	*Erythroxylum coca, Erythroxylum truxillense*	(Moor**,** 1994; Evans, 1981)
2.	Pyridine and Piperidine	 Nicotine	Piperine, coniine, trigonelline, arecaidine, guvacine, pilocarpine, cytisine, nicotine, sparteine, pelletierine, lobeline, arecoline, anabasine	*Piper nigrum, Areca catechu, Lobelia nicotianefolia*	(Parmar et al., 1997; Ravindran et al., 2000)
3.	Pyrrolizidine	 Cenecionine	Echimidine, senecionine, senesiphylline, symphitine	*Castanospermum australe, Senecio* sps.	(Molyneux, 1988; Hartmann and Witte, 1995)
4.	Tropane	 Atropine	Atropine, cocaine, ecgonine, scopolamine, hyoscine, meteloidine hyoscyamine, pseudo-pelletierine,	*Atropa belladonna, Dhatura stramonium, Erythroxylon coca, Schizanthus porrigens*	(Munoz and Cortes, 1998; Leete, 1988; Drager and Schaal, 1993)

5.	Quinoline	  Quinine  Prenylated quinolin-2-one	Quinine, quinidine, brucine, veratrine, dihydroquinine, dihydroquinidine, strychnine, cevadine, cinchonine, cupreine, cinchonidine, prenylated quinolin-2-one	*Cinchona officinalis, Cinchona calisaya, Almeidea rubra*	(Giroud, 1991; Phillipson et al., 1981; Santos et al., 1998; Scherlach and Hertweck, 2006)
6.	Isoquinoline	  Berberine	Morphine, codeine, thebaine, papaverine, narcotine, sanguinarine, narceine, hydrastine, berberine, d-tubocurarine, emetine, cephaeline, narcotine	*Papaver somniferum, Cephaelis ipecacuanha, Berberis aristata, Aristolochia elegans, Cocculus pendulus*	(Wu et al., 2000; Bisset, 1992; Schiff, 1991; Guinaudeau and Brunetonin, 1993; Bhakuni et al., 1976; Uprety and Bhakuni, 1975)
7.	Aporphine	  Phoebine	Boldine, phoebine, laurodionine, noraporphine, norpurpureine, nordelporphine	*Stephania venosa, Phoebe valeriana*	(Pharadai, 1985; Castro et al., 1986)

8.	Indole	  Lysergic acid	Psilocybin, serotonin, melatonin, reserpine, ergine, vincristine, vinblastine, strychnine, brucine, emetine, physostigmine, harmine, ergotamine, ergometrine, lysergic acid, yohimbine, hydroxygelsamydine	*Strychnos nuxvomica, Claviceps purpurea, Rauwolfia serpentine, Catharanthus roseus, Gelsemium elegans*	(Dembitsky et al., 2005; Lin, 1996)
9.	Imidazole	  Pilocarpine R= C_2H_5 Pilosine R= PhCHOH	Pilocarpine, pilosine	*Pilocarpus jaborandi, Maranham jaborandi, Ceara jaborandi*	(Negri, 1998)
10.	Norlupinane	  Sparteine	Cytisine, laburnine, lupanine, sparteine	*Punica granatum, Lobelia inflata*	(Yusuph and Mann, 1997)
11.	Purine	  Caffeine	Caffeine, theobromine, theophylline	*Theobroma cacao, Coffea arabica*	(Shervington, 1998)

12.	Steroidal	 Solasodine	Solasodine, solanine, solanidine, samandarin, protoveratrine, conessine, funtumine	*Solanum tuberosum, Veratrum album, Whithania somniferum, Holarrhena antidysenterica*	(Kumar and Ali, 2000; Shakirov, 1990; Rahman and Chaudhari, 1995; Rahman and Chaudhari, 1996)
13.	Diterpene	 Aconine - R_1=H, R_2=H Aconitine - R_1=COPh, R_2=COMe	Aconitine, aconine, hypoaconitine	*Aconitum napellus, Aconitum japonicum*	(Rahman and Chaudhari, 1995; Wang et al., 1992)
14.	Alkylamine/ Amino alkaloid		Ephedrine, pseudoephedrine, colchicine, demecolcine	*Ephedra gerardiana, Ephedra sinica, Colchicum autumnale*	(He et al., 1999)

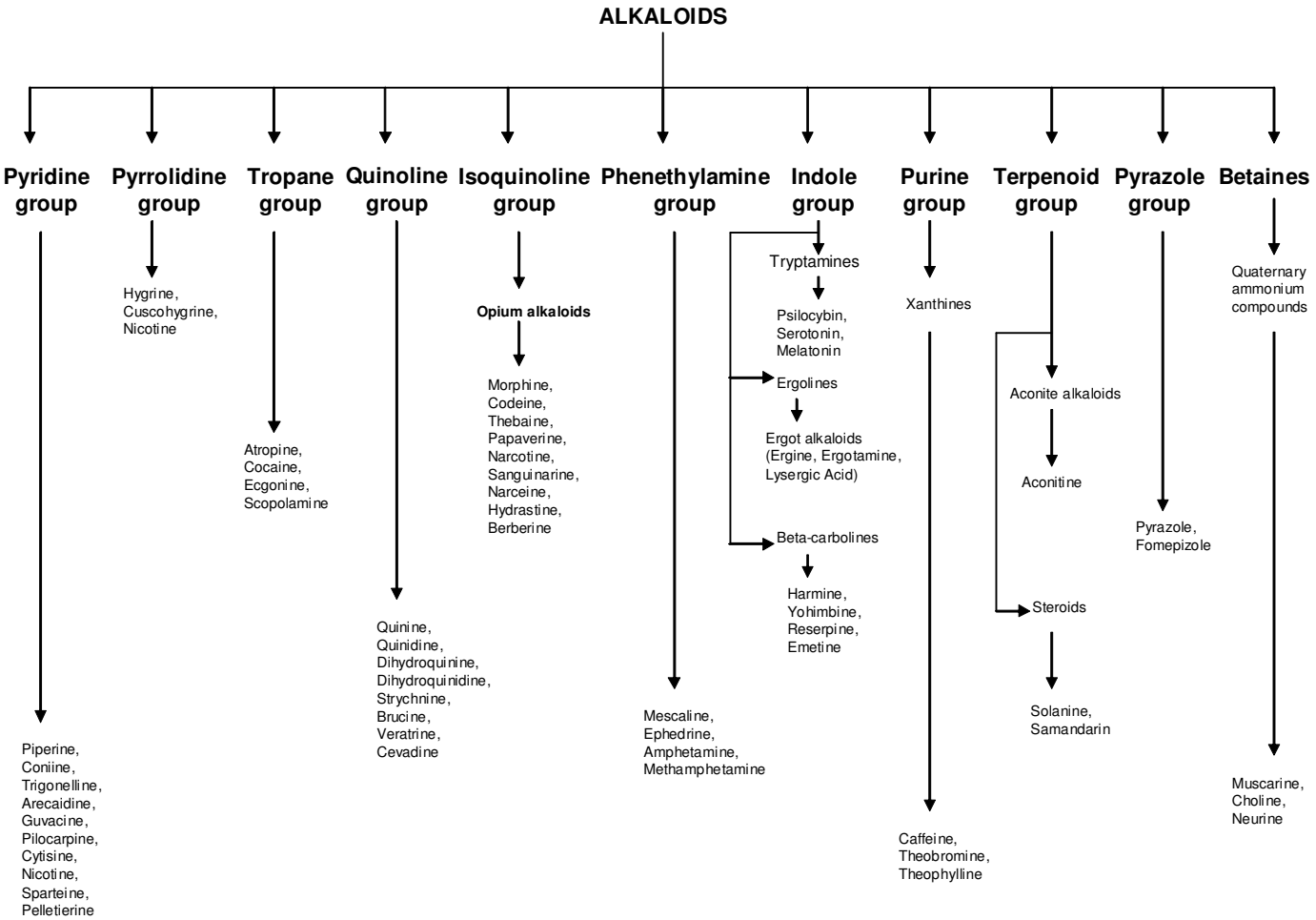


Fig. 3. General scheme for classification of alkaloids

TERPENOIDS- CLASIFICATION AND STRUCTURE

Introduction

There are many different classes of naturally occurring compounds. Terpenoids also form a group of naturally occurring compounds majority of which occur in plants, a few of them have also been obtained from other sources. Terpenoids are volatile substances which give plants and flowers their fragrance. They occur widely in the leaves and fruits of higher plants, conifers, citrus and eucalyptus.

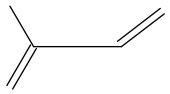
The term 'terpene' was given to the compounds isolated from terpentine, a volatile liquid isolated from pine trees. The simpler mono and sesqui terpenes are chief constituent of the essential oils obtained from sap and tissues of certain plant and trees. The di and tri terpenoids are not steam volatile. They are obtained from plant and tree gums and resins. Tertraterpenoids form a separate group of compounds called 'Carotenoids'

The term 'terpene' was originally employed to describe a mixture of isomeric hydrocarbons of the molecular formula $C_{10}H_{16}$ occurring in the essential oils obtained from sap and tissue of plants, and trees. But there is a tendency to use more general term 'terpenoids' which include hydrocarbons and their oxygenated derivatives. However the term terpene is being used these days by some authors to represent terpenoids.

By the modern definition: "Terpenoids are the hydrocarbons of plant origin of the general formula $(C_5H_8)_n$ as well as their oxygenated, hydrogenated and dehydrogenated derivatives."

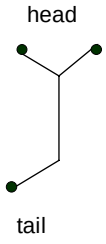
Isoprene rule: Thermal decomposition of terpenoids give isoprene as one of the product. Otto Wallach pointed out that terpenoids can be built up of isoprene unit.

Isoprene rule stats that the terpenoid molecules are constructed from two or more isoprene unit.



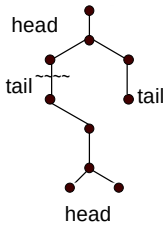
isoprene unit

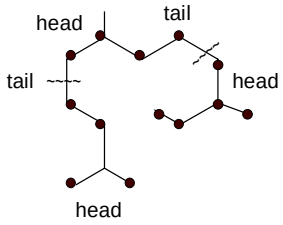
Further Ingold suggested that isoprene units are joined in the terpenoid via 'head to tail' fashion. **Special isoprene rule** states that the terpenoid molecule are constructed of two or more isoprene units joined in a 'head to tail' fashion.



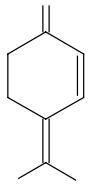
But this rule can only be used as guiding principle and not as a fixed rule. For example carotenoids are joined tail to tail at their central and there are also some terpenoids whose carbon content is not a multiple of five.

In applying isoprene rule we look only for the skeletal unit of carbon. The carbon skeletons of open chain monotrpenoids and sesqui terpenoids are,

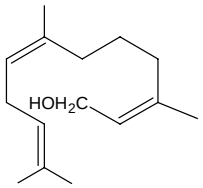




Examples.



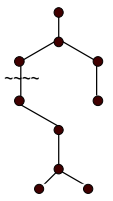
Myrcene
(monoterpene)

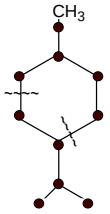


Farnesol (Sesquiterpene)

Ingold (1921) pointed that a gem alkyl group affects the stability of terpenoids. He summarized these results in the form of a rule called ‘gem dialkyl rule’ which may be stated as "Gem dialkyl group tends to render the cyclohexane ring unstable where as it stabilizes the three, four and five member rings.”

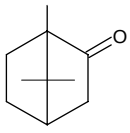
This rule limits the number of possible structure in closing the open chain to ring structure.Thus the monoterpenoid open chain give rise to only one possibility for a monocyclic monoterpenoid i.e the p-cymene structure.



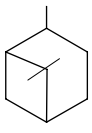


P-cymene structure

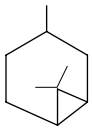
Bicyclic monoterpenodis contain a six member and a three member ring. Thus closure of the ten carbon open chain monoterpenoid gives three possible bicyclic structures.



Camphor
(6+5) system



Pinane
(6+4) system



Carane
(6+3) System

Classification of Terpenoids

Most natural terpenoid hydrocarbon have the general formula $(C_5H_8)n$. They can be classified on the basis of value of n or number of carbon atoms present in the structure.

Table-1: Classification of Terpenoids

S.No.	Number of carbon atoms	Value of n	Class
1.	10	2	Monoterpenoids($C_{10}H_{16}$)
2.	15	3	Sesquiterpenoinds($C_{15}H_{24}$)
3.	20	4	Diterpenoids($C_{20}H_{32}$)
4.	25	5	Sesterpenoids($C_{25}H_{40}$)
5.	30	6	Troterpenoids($C_{30}H_{48}$)
6.	40	8	Tetraterpenoids($C_{40}H_{64}$)
7.	>40	>8	Polyterpenoids$(C_5H_8)n$

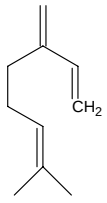
Each class can be further subdivided into subclasses according to the number of rings present in the structure.

i) **Acyclic Terpenoids**: They contain open structure.
ii) **Monocyclic Terpenoids:** They contain one ring in the structure.
iii) **Bicyclic Terpenoids:** They contain two rings in the structure.
iv) **Tricyclic Terpenoids:** They contain three rings in the structure.
v) **Tetracyclic Terpenoids:** They contain four rings in the structure.

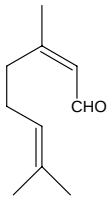
Some examples of mono, sesqui and di Terpenoids:

A) Mono Terpenoids:

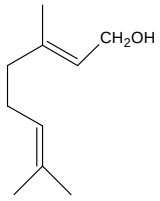
i) Acyclic Monoterpenoids



Myrcene

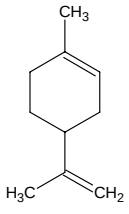


Citral

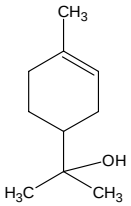


Geraniol.

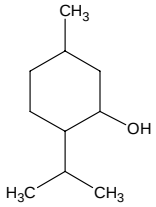
ii) Monocyclic monoterpenoids



Limonene



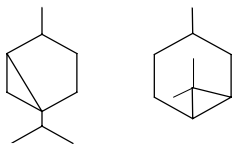
α-Terpineol



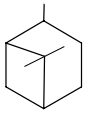
Menthol

iii)Bicyclic monoterpenoids: These are further divided into three classes.

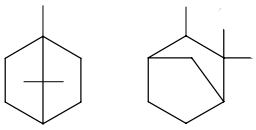
a) Containing -6+3-membered rings
b) Containing -6+4- membered rings.
c) Contining -6+5-membered rings.



Thujane Carane



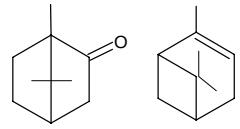
Pinane



Bornane (Camphane) non bornane (iso camphane)

Containing -6+3-membered rings -6+4-membered rings -6+5-membered rings

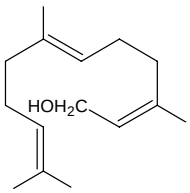
Some bicyclic monoterpenes are:



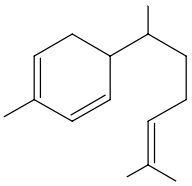
camphor α-pinene

B) Sesquiterpenoids:

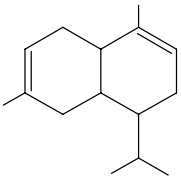
i) Acyclic sesquiterpenoids **ii) Monocyclic sesquiterpenoids** **iii) Bicyclic sesquiterpenoids.**



Farnesol



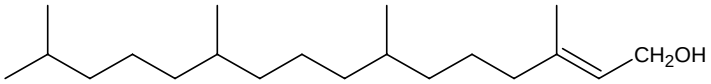
Zinziberene



Cadinene

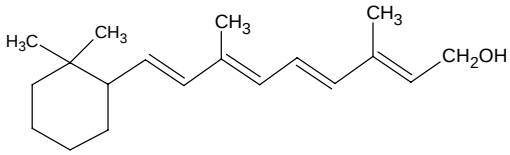
C) Diterpenoids:

i) Acyclic diterpenoids



Phytol

ii) Mono cyclic diterpenoids:



Vitamin A

Isolation of mono and sesquiterpenoids

Both mono and sesquiterpenoids have common source i.e essential oils. Their isolation is carried out in two steps:

i) Isolation of essential oils from plant parts
ii) Separation of Terpenoids from essential oils.

i) Isolation of essential oils from plant parts: The plants having essential oils generally have the highest concentration at some particular time. Therefore better yield of essential oil plant material have to be collected at this particular time. e.g. From jasmine at sunset. There are four methods of extractions of oils.

a) Expression method
b) Steam distillation method
c) Extraction by means of volatile solvents
d) Adsorption in purified fats

Steam distillation is most widely used method. In this method macerated plant material is steam distilled to get essential oils into the distillate form these are extracted by using pure organic volatile solvents. If compound decomposes during steam distillation, it may be extracted with petrol at 50^{o}C. After extraction solvent is removed under reduced pressure.

ii) Separation of Terpenoids from essential oil: A number of terpenoids are present in essential oil obtained from the extraction. Definite physical and chemical methods can be used for the separation of terpenoids. They are separated by fractional distillation. The terpenoid hydrocarbons distill over first followed by the oxygenated derivatives.

More recently different chromatographic techniques have been used both for isolation and separation of terpenoids.

General properties of Terpenoids

1. Most of the terpenoids are colourless, fragrant liquids which are lighter than water and volatile with steam. A few of them are solids e.g. camphor. All are soluble in organic solvent and usually insoluble in water. Most of them are optically active.

2. They are open chain or cyclic unsaturated compounds having one or more double bonds. Consequently they undergo addition reaction with hydrogen, halogen, acids, etc. A number of addition products have antiseptic properties.

3. They undergo polymerization and dehydrogenation

4. They are easily oxidized nearly by all the oxidizing agents. On thermal decomposition, most of the terpenoids yields isoprene as one of the product.

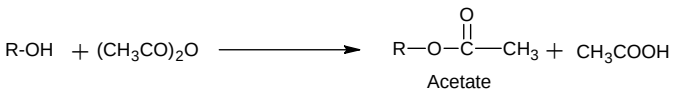
General Methods of structure elucidation

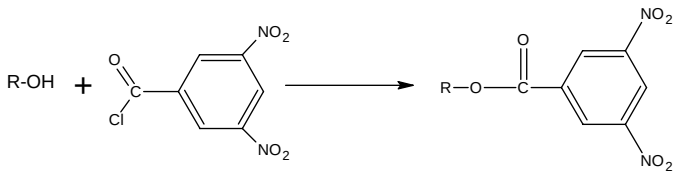
Terpenoids

i) **Molecular formula:** molecular formula is determined by usual quantitative analysis and mol.wt determination methods and by means of mass spectrometry. If terpenoid is optically active, its specific rotation can be measured.

ii) **Nature of oxygen atom present:** If oxygen is present in terpenoids its functional nature is generally as alcohol aledhyde, ketone or carboxylic groups.

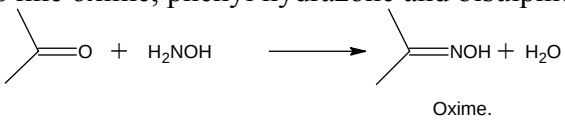
a) **Presence of oxygen atom present:** presence of –OH group can be determined by the formation of acetates with acetic anhydride and benzoyate with 3.5-dinitirobenzoyl chloride.



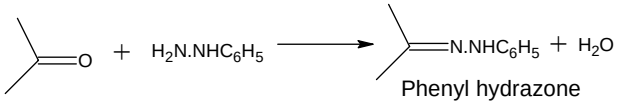


Primary alcoholic group undergo esterification more readily than secondary and tertiary alcohols.

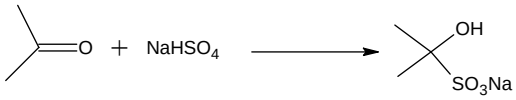
b) **Presence of >C=O group:** Terpenoids containing carbonyl function form crystalline addition products like oxime, phenyl hydrazone and bisulphite etc.



Oxime.



Phenyl hydrazone

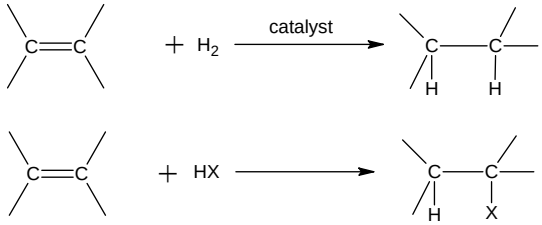


Sodium bisulphite derivative

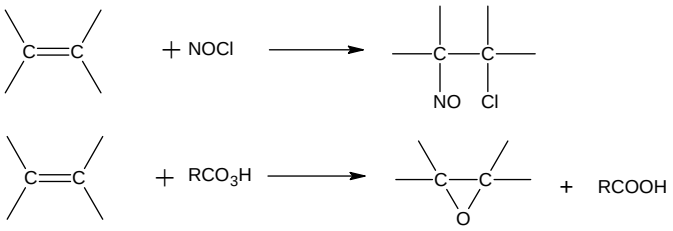
If carbonyl function is in the form of aldehyde it gives carboxylic acid on oxidation without loss of any carbon atom whereas the ketone on oxidation yields a mixture of lesser number of carbon atoms.

iii) **Unsaturation:** The presence of olefinic double bond is confirmed by means of bromine, and number of double bond determination by analysis of the bromide or by quantitative hydrogenation or by titration with monoperpthalic acid.

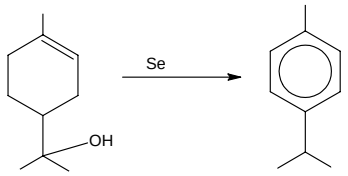
Presence of double bond also confirmed by means of catalytic hydrogenation or addition of halogen acids. Number of moles of HX absorbed by one molecule is equal to number of double bonds present.



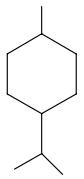
Addition of nitrosyl chloride(NOCl) (**Tilden's reagent**) and epoxide formation with peracid also gives idea about double bonds present in terpenoid molecule.



iv) **Dehydrogenation:** On dehydrogenation with sulphur, selenium, polonium or palladium terpenoids converted to aromatic compounds. Examination of these products the skelton structure and position of side chain in the original terpenoids can be determined.
For example α-terpenol on Se-dehydrogenation yields p-cymene.



Thus the carbon Skelton of terpenol is as follows.



v) **Oxidative degradation:** Oxidative degradation has been the parallel tool for elucidating the structure of terpenoids. Reagents for degradative oxidation are ozone, acid, neutral or alkaline potassium permanganate, chromic acid, sodium hypobromide, osmium tetroxide, nitric acid, lead tetra acetate and peroxy acids. Since oxidizing agents are selective, depending on a particular group to be oxidized, the oxidizing agent is chosen with the help of structure of degradation products.

vi) **Number of the rings present:** With the help of general formula of corresponding parent saturated hydrocarbon, number of rings present in that molecule can be determined.

Vii) **Relation between general formula of compound and type of compounds:** Table 2

Table-2: Relation between general formula of compound and type of compounds

General formula of parent saturated Hydrocarbon	Type of structure
C_nH_{2n+2}	Acyclic
C_nH_{2n}	Monocyclic
C_nH_{2n-2}	Bicyclic
C_nH_{2n-4}	Tricyclic
C_nH_{2n-6}	Tetrayclic

For example limonene (mol. formula. $C_{10}H_{16}$) absorbs 2 moles of hydrogen to give tetrahydro limonene (mol. Formula $C_{10}H_{20}$) corresponding to the general formula. C_nH_{2n}. It means limonoene has monocyclic structure.

viii) **Spectroscopic studies:** All the spectroscopic methods are very helpful for the confirmation of structure of natural terpenoids and also structure of degradation products. The various methods for elucidating the structure of terpenoids are;

a) UV Spectroscopy: In terpenes containing conjugated dienes or α,β-unsaturated ketones, UV spectroscopy is very useful tool. The values of λ_{max} for various types of terpenoids have been calculated by applying Woodward's empirical rules. There is generally good agreement between calculation and observed values. Isolated double bonds, α,β-unsaturated esters , acids, lactones also have characteristic maxima.

b) IR Spectroscopy: IR spectroscopy is useful in detecting group such as hydroxyl group (~3400cm^{-1}) or an oxo group (saturated 1750-1700cm^{-1}). Isopropyl group, cis and trans also have characteristic absorption peaks in IR region.

c) NMR Spectroscopy: This technique is useful to detect and identify double bonds, to determine the nature of end group and also the number of rings present, and also to reveal the orientation of methyl group in the relative position of double bonds.

d) Mass Spectroscopy: It is now being widely used as a means of elucidating structure of terpenoids. Used for determining mol. Wt., Mol. Formula, nature of functional groups present and relative positions of double bonds.

ix) **X-ray analysis:** This is very helpful technique for elucidating structure and stereochemistry of terpenoids.

x) **Synthesis:** Proposed structure is finally confirmed by synthesis. In terpenoid chemistry, many of the synthesis are ambiguous and in such cases analytical evidences are used in conjunction with the synthesis.

Citral

Citral is an acyclic monoterpenoid. It is a major constituent of lemon grass oil in which it occurs to an extent of 60-80%. It is pale yellow liquid having strong lemon like odour and can be obtained by fractional distillation under reduced pressure from Lemongrass oil.

Constitution:

i) Mol. formula $C_{10}H_{16}O$, b.p-77^oC

ii) Nature of Oxygen atom: Formation of oxime of citral indicates the presence of an oxo group in citral molecule.

$$\underset{\text{Citral}}{C_{10}H_{16}O} + H_2NOH \longrightarrow \underset{\text{Oxime}}{C_{10}H_{16}=N\text{-}OH}$$

On reduction with Na/Hg it gives an alcohol called geraniol and on oxidation with silver oxide it give a monocarboxylic acid called Geranic acid without loss of any carbon atom.

Polyphenols: Chemical Classification, Definition of Classes, Subcategories, and Structures

Polyphenols are natural compounds synthesized exclusively by plants with chemical features related to phenolic substances and eliciting strong antioxidants properties. *Objective:* The aim of this paper is to give a reliable overview of the chemical classification of natural polyphenols. *Methods:* Literature survey was done through google scholar, pubmed and scopus search engine. *Results and Discussion:* These molecules or classes of natural substances are characterized by two phenyl rings at least and one or more hydroxyl substituents. This description comprehends a large number of heterogeneous compounds with reference to their complexity. Therefore, polyphenols can be simply classified into flavonoids and non-flavonoids, or be subdivided in many sub-classes depending on the number of phenol units within their molecular structure, substituent groups, and/or the linkage type between phenol units. Polyphenols are widely distributed in plant tissues where they mainly exist in form of glycosides or aglycones. The structural diversity of flavonoid molecules arises from variations in hydroxylation pattern and oxidation state resulting in a wide range of compounds: flavanols, anthocyanidins, anthocyanins, isoflavones, flavones, flavonols, flavanones, and flavanonols.

Polyphenols are natural compounds synthesized exclusively by plants, with chemical features related to phenolic substances and strong antioxidant properties. These molecules or classes of substances are mainly present in fruits, vegetables, green tea, and whole grains.

As a brief premise, it should be considered that analytical research on natural foods and beverages worldwide concerns an increasing number of topics today, from the simple identification of chemicals with antimicrobial properties that are naturally found in certain foods to research on new additives for fortification and authenticity characterization. The addition of sugars, fructans, and natural oligosaccharides of different origins to certain recipes for special and normal consumers is well-known at present, while the analytical research is still growing in this ambit and should be enhanced and promoted (1–5). In addition, consumers are progressively worrying when speaking of new and improved foods and beverages, on the one hand, when considering, on the other hand, historical beverages for which properties were claimed without adequate and reliable demonstrations (6–9). Because of the notable importance of some of these compounds (polyphenols) among the whole group of "natural" healthy compounds, the present paper will give a general overview of the classification of these substances. The correlation between certain microbial menaces (with respect to humans and animals in many different conditions) and the possible use of antimicrobial strategies correlated with traditional or regional foods, beverages, products, and selected microorganisms, should be considered (10–13).

In detail, polyphenols are a well-known group of phenolic systems characterized by at least two phenyl rings and one or more hydroxyl substituents (14). This description comprehends a large number of heterogeneous compounds with reference to their complexity. Therefore, polyphenols can be simply classified into flavonoids and nonflavonoids or subdivided

into many subclasses depending on the number of phenol units within their molecular structure, substituent groups, and/or the linkage type between phenol units (15).

Polyphenols are synthesized by plants and widely distributed in plant tissues, where they mainly exist in the form of glycosides. Although basic structures of flavonoids are aglycones (the nonsugar fragment of the corresponding glycoside), flavonoids can be found as glycosides or aglycones. All flavonoids share the basic structure of diphenyl propanes (C6-C3-C6), in which phenolic rings (ring A and ring B) are usually linked by a heterocyclic ring (16). This heterocyclic ring (ring C) is usually a closed pyran, as shown in Figure 1.

The structural diversity of flavonoid molecules arises from variations in hydroxylation pattern and oxidation state of the central pyran ring, resulting in a wide range of compounds: flavanols, anthocyanidins, anthocyanins, isoflavones, flavones, flavonols, flavanones, and flavanonols (some of these structures are shown in Figure 2). Variations in hydroxylation pattern and oxidation state are based on the presence or absence of a double bond between C2 and C3, and the formation of carbonyl group by C4. Flavones, flavonols, flavanones, and flavanonols represent the largest subgroup among all polyphenols; consequently, they constitute the majority of flavonoid compounds (17).

Flavonols and Flavones

With relation to flavones, a carbonyl group is present at the C4 position, and ring B is attached to the heterocyclic ring at the C2 position; in addition, there is a double bond between the C2 and C3 atoms. The most important flavones are luteolin and apigenin (Figure 3). Moreover, an oxygen atom is placed on the C4 position, and ring B (in isoflavones) is attached to the heterocyclic ring (C3 position) instead of C2, as in other classes. Flavonols (dihydroflavonols) are the 3-hydroxy derivatives of flavanones. They differ from flavones by the presence of a hydroxyl group in the C3 position (18). Some of the most well-known and researched flavonols (in the aglycone form) are kaempferol and quercetin (Figure 3). Interestingly, these compounds can exist in more than 340 and 270 glycosidic forms, respectively (19).

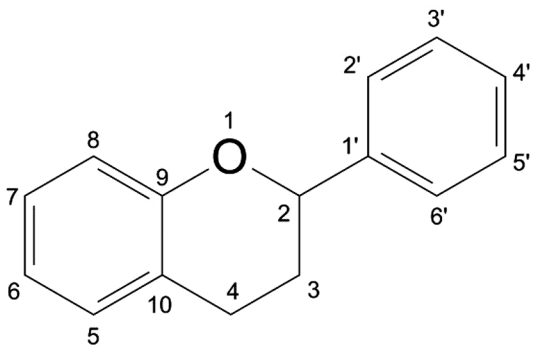


Figure 1. The common structure of flavonoids. Three rings are normally considered and labeled with letters A, B, and C. The heterocyclic ring is labeled C. The oxygen atom is numbered as the first position, and the remaining carbon atoms are numbered from C2 to C10. Ring B shows six positions from C1` to C6`. BKChem version 0.13.0 (2009; http://bkchem.zirael.org/index.html) was used to draw this structure.

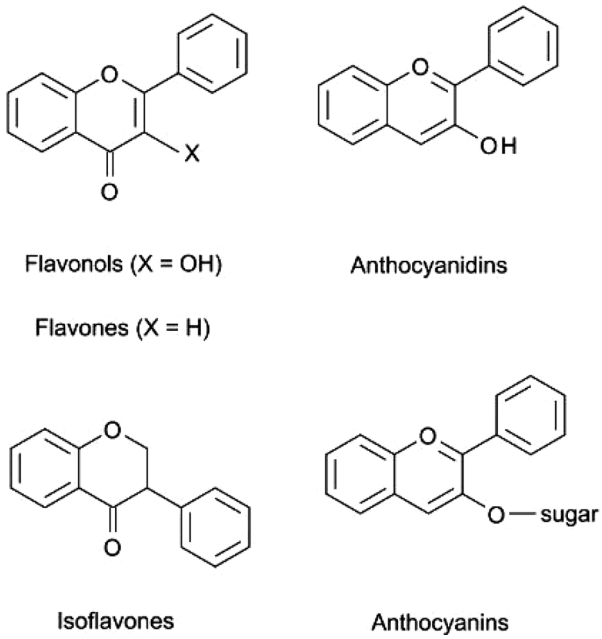


Figure 2. Flavonoids can be subdivided into different subclasses including flavonols, flavones and isoflavones, anthocyanidins, and anthocyanins. This classification concerns different structures. BKChem version 0.13.0 (2009; http://bkchem.zirael.org/index.html) was used to draw this structure.

Flavanones

Unlike flavonols and flavones, with the carbonyl group at the C4 position, the heterocyclic ring in flavanones has a saturated three-carbon chain without a hydroxyl group at the C3 position. Flavanones are characterized by a large number of substituted derivatives (e.g., prenylated flavanones and benzylated flavanones) because of their unique substitution patterns (20). The substitution in the C7 position by a disaccharide is the common form of glycosylated flavanones. Flavanones are mainly found in high concentrations in citrus fruits such as oranges (e.g., hesperidin) and lemons (e.g., eriodictyol; Figure 3), and most of these compounds are present as aglycone forms (18).

Anthocyanidins and Anthocyanins

Anthocyanidins and anthocyanins differ from other flavonoids by the presence of two double bonds in their heterocyclic rings. Anthocyanins are the glycosylated form of anthocyanidins and are characterized by the hydroxylation and methoxylation patterns on ring B (20). The variation in the number of hydroxylated groups and the nature and number of bonded sugar units to their structure result in a variety of anthocyanins. Bonded sugar units are usually monosaccharides, e.g., glucose, galactose, and arabinose (21). Glycosylated anthocyanins are water-soluble pigments present in colorful flowers and fruits; most exhibited colors are ascribed to these compounds (22).

Flavanols

Flavanols (flavan-3-ols) contain a saturated heterocyclic ring, no double bond between C2 and C3, and a hydroxyl group at the C3 position. Unlike other classes of flavonoids, flavanols

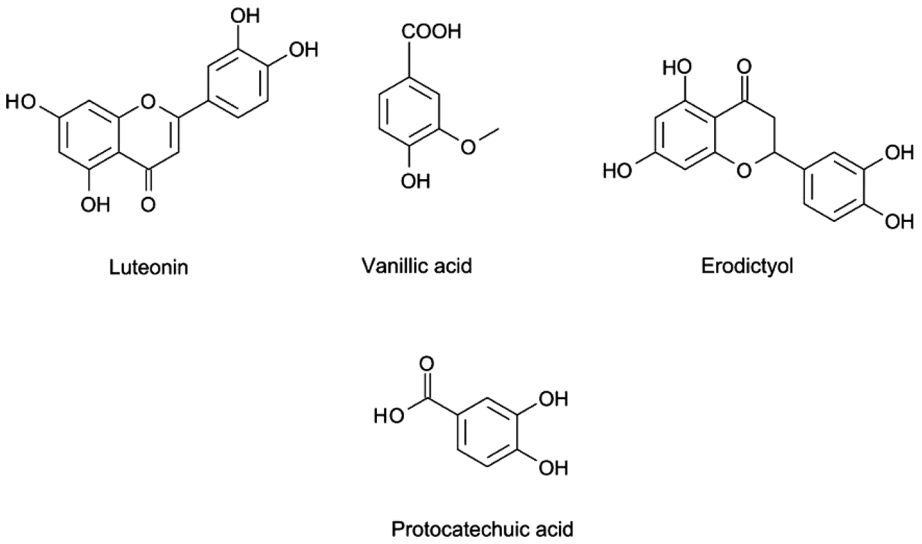


Figure 3. Structures of some of the known and important polyphenolic compounds: luteonin, vanillic acid, erodictyol, and protochatecuic acid. BKChem version 0.13.0 (2009; http://bkchem.zirael.org/index.html) was used to draw this structure.

are found in food as aglycones only (the glycosylated state is excluded). Additionally, they may be found as monomeric units, referred to as catechins and epicatechins (Figure 3), and as polymeric forms, referred to as tannins (23). The presence of a hydroxyl group attached to the C3 position can explain two chiral centers, with the consequent characterization of catechins and epicatechins as diastereoisomers. The catechin isomer shows the *trans* configuration of two stereoisomers, (+)-catechin and (–)-catechin, whereas epicatechin has the *cis* configuration of (+)-epicatechin and (–)-epicatechin stereoisomers (20). With relation to polymeric flavanols, these structures exhibit good water solubility and a relatively high molecular weight; they are known as tannins. Tannins, found in complexes with alkaloids, polysaccharides, and proteins, can be subdivided into hydrolysable tannins and condensed tannins (also known as proanthocyanidins; 22).

Polymers of catechin, epicatechin, and/or leucoanthocyanidin, traditionally called condensed tannins, are the most abundant polyphenols in woody plants. Condensed tannins gained their name as proanthocyanidins because of their ability to convert to anthocyanidins under oxidative conditions (24). Esters of gallic and ellagic acids are known as hydrolysable tannins. A glucose molecule is the center of the hydrolysable tannin molecule, where the hydroxyl group is esterified with gallic or ellagic acid, yielding allotannins or ellagitannins, respectively (21).

Nonflavonoids

Although the polyphenol structural skeleton contains several hydroxyl groups on aromatic rings, the basic structure of nonflavonoids is a single aromatic ring. Nonflavonoid compounds include phenolic acids, stilbenes, and lignans. The main class in this group is represented by phenolic acids, predominantly benzoic acid and cinnamic acid derivatives. These molecules rarely exist in their free form but are most commonly found in conjugation with other polyphenols, glucose, quinic acid, or structural components of the original plant (25). Although the variation in the number and location of hydroxyl groups on the aromatic rings of these acids has to be considered, there are two distinguishing parent skeletons of phenolic acids: hydroxycinnamic and hydroxybenzoic acids.

Gallic, *p*-hydroxybenzoic, protocatechuic, syringic, and vanillic acids (Figure 3) are the most common hydroxybenzoic acids (26). They are found either as free acids or in their conjugated forms (glycosides or esters). On the other hand, the aromatic ring in hydroxycinnamic acids has a three-carbon side chain (C6–C3). Caffeic, ferulic, *p*-coumaric, and sinapic acids are the most common hydroxycinnamic acids widely found in foods (18, 27).

Another relatively small class of nonflavonoids, stilbenes (1,2-diarylethenes), is characterized by two phenyl moieties linked together by a two-carbon methylene group (Figure 4). In stilbenes, the *m*-positions in ring A are usually substituted by two hydroxyl groups, while various positions in ring B may be substituted by hydroxyl and methoxyl groups. On the other hand, stilbenes exist in both isomeric forms [*cis* and *trans* configurations (Figure 4)], as well as occur as free (minor) and glycosylated (major) forms (25). One of the most well-known stilbenes is resveratrol (3,5,4'- trihydroxystilbene), which is produced by several plants such as grapes, peanuts, and berries (14).

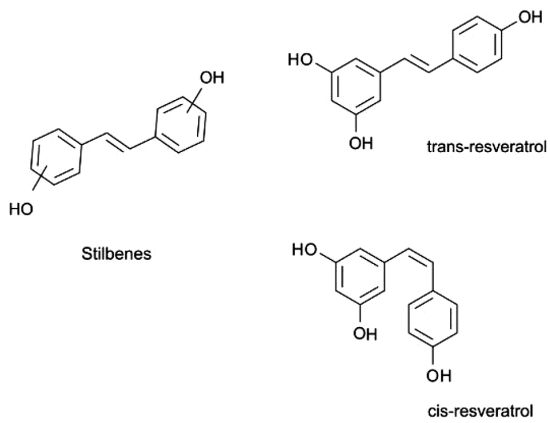


Figure 4. The basic structure of stilbenes (1,2-diarylethenes; left) and *cis*- and *trans*-resveratrol (right).

Lignans represent a nonflavonoid class featuring two propylbenzene units (C6-C3) linked together between the β-position in C8 of the propane side chains. The C9 and C9` positions of lignans are substituted in different patterns, resulting in a wide range of different structural forms. For this reason, lignans are classified in eight subgroups, including furan, dibenzylbutane, and aryltetralin (28, 29). Lignans are found in legumes, seeds, and vegetable oils; they are mainly found in their free forms, while the glycosylated structure is not abundant (30, 31).

Polyphenol Amides

Polyphenols may exhibit an amide group. Avenanthramides and capsaicinoids are reported to have some significant features in terms of antioxidant and anti-inflammatory properties. Interestingly, these compounds are associated with peculiar foods: chili peppers when speaking of capsaicinoids and oat products with relation to avenanthramides. Consequently, the interest in these compounds has grown in recent years (18).