PLANT CELLS AS PRODUCER OF SECONDARY METABOLITES

Secondary metabolites are described as organic compounds which were not directly involved in the normal growth, development, or reproduction of an organism (Fraenkel and Gottfried, 1959).

Plant kingdom (250,000 species) is the factory and storehouse of about hundreds of thousands of secondary compounds whereas the compounds are also produced in fungi, bacteria, sessile marine animals. About 25-28% of modern medicines and particularly 60% of anticancer drugs are derived from plants directly or indirectly.

1. Secondary metabolism and compounds

Secondary metabolism refers to the biosynthesis, transformation, and degradation of a wide array of natural products. Secondary compounds are classified into biosynthetic families (terpenes, polyketides, polyacetylenes); on the basis of biological activity (antibiotics, anticancer agents, hallucinogens, toxins); or into other miscellaneous categories based on attributes such as light absorption (pigments), industrial applications (surfactants, emulsifying agents) or agricultural potential (herbicides, growth promoters).

2. Functions of secondary compounds in plants

i. Chemical signaling

Behavioural messages are delivered by a wide array of chemical compounds. In some cases, they may facilitate communication between the members of a single species (e.g., pheromones) or between members of different species (e.g., allelochemicals). These interactions include largely a negative effect on germination, growth, development, distribution and behaviour of other organisms. The signals are used for entomophily, zoochory, tritrophic interactions and root nodulation.

ii. Defense against pathogens (Bacteria, fungi and viruses)

Secondary metabolites with antimicrobial activity derived from plants may be preformed inhibitors that are present constitutively in healthy plants (phytoanticipins), or they may be synthesized de novo in response to pathogen attack or another stress conditions (phytoalexins). The same compound can be a phytoalexin or a phytoanticipin in different organs of the same plant e.g. production of caffeine in plants act as a pesticide.

iii. Defense against predators

Herbivores and insects feed on plants. Alkaloids present in plants have potential to block ion channels, inhibit enzymes, and interfere with neurotransmission causing hallucinations, loss of coordination, convulsions, vomiting and death in animals. Phenolics interfere with digestion, slow growth, block enzyme activity and cell division, or just taste awful. Glucosinolates (Brassicaceae) protects against herbivory.

iv. Protection against abiotic factors

Specific flavonoids protect plants from UV-B irradiation. Carotenoids protect cell or organelles against photodestruction. Abscisic acid (Terpenes) makes plant tolerant to drought conditions.

v. As Plant hormones

Terpenes act as plant hormones e.g. Abscisic acid and Giberellins.

vi. Protect the dormant spores

Since, synthesis of SMs and spore formation are regulated by similar factors. Therefore, SM production slows down the process of spore germination and they protect the dormant or initiated spore from being consumed by the *Ameobae*.

vii. Metal transporting agents

viii. As agents of symbiosis between plants and microbes, nematodes, insects and higher animals

ix. As differentiation effectors

3. Distribution of Secondary Compounds in Space and Time

Secondary metabolites are not distributed evenly throughout the plant, either quantitatively or qualitatively, in space and time. Usually the amounts are greatest in epidermal tissue, as might be expected if these compounds function in defense. Nor are secondary products synthesized or accumulated in any group of cells at all times during the life of the plant. For example, seeds often contain high concentrations of such compounds which disappear relatively quickly after germination and early seedling development. Thus, they perform a protective function at the most vulnerable period of growth. The concentration of secondary compounds in many if not all plants also varies diurnally, and in some cases gross changes have been shown to occur over periods as short as an hour.

Glycolysation (Sugar conjugation) determines compartmentalization in plants e.g., glycosylated forms of anthocyanins and monolignols are stored in vacuoles. Similarly, Phenylalanine biosynthesis occurs in plastids and is further converted to volatile compounds (Phenylpropanoids and benzenoids) outside the plastids.

4. Accumulation

The site of synthesis for SM is not certainly the site of accumulation. Hydrophilic compounds (alkaloids, NPAAs, cyanogenic glucosides, glucosinolates, saponins, anthocyanins, flavonoids and cardenolides) are mainly stored in the vacuole while the lipophilic SM are commonly sequestered in resin ducts, laticifers (store latex), oil cells, trichomes, or in the cuticle. Phenolics compounds are secreted to apoplast or accumulate in vacuoles. Most of the biosynthetic pathways completely or partially proceed in the **cytoplasm**. Some alkaloids (coniine, caffeine

and quinolizidines), furano-coumarins and some terpenes (monoterpenes, diterpenes, phytol and carotenoids) are synthesized in the **chloroplast.** Sesquiterpenes, sterols and dolichols are manufactured in **ER** or **cytosolic compartment**. Coniine and amine are produced in **mitochondria**. **Vesicles** serve as the biosynthetic compartments of protoberberine.

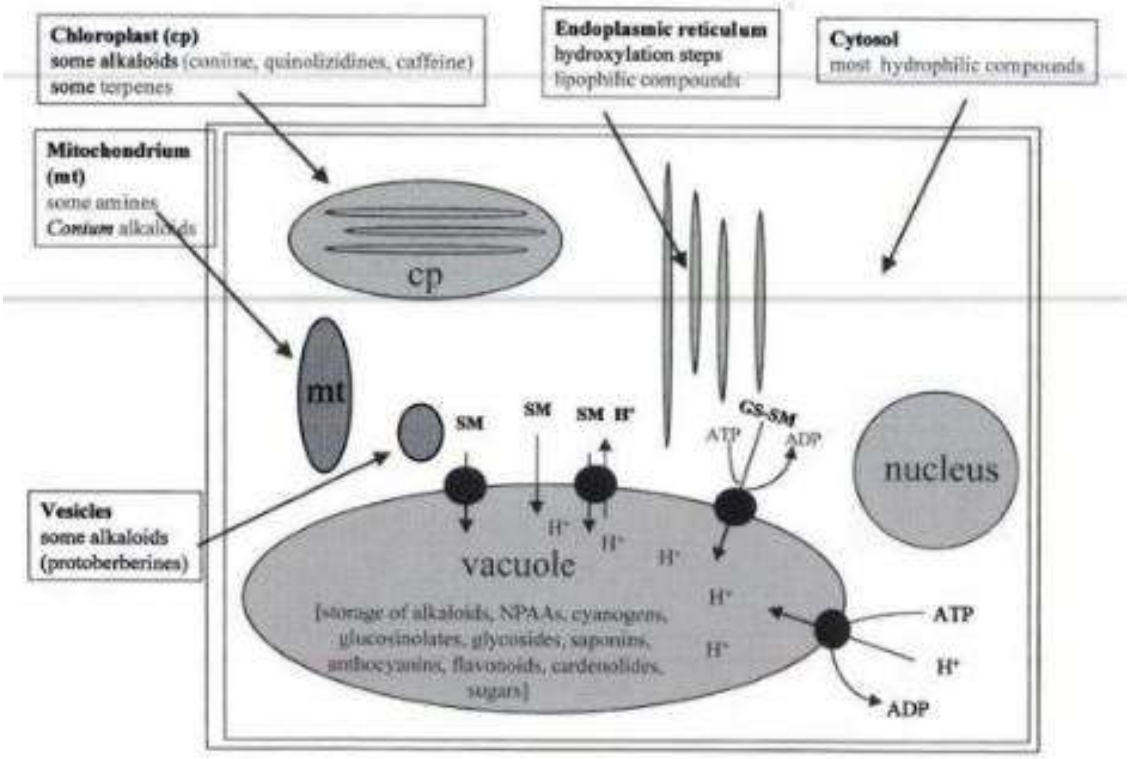

Figure 1. *Compartmentation of biosynthesis and accumulation. Abbreviations: SM, Secondary metabolites; GS-MS, conjugate of SM with glutathione; NPAAs, nonprotein amino acids; ATP, Adenosine triphosphate; ADP, Adenosine disphophate*

5. Storage

Glucosinolates are synthesized in roots and vegetative tissues but get stored in elevated amounts in embryos of the seeds. In a number of plants, specific idioblasts have been detected that contain tannins, alkaloids or glucosinolates. More often, SMs are concentrated in trichomes or glandular hairs (many terpenoids in Labiatae, Asteraceae), stinging hairs (many amines in *Urticaceae*) or the epidermis itself (many alkaloids, flavanoids, anthocyanins, cynogenic glycosides, coumarins etc.) flowers, fruits and seeds are usually rich in SMs, especially in annual plants. In perennial species, high amounts of SMs found in bulbs, roots, rhizomes and the bark of roots and stems. It is well-established that profiles of SMs vary with time, space and developmental stage. Since related plant species often show similarities in the profiles of their SMs, they have been used as taxonomic tool in plant systematic (Figure 2.).

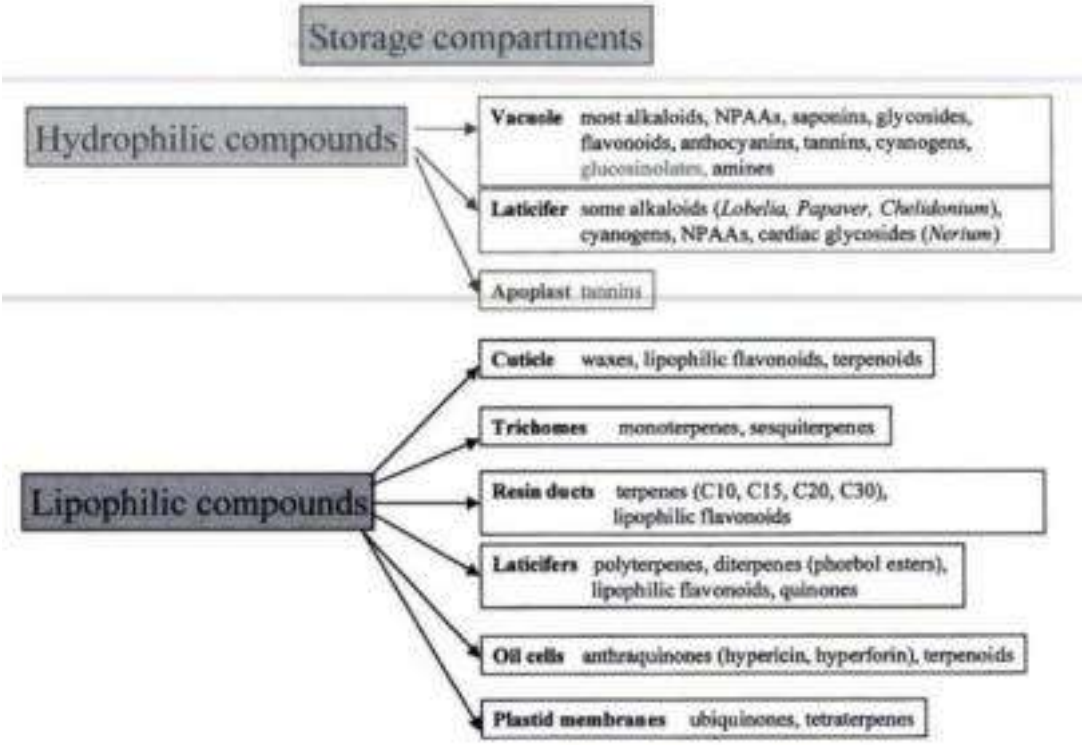

Figure 2. *Storage compartments for hydrophilic and lipophilic compounds*

6. Synthesis

When plants are under stress, an exchange occurs between carbon to biomass production or defensive secondary compounds. A stress response is induced when plants recognizes stress at the cellular level. Secondary compounds are involved in protective functions in response to both biotic and abiotic stress conditions.

Secondary metabolites in plants can be divided into three main groups according to their biosynthetic origin: terpenoids, nitrogen-containing compounds (alkaloids, glucosinolates and cyanohydrins) and phenylpropanoids also known as phenolic compounds. The most important building blocks employed in the biosynthesis of SM are derived from: acetyl coenzyme A, shikimic acid, mevalonic acid, and 1-deoxyxylulose-5-phosphate and, these are utilized respectively in the acetate, shikimate, mevalonate and deoxyxylulose phosphate pathways (Figure 3).

i. **Acetate pathway** for the synthesis of polyketides.
ii. **Acetate-mevalonate pathway** for the biosynthesis of terpenoids and steroids.
iii. **Acetate-malonate pathway** for the synthesis of fatty acids, Plumbagin (*Plumbago*, Plumbaginaceae).
iv. **Shikimate pathway** for the synthesis of Lignin and ligans, stilbes, glucosinolates, cyanohydrins, hydroxyl benzoic acid, Juglon (*Juglans*, Juglandaceae).
v. **Homogentisate pathway** for the synthesis of Chimaphylline (*Chimaphila*, Pyrolaceae).

vi. **Hydroxybenzoate pathway** for the synthesis of Alkannin (*Plagiobothrys*, Boraginaceae).

vii. **Phenylpropanoid pathway** for the synthesis of Lignin, and starting point of biosynthesis of flavonoids, coumarins and lignans.

viii. **Mevalonate/ Isoprenoid pathway** for biosynthesis of sterol isoprenoids (cholesterol) and non-sterol isoprenoids (dolichol, haem A, isopentyl tRNA and ubiquinone).

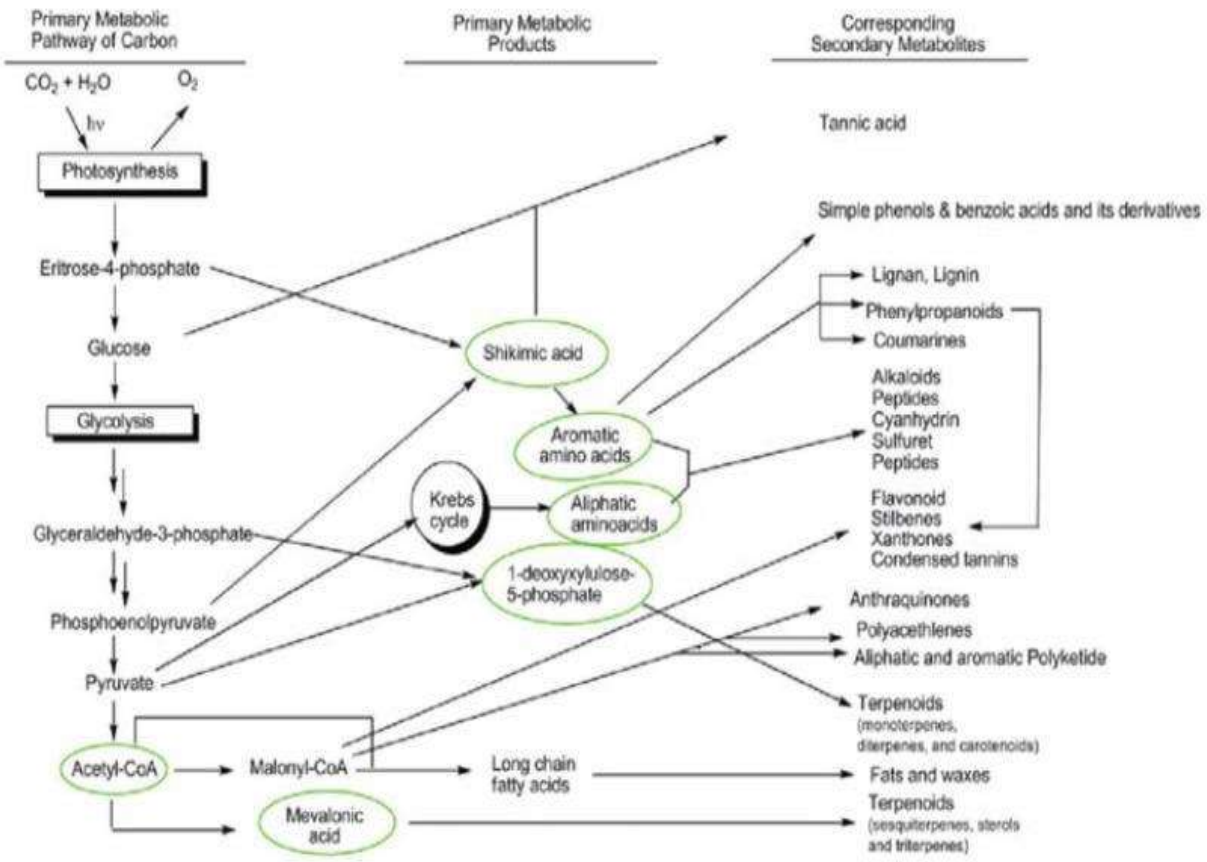

Figure 3. *Biosynthetic pathways of primary and secondary metabolites production*

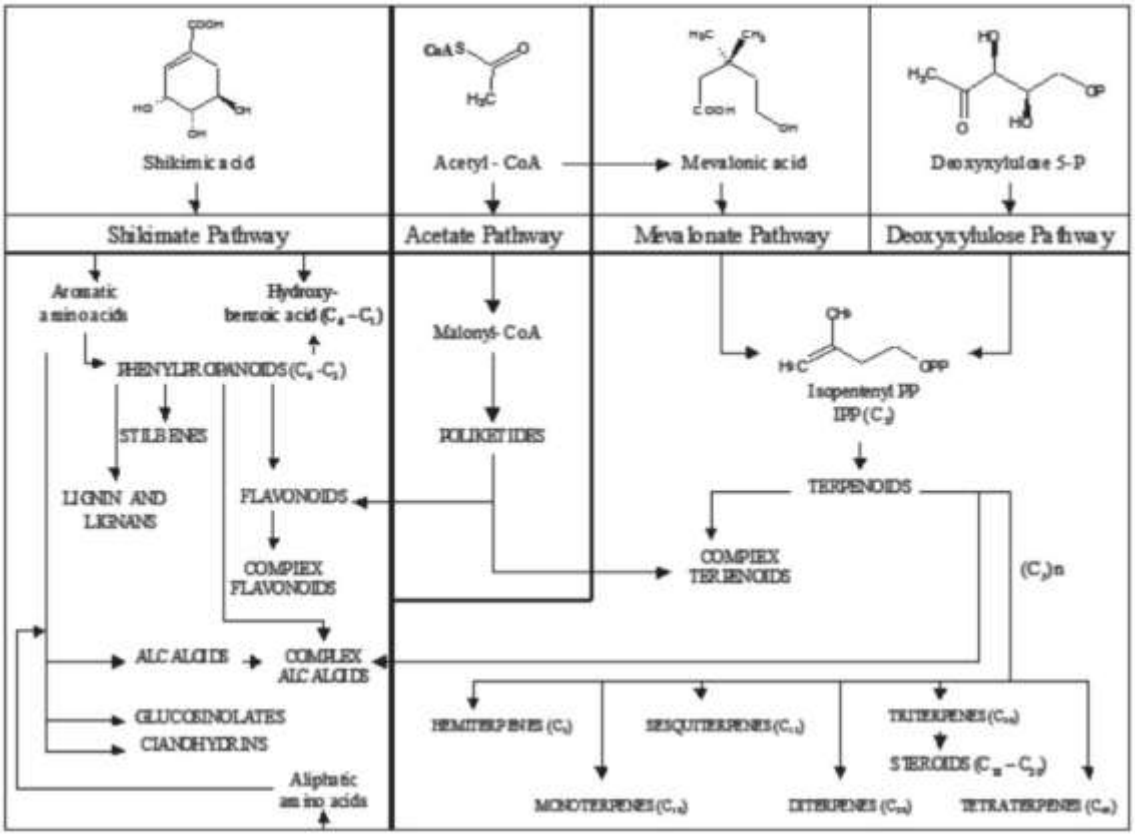

Figure 4. *Plant biosynthetic pathways for secondary compounds production (Ribera and Zuniga, 2012)*

7. Transport

Long distance transport of metabolites between source and sink tissues of plants is facilitated by transport pathways in two tissues: the xylem and the phloem. These consist of, respectively, dead xylem vessels facilitating upward movement of water and compounds from roots, and the sieve elements facilitating phloem movement from source to sink tissues. The source–sink transport route utilized predicts which membrane barriers metabolites must cross in order to access the long distance transport pathways and consequently the number and kind of transport proteins involved. Membrane barriers do not restrict the interface between the apoplast (extracellular space) and the xylem vessels, and therefore metabolites need only to be exported from cells into the apoplast adjacent to the xylem to access this transport pathway. In comparison, sieve elements and the associated companion cells are separated from the apoplast by a plasma membrane and entry into the phloem pathway typically requires the additional activity of a plasma membrane-localized importer.

Translocation of SMs from source to sink site is achieved via transport mechanism. Following mechanisms have been proposed through studies:

1. **Simple diffusion**

2. **Vesicle-mediated transport**
3. **Transporter-mediated membrane transport**

The identified transporter proteins for secondary metabolites can be roughly classified into 4 families:

1. ABC (ATP-binding cassette) transporter: The transporters export or import substrates using energy obtained from ATP hydrolysis. Plants use these transporters for the transport of primary metabolites, hormones, heavy metals, inorganic acids, xenobiotics etc. Usually the transportations are involved in detoxification and other physiological functions of the plant throughout their development.

2. NRT (nitrate-peptide transporter): Originally, the transporters were identified as transporters for nitrate or peptides, and probably function as proton symporters. Recently, they are reported as transporters of glucosinolates and plant hormones.

3. MATE (multidrug and toxic compound extrusion): These were presumed to function as proton antiporters. They are also reported to be involved in xenobiotic efflux, Fe translocation, Al detoxification, and hormone signaling.

4. PUP (purine permease): Transports substance through proton symporters. Substances include purine ring containing compounds (adenine, cytosine and cytokinins) and pyridine ring containing compounds (pyridoxine and pyridoxal).

8. Role of individual development stage

Concentration of SMs varies in different developmental stages of the plant. For example,

i. Alkaloid levels are higher in leaves as compared to other parts of the plants. It has also been observed that older parts contain lesser amounts as compared to younger parts of the same plant. Alkaloids store waste nitrogen, involves in cationic balance and protection against the parasites.
ii. Leaves in older trees of Eucalyptus globulus have been demonstrated to have higher essential oil content than leaves in younger trees. These patterns correspond well to the observed susceptibility of eucalypts to insect damage, with younger leaves and younger trees with lower essential oil content being more susceptible to insect herbivory than older leaves and older trees.
iii. The abundance of glucosinolates is highest in dormant and germinated seeds, followed by inflorescence, fruit, leaves, and roots. Higher concentrations of aliphatic glucosinolates, including methylthioalkyl and hydroxyalkyl glucosinolates, are present in the seed compared with other organs. Aliphatic glucosinolates are the predominant form in most organs, and indole glucosinolates are higher in roots and late-stage rosette leaves. During seed germination and leaf senescence, the content of glucosinolates decreases significantly.

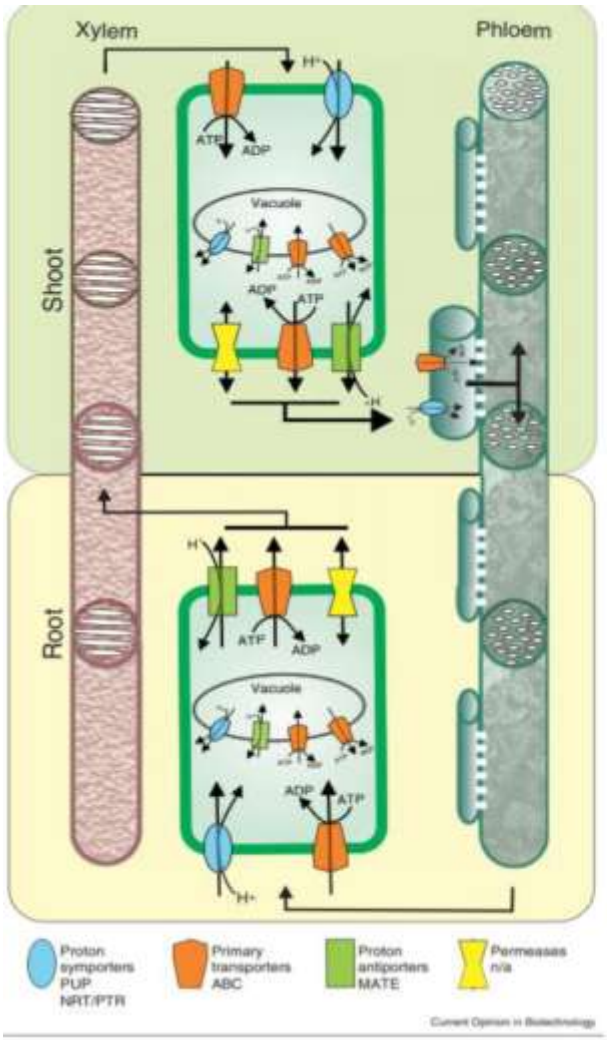

Figure 5. *Mechanism of transport of SM in plants*

Compounds	Xylem	Phloem
Quinolizidine alkaloids	–	+
Pyrrolizidine alkaloids	–	+
Aconitine	–	+
Polyhydroxy alkaloids (swainsonine)	–	+
Glucosinolates	–	+
Cardiac glycosides	–	+
Cyanogenic glycosides	–	+
Nicotine	+	–
Tropane alkaloids	+	–

Table 1. *Examples of xylem and phloem transport of SMs*

9. Selection process

For selection or isolation of pure compound or SM from the plant material, its extraction is must. **Extraction from the secretory tissues**

Tapping: The wounding of the plant in the area where secretory tissue is located to ensure sustained yields and tree health maintenance e.g. extraction of resins and latex.

i. Extraction of SM from plant material:

Extraction of SM from plants consists of classical and modern techniques. Both techniques use organic fluid (hexane, acetone, methanol, ethanol etc.) or water for extraction, but, the former approaches are carried out at normal atmospheric pressure, whereas, the latter techniques use high pressure and temperature.

The choice of solvent is an important step for differentiation of active components. The solvents move into the solid plant material and solubilize the compounds with similar polarity. The polar solvents extract out polar active compounds and the non-polar solvents extract out non-polar solvents. For better extraction, mixture of solvents is preferred.

A. Classical techniques

The traditional solid-liquid extraction methodologies are as follows:

a. **Decoction:** Distilled water is added to the dried extract and the mixture is subjected to heating continuously for a period of time at a temperature of 100°C. Then it is allowed to cool to room temperature and filtration is performed to obtain the filtrate. That filtrate is concentrated to obtained extract. The method is not advised for the extraction of heat sensitive compounds.
b. **Infusion:** In this method, extraction consist in soaking the solids plants powder either cold or boiling water for a short period of time.
c. **Soxhlet extraction:** During extraction with soxhlet, the process of distillation is implicated. It consist of heating a solution up to boiling and then condensed send back to the original flask. Practically, a limited quantity of dry material is introduced in a thimble. This thimble is then deposited in a distillation flask fill with specific solvent. After reaching to a submersion level, a siphon absorb the solvent in the thimble-holder and then release it back into the distillation flask. This solution contain the extracted solutes. This process is done continuously until the extraction is completed. The separation of the extract to the solvent is made using the device called Rotavapor. In this apparatus a vacuum evaporation is carried out using a vacuum pump with a check valve. During evaporation the ball is rotated and immersed in a heated liquid bath. The apparatus is fitted with a condensate collecting flask. Rotation of the balloon creates a greater exchange surface and therefore renewed for performing rapid evaporation.
d. **Maceration:** Maceration involve three principals' steps. Firstly, plant materials is convert to powder form by grinding. This allow good contact between solvent and material the surface area for proper mixing with solvent. After grinding, a chosen solvent is added in a closed vessel. Then, the liquid is strained off but the solid residue of this extraction process is pressed to recover large amount of occluded solutions. During the process of maceration occasional shaking facilitate extraction by increasing diffusion and remove concentrated solution from the sample surface for bringing new solvent to the menstruum for more extraction yield.
e. **Hydrodistillation:** Plant materials are packed in a still compartment and water is added in sufficient amount, and then brought to boil. Alternatively, direct steam is injected into the plant sample. Hot water and steam act as the main influential factors to free bioactive compounds of plant tissue. Indirect cooling by water condenses the vapor mixture of water and oil. Condensed mixture flows from condenser to a separator, where oil and bioactive compounds separate automatically from the water.

B. Modern techniques

a. **Ultrasound assisted extraction (UAE):** The enhancement in extraction obtained by using ultrasound is mainly attributed to the effect of acoustic cavitations produced in the solvent by the passage of an ultrasound wave. Ultrasound also exerts a mechanical effect (ultrasonic waves break the cell walls), allowing greater penetration of solvent into the tissue, increasing the contact surface area between the solid and liquid phase. Then we assist in a situation where the solute quickly diffuses from the solid phase to the solvent.

b. **Microwave assisted extraction (MAE):** The principle of heating using microwave is based upon its direct impacts on polar materials. Electromagnetic energy is converted to heat following ionic conduction and dipole rotation mechanisms. Microwaves penetrate into biomaterials and generate heat by interacting with polar molecules such as water inside the materials. Then the penetration of microwaves depth into plant matrix depends on dielectric constant, moisture content, temperature, and the frequency of the electrical field. The water contained in a plant material is responsible for the absorption of microwave energy which led to internal superheating and cell structure disruption. This action created the diffusion of bioactive compound from the plant matrix. The surrounding extraction solvent can remain cold if it dielectric constant is low and this can be advised for extraction of heat sensitive compounds. Toluene and hexane are suggested for MAE because of their low dielectric constant compare to water, ethanol and methanol which are polar enough and able to strongly absorb microwaves energy. Also mixture of solvent is possible if the extracting selectivity and modulation of interaction between solvent and microwave energy is objected. The extraction mechanism of microwave assisted extraction involves three sequential steps. Firstly, solutes from active sites of sample matrix are separated under increased temperature and pressure; secondly, solvent diffused across sample matrix and thirdly, solutes are released from sample matrix to solvent. The frequency range of non-ionizing electromagnetic fields is 300 MHz to 300 GHz.

c. **Supercritical fluid extraction (SFE):** The supercritical fluid extraction, particularly by supercritical Carbon dioxide (CO2) (because CO2 is close to room temperature, and it has low critical pressure that offers the possibility to operate at moderate pressures, generally between 100 and 450 bar) was introduced as an alternative to the extraction methods using solvent. Several solvents can be used for SFE, such as, hexane, pentane, butane, nitrous oxide, sulfur hexafluoride, and fluorinated hydrocarbons. Carbon dioxide is the most commonly used extraction solvent in SFE. CO2 alone is non selective but its capacity and selectivity of extraction can be improved by using a co-solvent or modifier. After the extraction co-solvent can easily be removed.

10. Downstream process

Isolation of pure compounds

Chromatographic and non-chromatographic techniques are employed to get pure compound from complex matrices.

A. Chromatographic techniques

The basic principle of these techniques is that components in a mixture have different tendencies to adsorb onto a surface or dissolve in a solvent. It is a powerful method in industry, where it is used on a large scale to separate and purify the intermediates and products in various syntheses. Following are the chromatographic techniques:

a. **Gas chromatography (GC):** GC is most useful for the analysis of trace amounts of organically extractable, non-polar, volatile compounds and highly volatile compounds. Moreover, the use of GC-MS in the scan mode allows for non-targeted metabolic profiling and the discovery of novel compounds and metabolites (Krone et al., 2010). In GC mobile phase is gaseous. The mixture to be analyzed is vaporized into the column. The stationary phase in the column can be solid or liquid. Gas chromatography (GC) and GC-MS with high specificity, high sensitivity, stability and small amount of sample characteristics, are unanimously accepted as the method for the analysis of volatile constituents. Moreover, the high selectivity of capillary columns enables separation of many volatile compounds simultaneously within very short time. GC-MS has limitations in the analysis of highly polar compounds due to their thermolability and low volatility.
b. **High performance liquid chromatography (HPLC):** Using HPLC, the compound of interest is separated or extracted to the target compound from other compounds or contaminants. To get an optimum separation of each compound, the chromatographer may choose the appropriate conditions, such as the proper mobile phase, flow rate, suitable detectors and columns base in the fact that any compound have a characteristic peak under certain chromatographic condition.
c. **Thin-layer chromatography (TLC):** TLC is a common method for herbal analysis because of its simplicity, rapidity and economy. A major advantage of TLC is that it can provide the light images and fluorescence images, which is one more visual parameter than Chromatograms, and also give different levels of profiles and corresponding integral data with chromatography scanning and digital processing. But TLC analysis also has short comings: low resolution, low sensitivity and the difficulty of detection of trace components, etc.

B. Non-chromatographic techniques

a. **Phytochemical screening assay:** By employing different chemical tests, phytochemicals present in crude extract or active fraction from parent material can be detected.

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