In Vitro Production of Alkaloids

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Abstract

Plants are considered as a potent source of a wide variety of bioactive molecules that can be used for the development of the various pharmaceutical drugs. Alkaloids are the important class of secondary metabolites, known to exhibit therapeutic properties including anti-tumor, anti-viral, anti-inflammatory, and anti-malarial activities. Alkaloids are able to prevent various degenerative diseases by binding with the oxidative reaction catalyst or free radicals. The commercial extraction of alkaloids is reported from some major families like Apocynaceae, Papaveraceae, Rubiaceae, and Solanaceae. By this system, the yield of alkaloids is inconsistent due to genetic and geographical variations. Chemical synthesis is still not feasible system due to complex molecular structure of various metabolites. Therefore, in vitro system for production of alkaloids has become a promising biotechnological approach from a range of medicinal plants. Some of the medicinal plants such as *Nicotiana tobaccum* (nicotine), *Erythroxylum coca* (cocaine), *Cinchona officinalis* (quinine and quinidine), *Rauwolfia serpentina* (reserpine), and *Pilocarpine microphyllus* (pilocarpine) have been explored for in vitro production of their respective alkaloids. The present chapter provides brief information on various in vitro production systems and scale-up techniques used for alkaloid production.

Keywords

Alkaloids · Biosynthesis · Extraction · Biological activities · Bioreactor · Plant cell culture

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6.1 Introduction

Plants have always been the major source for the traditional medicine systems, and they have provided various remedies in therapeutic application for thousands of years (Ramawat et al. 2009). Generally, plants are under selection pressure to protect themselves from pathogenic microbes and insects, whereas these pathogens struggle for their survival from plant defense to obtain food and reproduction site. Therefore, both plants and pathogens need to develop tactics to adapt or adjust with each other and the changing environment. Then the co-evolution of secondary metabolites is a consequence of the biological processes in plants that regulate defense mechanism (Ramawat and Goyal 2019). Alkaloids are one of the major secondary metabolites obtained from plants. Ubiquitous distribution of alkaloids is found in the plant kingdom mainly in higher plants, such as those belonging to Ranunculaceae, Leguminosae, Papaveraceae, Menispermaceae, and Loganiaceae (Ramawat and Mérillon 2013; Kandar 2021).

These compounds have been classified into various categories which include indole, piperidine, tropane, purine, pyrrolizidine, imidazole, quinolizidine, isoquinoline, and pyrrolidine alkaloids on the basis of their biosynthetic precursor and heterocyclic ring system (Waller 2012). In vitro cell, tissue, or organ culture has been employed as a probabilistic alternative to produce such industrial compounds. Tissue culture techniques can be used for the large-scale culture that provide continuous, reliable, and renewable source of valuable plant pharmaceuticals to extend commercial importance of plants to emerge or identify their biological activities which includes anti-tumor, anti-viral, anti-inflammatory, anti-malarial activities (Debnath et al. 2018). There are up to 80% of people in developing countries who are totally dependent on herbal drugs for their primary healthcare, and over 25% of prescribed medicines in developed countries are derived from wild plant species (Hamilton 2004). As there is an increasing demand of medicinal plants for herbal drugs, natural health products, and secondary metabolites, the use of medicinal plants is growing rapidly throughout the world. Consequently, some of them are increasingly being threatened even in their natural habitats, and some also face natural extinction (Chen et al. 2016). Therefore, in the production of desirable medicinal compounds from plants in search for alternatives, cell and tissue culture technologies were emerged as a possible tool for studying, and producing plant secondary metabolites as in vitro regeneration holds enormous potential for the synthesis of high-quality plant-based medicines (Rohini 2020). Techniques like somaclonal variations and genetic manipulations may be utilized to improve the production of alkaloids. The in vitro cell culture system is more beneficial than the conventional in vivo cultivation of whole plants in context of production of desirable compounds under controlled conditions independent to climatic factors or soil compositions and reduces labor costs and improves productivity as automated control of cell growth and rational regulation of metabolite processes. These cultured cells would be free of microbes and insects. Another benefit of cell culture is that the cells of any plants, tropical or alpine, could be easily multiplied to yield their specific metabolites in any artificial conditions. In vitro production of alkaloids is an

impactful technique to develop the large-scale scenario in pharmaceutical industries. Callus culture assisted the optimization of alkaloid production. Media composition is significant for the callus induction to enhance the alkaloid content and conservation of threatened genotype (Hussain et al. 2012).

6.2 Biosynthetic Pathway

According to Ramawat and Mérillon (2013), alkaloids are classified into three major categories on the basis of their origin and structure:

1. **True-alkaloids**: These are derived from amino acids and containing nitrogen moiety in their heterocyclic ring and found to be basic in nature. These alkaloids are highly reactive molecules with biological activity even in low doses.
   Examples: Nicotine, morphine, ergotamine, quinine, atropine, etc.
2. **Proto-alkaloids: These are also derived from amino acids**, but nitrogen is absent in their heterocyclic ring.
   Examples: Ephedrine, mescaline, etc.
3. **Pseudo-alkaloids**: Alkaloid-like compounds that do not originate from amino acids. It includes mainly terpenoid, steroid, and purine-like alkaloids. So they are also called as steroidal alkaloids.
   Examples: caffeine, pinidine, coniceine, etc.

The precursors of alkaloids biosynthesis are mainly amino acids (Fig. 6.1). Furthermore, the diversity of alkaloids depends on their precursor molecule and their structure. Alkaloid biosynthesis is a sequenced process in context of plant development, controlling the expression of genes in pathways, inside specific cells or organelles. Their biosynthesis and accumulation depends on developmental stage and environmental conditions and also have cell or tissue specific regulations. The degree of expression of the genes involved in the biosynthetic pathway of a particular alkaloid affects the accumulation of that metabolite (Ziegler and Facchini 2008). Alkaloid biosynthesis and accumulation is increasing with the number of genes involved in it. Generally, biosynthesis starts from the fixation of atmospheric CO_2 in primary carbon metabolism. The erythrose-4-phosphate (PPP pathway intermediate) and phosphoenolpyruvate (glycolysis intermediate) go through the shikimic acid pathway (Fig. 6.2) to form aromatic amino acids and pyruvate (end product of glycolysis) followed by acetyl coA and then go through TCA cycle to form aliphatic amino acids. These two types of amino acid constitute for synthesis of a range of N-containing compounds (alkaloids). This is followed by a series of reactions such as bond formations, breakages, rearrangements, addition, and modification of functional groups, yielding a vast range of various alkaloids. The pathway begins with two substrates, phosphoenolpyruvate and erythrose-4-phosphate, and ends with chorismate (substrate for the three aromatic amino acids – Tyr, Phe, Trp). This pathway includes seven steps regulated by seven enzymes: DAHP (3-deoxy-D-arabino-heptulosonic acid-7-phosphate) synthase, 3-dehydroquinate synthase,

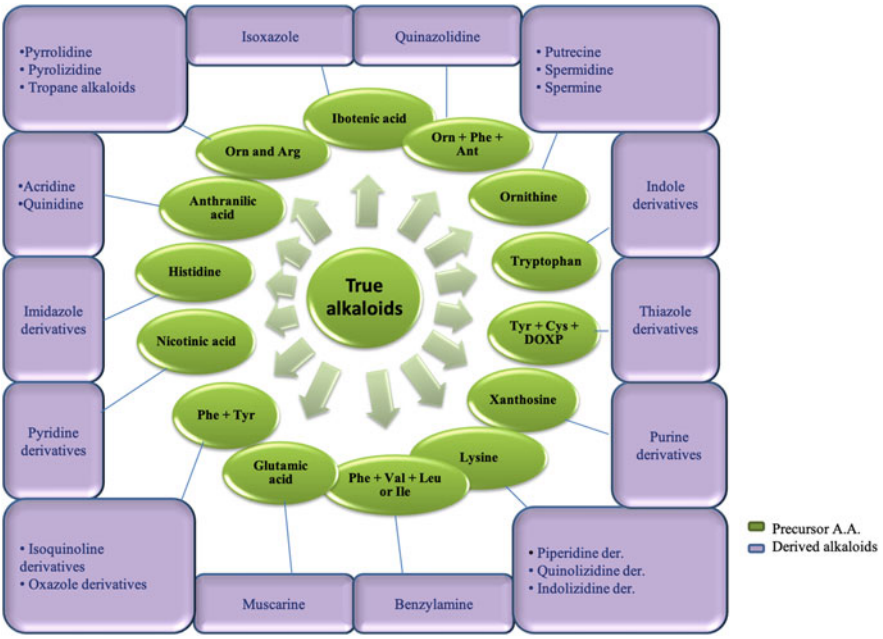

Fig. 6.1 Precursors of some true alkaloids for biosynthesis

3-hydratase, shikimate dehydrogenase, shikimate kinase, EPSP (3-enolpyruvylshikimate-5-phosphate) synthase, and chorismate synthase (Pathak et al. 2019). After that, a particular group of alkaloid followed a different pathway to complete its biosynthesis. Here are some examples of complete biosynthesis of alkaloid category.

1. **Amaryllidaceae** alkaloids use L-tyrosine and L-phenylalanine as precursors. From phenylalanine, the phenylpropanoid pathway leads the formation of 3, 4-dihydroxybenzaldehyde (3, 4-DHBA) to synthesize aldehyde (-CHO) moiety of Amaryllidaceae alkaloid. The other pathway to synthesize tyramine (the amine $–NH_2$ moiety of Amaryllidaceae alkaloid) is from tyrosine and its concretion with 3, 4-DHBA to form the central precursor norbelladine as well as its pursuant *O*-methylation occurs. The phenol coupling of the 4-*O*-methylnorbelladine in intermediate pathway ensued by a reduction step will progress in a series of unstable intermediates. The pathway(s) for the different Amaryllidaceae alkaloid's biosynthesis found in different plant species and their pathways remain still uncharacterized (Desgagné-Penix 2020).
2. The biosynthesis of **quinolizidine** alkaloids (QAs) starts with the formation of cadaverine as intermediate by the decarboxylation of L-lysine. The cadaverine then undergoes oxidative deamination, by a copper amine oxidase enzyme to yield 5-aminopentanal which is then spontaneously cyclized to 11-piperidine Schiff base. In addition to these reactions, a chain of reactions including Schiff

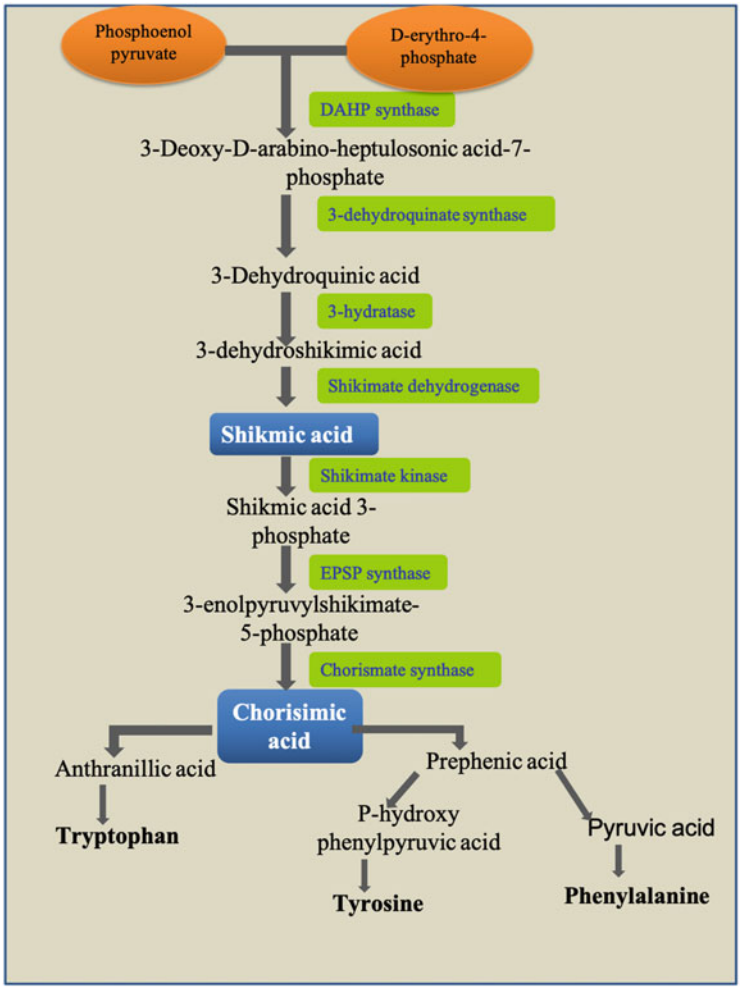

Fig. 6.2 Shikimic acid pathway

base formations, aldol-type reactions, hydrolysis, oxidative deamination, and coupling generates the major structural QAs. The alkaloidal group can be further modified by dehydrogenation, oxygenation, hydroxylation, glycosylation, or esterification to form a wide range of structurally related QAs (Frick et al. 2017).

3. The biosynthesis of **benzylisoquinoline alkaloids** (BIAs) initiates the formation of dopamine and 4-hydroxyphenylacetaldehyde by using L-lysine as precursor amino acid, which are then condensed to (S)-norcoclaurine by (S)-norcoclaurine synthase (NCS). One cytochrome P450 [(S)-*N*-methylcoclaurine 30-hydroxylase] and three methyltransferases [(S)-norcoclaurine/norlaudanosoline 6-Omethyltransferase, (S)-coclaurine-*N*-methyltransferase, and (S)-30-hydroxy-*N*-methylcoclaurine-40-omethyltransferase] are involved in

catalyzing the conversion of (S)-norcoclaurine to a central intermediate, (S)-reticuline, for the production of different BIAs, i.e., protoberberine, benzophenanthridine, and morphinan alkaloids (Beaudoin and Facchini 2014; Yamada et al. 2017). BIA biosynthesis consists of several steps (describes above), followed by multistep transformations that yield structurally different end products (Beaudoin and Facchini 2014; He et al. 2018).

In spite of these, biosynthetic pathway(s) of tropane and granatane alkaloids have also been reported. They belong to the pyrroline and piperidine classes of plant alkaloids, respectively (Kim et al. 2016).

6.3 In Vitro Production Methodology

Many plant secondary metabolites have been commercially produced by extraction and purification from plant materials either they are naturally present or field cultivated. It is known that the amount of produced alkaloid in naturally growing plant is very low, due to environmental or seasonal variations, nutrient availability, and stress conditions, and it is often restricted to a genus or species and might be activated only during a particular growth or developmental stage, which hinders the biological study of the compounds (Bagnères and Hossaert-McKey 2016). On the other hand, using wild plant materials has considerable risk related to extinction of many valuable and even endemic species. Hereupon, to increase the production of various alkaloids for remedial applications, two approaches have been proposed. The primary one is a total chemical synthesis that is complex and not much effective. The second is in vitro culture by using different plant parts (explants) to enhance the secondary metabolites content (Fig. 6.3). So, plant cell and tissue culture techniques have been investigated extensively as an alternative method for production of secondary metabolites of commercial interest since the end of the 1950s (Davies and Deroles 2014; Ramawat 2021). Plant secondary metabolites can be produced by two major groups of in vitro cultures: organized cultures of differentiated tissues (i.e., organ cultures as root, shoot, and embryo cultures) and unorganized cultures of undifferentiated cells (i.e., cell suspension and callus cultures) (Fig. 6.4). Investigations have showed that differentiated plant tissues produce the identical product as the plant produce itself, and they were relatively more stable in the production of secondary metabolites than the undifferentiated cells (Nielsen et al. 2019). Shoot cultures are used for many medicinal plants, which accumulate secondary metabolites much greater than that of natural plants. Besides, many of the valuable secondary metabolites like tropane alkaloids, hyoscyamine, and scopolamine are produced quite well in the root cultures (Filova 2014). However, plant roots in cultures generally grow slower than undifferentiated plant cells, and their harvesting is difficult. Therefore, plant hairy root cultures have been applied as an alternative method for the production of compounds synthesized in the plant roots. Hairy roots obtained by *Agrobacterium rhizogenes*-mediated transformation exhibits higher growth rates than cell suspension cultures and produce secondary

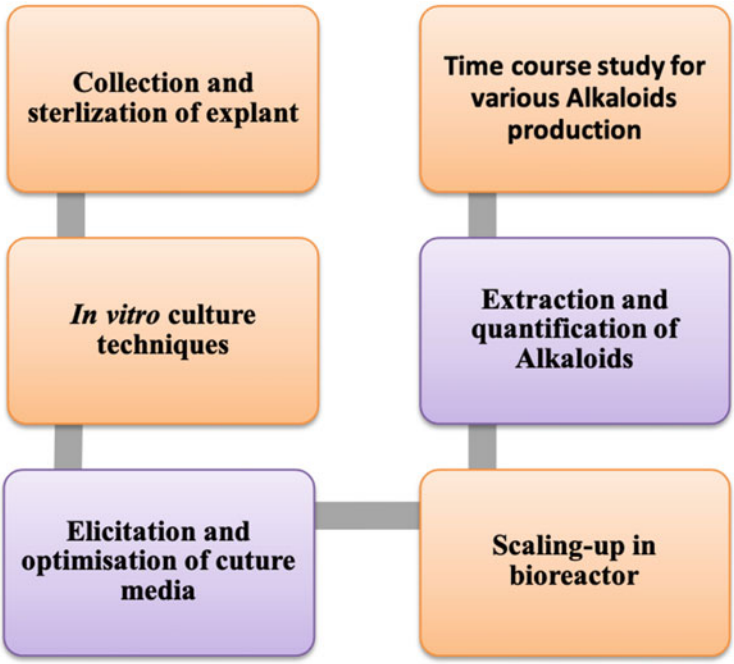

Fig. 6.3 Schematic presentation of in vitro alkaloids production

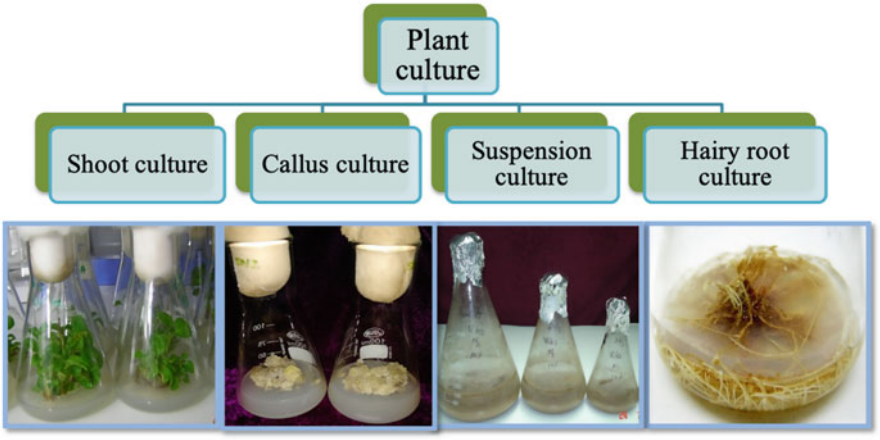

Fig. 6.4 Types of in vitro cultures used in alkaloids production from certain medicinal plants

metabolites over successive generations without losing genetic or biosynthesis stability (Mehrotra et al. 2015a, b). Huang et al. (2018) reported a sanguinarine alkaloid from *Macleaya cordata* hairy root cultures by co-cultivating leaf and stem explants with *A. rhizogenes*. Furthermore, production of two different secondary

metabolites is feasible by simultaneously co-culturing of adventitious roots. Natural adventitious roots are induced in many medicinal plants via flask scale to large-scale bioreactor cultivation for the production of several bioactive compounds (Baque et al. 2012). Non-embryogenic plant callus cultures, consisting of more or less homogeneous clumps of dedifferentiated cells, are used for production of secondary metabolites. During the past decades, plant cell suspension cultures initiated from callus cultures have been extensively studied and have emerged as attractive alternatives for production of the range of valuable compounds found in the whole plant. However, production of many of the pharmaceuticals is just too low or may be zero in cultured cells due to controlling their production by a tissue-specific manner and loss of production capacity resulting from dedifferentiation.

In recent years, various strategies have been developed for enhanced biomass accumulation and regulation of biosynthesis of secondary metabolites, such as selection of cell lines, optimization of medium and culture conditions, elicitation, immobilization, nutrient and precursor feeding, permeabilization, and biotransformation techniques (Filova 2014; Bagnères and Hossaert-McKey 2016). Secondary metabolite accumulation in plants is genotype- and tissue-specific, so explants should be selected from elite parent plants and tissues, which have higher contents of desired compound, to initiate cell and organ cultures. Levels and types of various chemical components, such as carbohydrates, nitrate, phosphate, and growth regulators which could affect biomass accumulation and biosynthesis of secondary metabolites in plant cell and organ cultures, have been taken into consideration to optimize the medium. Agitation and aeration are also important factors that ought to be controlled in flask-scale to large-scale bioreactor cultures for optimization of biomass growth and secondary metabolite production (Murty et al. 2014). Secondary metabolites are being synthesized in plant cells to retort various abiotic (e.g., temperature, salinity, water, heavy metal, etc.) and biotic (e.g., pathogen or insects) stresses. Therefore, these stress factors have been designated as "elicitors" to induce biosynthesis of secondary metabolites. The number of parameters, such as concentration of elicitors, types, exposure duration, cell line, nutrient composition, and culture age or stage, is also crucial factors influencing the successful production of biomass and alkaloid accumulation (Naik and Al-Khayri 2016). Unfortunately, elicitation does not always result in product of interest because it activates certain plant species in the specific pathway. By utilizing preexisting enzyme systems, many plant cell cultures have also been used to convert precursors into products. Biotransformation is another technique which can be utilized for the high production of selected metabolites using plant cell and organ cultures (Bhatia and Bera 2015). In spite of this, the yield of secondary metabolites continues to be economically insufficient and expensive in many cases. Therefore, metabolic and genetic engineering techniques have also been incorporated into plant cell cultures to boost the production of secondary metabolites via regulation of their biosynthesis (Wilson and Roberts 2012). Some alkaloidal plant sources that have been used for in vitro cultures are mentioned in Table 6.1.

Table 6.1 Types of in vitro cultures of alkaloid producing various medicinal plants

Plant name	Active alkaloids	Culture medium	Culture type	Results	Reference
Atropa belladonna	Tropane alkaloids	Seeds + MS medium with 3% sucrose, 0.1 mg/l indole acetic acid (IAA) and 1 mg/l benzyladenine (BA)	Hairy root culture	The highest amount of atropine was observed; however, there were no differences in the amount of scopolamine, where the scopolamine content was significantly decreased	Chashmi et al. (2010)
Alstonia scholaris (Apocynaceae)	Indole alkaloids (echitamine, acetylechitamine, tubotaiwine, and picrinine)	Leaf + MS medium, 0.3 mg/l 2,4-D, 0.5 mg/l FAP and 3% sucrose. Elicited by MJ, PEG, and CHI	Callus culture	Enrichments of acetylechitamine (6.3780 mg/g DW, i.e., ~ 15-fold) and echitamine (1.6513 mg/g DW, i.e., ~ 12-fold) were found with 4.5 g/L KCl in 10 days incubation period, followed by tubotaiwine (0.0952 mg/g DW, i.e., ~ fourfold) with 3.0 g/L KCl in 10 days and picrinine (0.3784 mg/g DW, i.e., ~ fourfold) with 4.5 g/L KCl	Jeet et al. (2020)
Nicotiana rustica (Solanaceae)	Nicotine	Gamborg's B5 medium with 20 g/l sucrose and 3 g/l phytagel	Hairy root culture	Alteration of aeration results decreases nicotine accumulation	Zhao et al. (2013)
Papaver orientale (Papaveraceae)	Morphine, thebaine, codeine	Seed + Gamborg's B5 liquid medium, 3% sucrose, 300 mg/l cefotaxime, 1.0 g/l PVP, 15 mg/l ascorbic acid. Elicited by methyl jasmonate	Hairy root culture	Enhanced thebaine, codeine, and morphine by 2.63-fold (3.08 mg g-1), 3.67-fold (2.57 mg g-1), and 6.18-fold (5.38 mg g-1), respectively	Hashemi and Naghavi (2016)
Catharanthus roseus (Apocynaceae)	Vinca alkaloids	Leaf + MS medium supplemented with 1.5 mg/L BAP and 1.5 mg/L 2,4D	Callus culture	Vinblastine showed a 3.39-fold increase compared to the wild plant	Mekky et al. (2018)

(continued)

Table 6.1 (continued)

Plant name	Active alkaloids	Culture medium	Culture type	Results	Reference
Hyoscyamus niger (Solanaceae)	Tropane alkaloids	Leaf explants + MS medium, 3% sucrose supplemented with antibiotics (cefotaxime and amoxicillin, 500 mg/l)	Hairy root culture	The amount of cuscohygrine was (7.079 mg/g dry wt) more than 20-fold higher than the concentrations of anisodamine, therefore in order to determine the anisodamine content	Jaremicz et al. (2014)
Securinega suffruticosa (Phyllanthaceae)	Indolizine alkaloids	Callus + SH medium, 3% sucrose, 5.0 mg/l 2,4-D, 5.0 mg/l kinetin	Callus culture	The highest concentrations of securinine (1.73 mg g-1 DW) and allosecurinine (3.11 mg g-1 DW) were observed	Raj et al. (2015a, b)
Macleaya cordata (Papaveraceae)	Benzylisoquinoline alkaloids (protopine, sanguinarine, dihydrosanguinarine)	Leaf and stem + MS solid medium, 30 g/L sucrose, 8 g/L agar	Hairy root culture	The contents of 3 alkaloids (PROT, DHSAN, SAN) were significantly higher in hairy root cultures than in wild plant	Huang et al. (2018)
Hyoscyamus reticulatus (Solanaceae)	Hyoscyamine and scopolamine	Seeds + MS medium, 3% sucrose, 7.2 g/l agar, and 0.1 g/l myo-inositol and 200 mg/l cefotaxime elicited by iron oxide nanoparticles (FeNPs) at different concentrations (0, 450, 900, 1800, and 3600 mg L^{-1})	Hairy root culture	Highest hyoscyamine and scopolamine production (about fivefold increase over the control) was achieved with 900 and 450 mg L^{-1} FeNPs	Moharrami et al. (2017)
Hyoscyamus muticus (Solanaceae)	Hyoscyamine	Shoot tip + MS media +0.5 mg/l BAP, 0.5, 1 and 2 mg/l NAA, pH (5.7–5.8)	Callus culture	Total alkaloids increased by twofold at 10 dS/m compared to control or wild leaves	Abdelrazik et al. (2019)
Pancratium maritimum (Amaryllidaceae)	Amaryllidaceae alkaloids	Fruit slice + MS medium, 3% sucrose, 1.15 mg/L NAA and 2.0 mg/L BAP	Shoot culture	Twenty-two compounds of different structural types of the Amaryllidaceae alkaloids (tyramine, narciclasine, galanthamine, haemanthamine, lycorine, pancracine, tazettine, and homolycorine types) were detected in the studied samples	Georgiev et al. (2011)

6.4 Scale-Up Techniques and Bioreactors

The extraction method of alkaloids from the plant sources merely depends upon the objective and scale of the operation (pilot-scale or laboratory scale). It is also based on the quantum and bulk of stuff to be employed in the operation. For using commercially, it is required to develop the sufficient amount of alkaloids. A scale-up technique must be needed to obtain the plant's by-products, which is accomplished with no reduction in alkaloid productivity and bioactivity. A bioreactor (Figs. 6.5 and 6.6) is a device that supports a biologically active environment

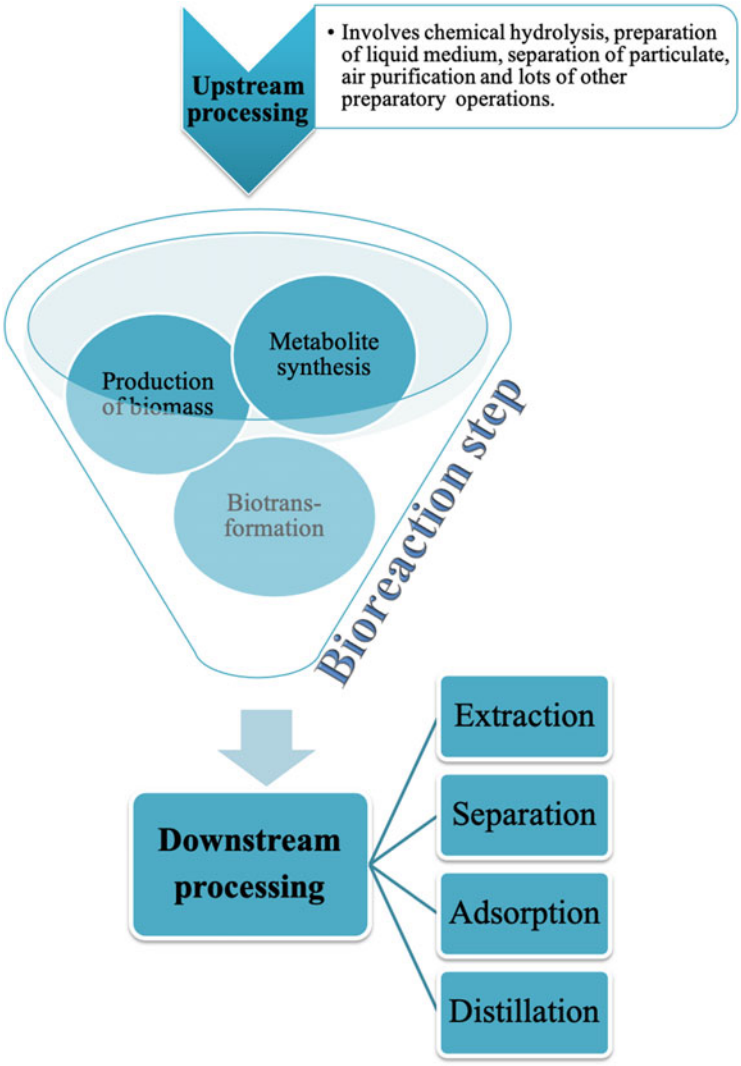

Fig. 6.5 Bioreactor processing

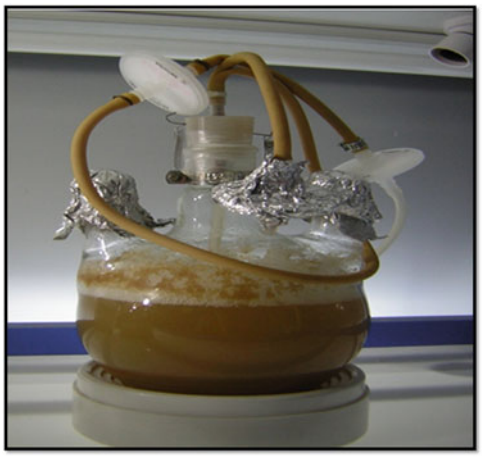

Fig. 6.6 Scale-up techniques for enhanced production of secondary metabolites

(aerobic or anaerobic) and allowing continuous extraction of alkaloids by using tissue culture techniques, i.e., hairy roots, suspension culture, etc. Various capacity and designs of bioreactors (Table 6.2) have been widely used for growing cell cultures of different plants, but growth of organized cultures should be started from a smaller capacity bioreactor (shake flasks). Cell cultures have been grown in both static and liquid cultures. These cell cultures are exposed to biotic and abiotic elicitors to increase alkaloid production. The biotransformation of added precursors and exploitation of variant cell strains can surely improve the employment of cell cultures for the production of desired compounds. The two facets of the examined bioreactor are upstream (elicitation, scale-up experiments) and downstream processing which involves permeabilization and in situ extraction (Ruffoni et al. 2010). In upstream processing, the raw material of alkaloidal source becomes more suitable for the processing which involves chemical hydrolysis, preparation of liquid medium, particulate separation, air purification, and lots of other preparatory operations. After that, the resulting feed is transferred to multiple bioreaction stages (Rosser and Thomas 2018). Three operations, production of biomass, metabolite biosynthesis and biotransformation, are included in the bioreaction step. Finally, the produced material must be further processed in the downstream section to transform it into a more beneficial form. The downstream process mainly comprises of physical separation operations such as solid-liquid separation, adsorption, distillation, liquid-liquid extraction, drying, etc. (Hatti-Kaul 2010).

A research study on production of tropane alkaloids by transformed hairy root cultures of *Atropa belladonna* in stirred bioreactors is reported. In this, the transformed roots of *A. belladonna* conserved the ability of growth and tropane alkaloid biosynthesis after a random cut treatment. Cut roots were inoculated and immobilized on a stainless-steel mesh, which resulted in the good distribution in the modified stirred bioreactor for a scale-up culture. This sort of bioreactor would help provide a sufficient supply of oxygen and nutrition for root growth and alkaloid production (Lee et al. 1999). Hairy root cultures of *Hyoscyamus niger* (black

Table 6.2 Different types of bioreactor used for enhancement of the alkaloid content

Bioreactor type		Plant source	Bioreactor conditions	Enhanced alkaloid	Reference
Liquid phase Submerged connective flow bioreactor	Stirred tank bioreactor Bubble column bioreactor	*Catharanthus roseus*	Air flow rate 4 vvm and stirring speed 100–120 rpm	Ajmalicine, catharanthine, serpentine	Verma et al. (2012)
		Datura stramonium	Aeration rate 15.0 vvm	Tropane alkaloids	Marchev et al. (2012); Pavlov (2012)
		Papaver somniferum	Air flow rate 2 vvm and rotation speed 70–100 rpm	Sanguinarine	Verma et al. (2014)
		Uncaria tomentosa	Impeller tip speed 95 cm/s and agitation speed 400 rpm	Monoterpenoid oxindole alkaloid	Trejo-Tapia et al. (2005, 2007)
		Brugmansia candida	Air flow rate 0.5 vvm and agitation speed 50 rpm	Scopolamine, anisodamine, and hyoscyamine	Cardillo et al. (2010)
	Bubble column bioreactor	*Stephania glabra*	Air flow rate 0.1–1.0 vvm and agitation speed 30–65 rpm	Stepharine alkaloid	Titova et al. (2012)
		Securinega suffruticosa	Aeration rate 800 ml/min	Indolizidine alkaloids	Raj et al. (2015a, b)
		Catharanthus roseus	Aeration rate 0.3 vvm	Ajmalicine	Thakore et al. (2017); Fulzele and Namdeo (2018)
		Tripterygium wilfordii	Air flow rate 5 L/min, pressure 0.05 MPa	Wilforgine and wilforine (sesquiterpene)	Miao et al. (2013)
		Leucojum aestivum	Shaking at 50 rpm, immersion and gassing (continuous and discontinuous)	Galanthamine	Georgiev et al. (2012); Schumann et al. (2012); Ptak et al. (2013)
	Bubble column and spray bioreactor	*Hyoscyamus niger*	Aeration rate 0.8 vvm	Tropane alkaloids (scopolamine, cuscohygrine, anisodamine)	Jaremicz et al. (2014)

(continued)

Table 6.2 (continued)

Bioreactor type		Plant source	Bioreactor conditions	Enhanced alkaloid	Reference
	Air sparged and mechanically agitated bioreactor	*Rauwolfia serpentine*	Aeration rate 0.2–1.2 vvm and agitation rate 50–200 rpm	Indole alkaloids	Mehrotra et al. (2015a, b)
Gas phase	Balloon-type airlift bioreactor	*Dendrobium candidum*	Aeration rate 0.1 vvm, temp. 25 ± 2 C with 70% relative humidity	Alkaloids	Yang et al. (2015)
	Liquid-liquid impelled loop bioreactor	*Atropa belladonna*	Agitation (40, 70, 110 rpm) and aeration (0.75, 1.25, 1.75 vvm)	Scopolamine	Habibi et al. (2015)
	Siphon-mist bioreactor	*Pseudostellaria heterophylla*	Air flow rate 0.1 to 0.7 vvm by adjusting the gas pump	Alkaloids	Wang and Qi (2010)

henbane) were cultivated in shake flasks, a bubble-column bioreactor, and a hybrid bubble-column/spray bioreactor for anisodamine, scopolamine, hyoscyamine, and cuscohygrine alkaloids production (Jaremicz et al. 2014). *Brugmansia candida* produces tropane alkaloids (hyoscyamine, 6β-hydroxyhyoscyamine (anisodamine), and scopolamine) that have been widely applied in medicines (Cardillo et al. 2016). The chemical synthesis of alkaloids is complex and expensive; thereby the in vitro production of alkaloids by hairy roots cultures in bioreactor presents certain advantages over the natural source and chemical synthesis. Besides, the scaling-up of hairy root cultures makes this technology an attractive tool for industrial or commercial scale. The production of alkaloids in bioreactor guarantees that the process has been done under defined and controlled conditions, thus preventing or reducing the variations in the quality and yield of alkaloid compounds.

6.5 Extraction and Detection Techniques

Due to the high value of alkaloids, the worldwide researchers have tried to search out new and reliable methods for the extraction and detection of those compounds. Special methods have been developed for isolating commercially useful alkaloids.

In most cases, plant tissue is processed to get aqueous solutions of the alkaloids. The alkaloids are then recovered from the solution by a process called extraction, which involves dissolving some components of the mixture with compatible or suitable solvents/reagents that may be polar or nonpolar. This process requires either an acidic or alkaline/basic environment. Extraction techniques (Fig. 6.7), such as solid-liquid extraction (SLE), supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), solid-phase microextraction (SPME), supercritical carbon dioxide extraction method, and ultrasound-assisted method, have been used. Then, different alkaloids can be separated and purified from the mixture. A range of chromatographic techniques may be used for the efficient quantitative and qualitative analysis of alkaloids. Alkaloids in crystalline form are also obtained using certain solvents (Gupta et al. 2012; Zhu et al. 2018). Extraction of pure alkaloids from crude extract needs to be performed with multi-step chromatographic techniques.

It can be started with paper chromatography that is the easier way for the quantification of alkaloids. This method is rapid and cheaper. Further thin-layer chromatography is used. It is a reproducible method and has a low detection limit as compared to paper chromatography. After that, highly efficient chromatographic techniques can be employed, i.e., gas chromatography (GC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), etc. (Maciel et al. 2019). These techniques are chosen accordingly to the nature of the alkaloidal sources.

Detection/analysis of the particular alkaloid with some specifications, mass spectrometry techniques can be used. MS (mass spectrometry) technique now plays a valuable role in the analysis of biomolecules, i.e., alkaloids, flavonoids, terpenes, etc. This revolution is realized by ESI-MS (electrospray ionization mass

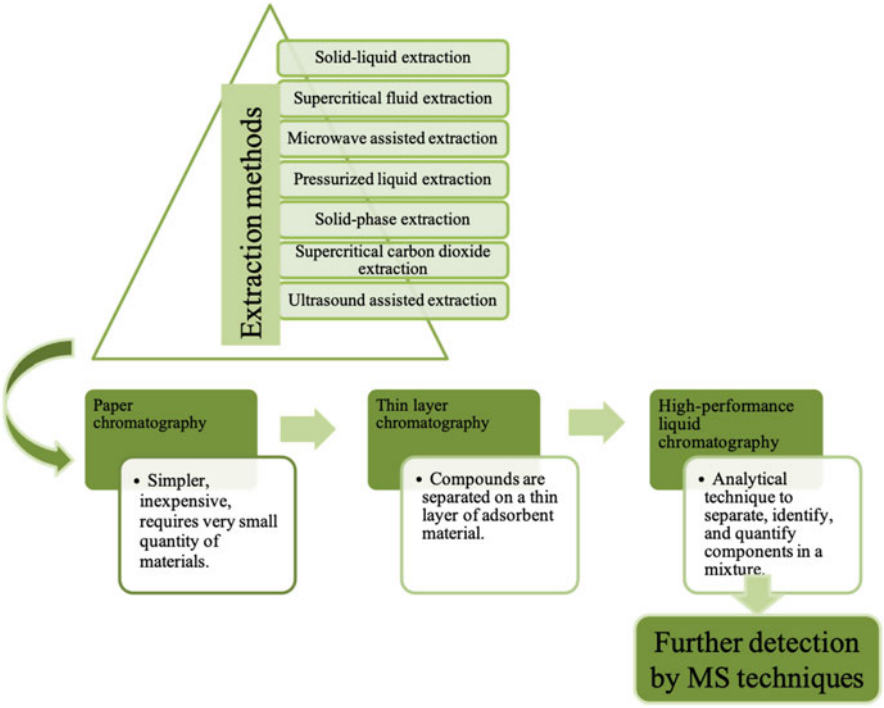

Fig. 6.7 Methods of extraction, purification, and detection of alkaloids

spectrometry) and MALDI-MS (matrix-assisted laser desorption ionization mass spectrometry) in the analysis of bio-polymeric products (Sasidharan et al. 2011). A completely unique method was developed for extraction and enrichment of the four alkaloids (nuciferine, *O*-nornuciferine, armepavine, and *N*-nornuciferine) from lotus leaf by coupling microwave-assisted extraction (MAE) with solid-phase microextraction (SPME) before ultra-high-performance liquid chromatography (UHPLC) analysis (Zou et al. 2020). In this recent report, the newly MAE-SPME is concluded as an efficient method for the extraction and enrichment for alkaloids from herbs (Zou et al. 2020). A two-dimensional analysis method endorsed high-performance liquid chromatography (HPLC) separation and electrospray ionization-ion mobility spectrometry (ESI-IMS) detection was developed for the evaluation of alkaloid compounds from *Peganum harmala* L. seeds. Their results reveal that this method is recognized to be advantageous over traditional absorbance detection methods for resolving complex mixtures due to complementary separation steps, elevated peak capacity, and better sensitivity (Wang et al. 2018). A simple, cost-effective salting-out assisted liquid-liquid extraction-based method for HPLC–DAD determination of khat (*Catha edulis*) alkaloids has been found to endow cleaner chromatogram with good selectivity and reproducibility. The salting-out assisted liquid-liquid extraction (SALLE)-based protocol provided good results as the

conventional extraction method (ultrasonic-assisted extraction followed by solid-phase extraction, UAE–SPE), and hence the method can be applied in forensic and biomedical sectors (Atlabachew et al. 2017).

6.6 Biological Activities

The medicinal properties of alkaloids are quite diverse. Alkaloids generally exert biological activities particularly in humans (Koleva et al. 2012). Even today, many of the used drugs are natural alkaloids or made by them, and new alkaloidal drugs are still being developed for clinical uses. The activity of alkaloids against herbivores, cytotoxic activity, the molecular targets of alkaloids, mutagenic or carcinogenic activity, antibacterial, antifungal, and antiviral properties and their possible roles as phytoalexins have been evaluated (Debnath et al. 2018). Some alkaloids, i.e., morphine, codeine, nicotine, cocaine, etc., can be extremely harmful to animals/humans to cause death due to their dose-dependent toxicity if taken orally (Matsuura and Fett-Neto 2015).

6.6.1 Biological Activities of Pyridine Alkaloids Group

Nicotine obtained from the tobacco plant (*Nicotiana tabacum*) is the principal alkaloid and main ingredient of the tobacco smoked in cigarettes, cigars, and pipes. Nicotine binds to nicotinic cholinergic receptors. It facilitates neurotransmitter release and is liable for behavior modifying effects in individual. Stimulation of central nAChRs (nicotinic acetylcholine receptor) by nicotine leads to the release of a range of neurotransmitters in the brain. Nicotine-containing product is obtainable in the market to interrupt the habit of smoking. Nasal mucosa irritation, arthralgia, nausea, vomiting, and mild headache are most common adverse effect of nicotine (Benowitz 2009; Pang et al. 2016). Cytisine could be a selective nicotinic cholinergic agonist obtained from the seeds of *Laburnum anagyroides* of Leguminosae family. It also acts as nicotine for smoking cessation (Perez et al. 2012).

6.6.2 Biological Activities of Tropane Alkaloids Group

Many tropane alkaloids possess local anesthetic properties. **Atropine** (obtained from *Atropa belladonna*) is anticholinergic (Çaksen et al. 2003). It reduces the secretion such as sweat, saliva, and gastric juice. It competitively inhibits muscarinic acetylcholine receptor. The most prominent effect of atropine is tachycardia due to blockade of the M2 receptor present on SA node through which vagal tone decreases (Tripathi 2013). **Scopolamine** is available in the leaves of plant *Hyoscyamus niger* (Solanaceae). It is also known as hyoscine. It competitively inhibits muscarinic receptors and acts as a nonselective muscarinic antagonist. It produces both peripheral anti-muscarinic properties and also sedative, antiemetic, and amnestic effects

(Ullrich et al. 2017). **Cocaine** is isolated from the dried leaves of *Erythroxylum coca* and *Erythroxylon truxillense*, belonging to the family Erythroxylaceae, a very potent local anesthetic. The central action of cocaine is sympathetic and works as a CNS stimulant agent. Loss of sense in taste and smell (after given in the nose or mouth) are the most common side effects of cocaine (Manna et al. 2020). **Catuabine** is a tropane alkaloid obtained from the bark of *Trichilia catigua* belonging to the family Meliaceae. A pure catuabine found antidepressant-like effects on forced swim model of depression in mice and rats (Campos et al. 2005).

6.6.3 Biological Activities of Quinoline Alkaloids Group

Quinine and **quinidine** are obtained from the bark of *Cinchona officinalis* belonging to the family Rubiaceae. Quinine is used to treat malaria. It was the first anti-malarial drug used in the early 1600s (Achan et al. 2011). It has rapid schizonticidal action against intra-erythrocytic malaria parasites. Quinidine is the dextro isomer of the quinine alkaloid. It blocks myocardial Naþ channels and acts as antiarrhythmic drug to treat irregular rhythms of the heartbeat. It is effective antimalarial drug against *Plasmodium falciparum*. Reported adverse reactions of quinidine are diarrhea, nausea, and vomiting (Diaz et al. 2015). **Dihydroquinine** is a natural impure compound found in commercial pharmaceutical formulations of quinine. **Dihydroquinidine** also have similar bioactivity (antimalarial). Both alkaloids inhibit the actions of parasympathetic nervous system. Therefore, biological source of dihydroquinine and dihydroquinidine are same with quinine as these are obtained from the bark of *Cinchona officinalis* (Mehrotra et al. 2018).

6.6.4 Biological Activities of Isoquinoline Alkaloids Group

Papaverine is a benzylisoquinoline alkaloid that occurs in the plant *Papaver somniferum* belonging to family Papaveraceae. It acts on smooth muscle throughout the body and causes vasodilation and relaxation of smooth muscle tone (Shimizu et al. 2000). **Berberine** occurs in roots and stem bark of different species of *Berberis* belonging to the family Berberidaceae. *Berberis aristata, B. lyceum, B. petiolaris,* and *B. tinctoria* are the main sources of berberine (Srivastava et al. 2015). The most important biological activity of berberine is its anti-diabetic effect. It activates AMPK and improves insulin sensitivity in rodent models of insulin resistance (Turner et al. 2008). Berberine-induced apoptosis is associated with upregulated expressions of p53, and decreased vimentin expression. These results suggest that berberine can suppress cell growth (Han and Qi 2012). Other important pharmacological activities are anti-hypertensive, anti-inflammatory, antioxidant, antidepressant, and hepatoprotective activities (Amritpal et al. 2010).

6.6.5 Biological Activities of Phenanthrene Alkaloids Group

Codeine and morphine are present in dried latex of unripe capsules of *Papaver somniferum*. These are used as opioid analgesic. Morphine (10%) and codeine (0.5%) are present in opium. Morphine and codeine depress respiratory center in a dose-dependent manner. Morphine is an egregious narcotic used for the pain relief, though its addictive properties limit its usefulness. Codeine is a wonderful analgesic that is relatively nonaddictive. Death may occur due to respiratory failure at its high doses (Dehghan et al. 2010).

6.6.6 Biological Activities of Phenylethylamine Alkaloids Group

Ephedrine is obtained naturally from the plants *Ephedra vulgaris*, *E. sinica, E. major*, *E. gerardiana*, etc. of genus ephedra (Family: Ephedraceae). It is a sympathetic stimulant that directly acts on α- and β-receptor. It can be used to prevent low blood pressure during spinal anesthesia. It is also used as bronchodilator in asthmatic condition. Allergic condition like hay fever can be treated with ephedrine (Ma et al. 2007). **Hordenine** is a natural phenethylamine compound that occurs in barley grass (*Hordeum vulgare*), a cereal crop belonging to the family Poaceae. It is a nootropic (non-pharmaceutical cognitive enhancers) compound that enhances cognitive ability. It is an effective MAO-B inhibitor. Since it helps to increase the level of norepinephrine, it is considered as norepinephrine and noradrenaline uptake inhibitor (Debnath et al. 2018).

6.6.7 Biological Activities of Indole Alkaloids Group

Reserpine is isolated mostly from the root of *Rauwolfia serpentina* and *Rauvolfia vomitoria*. It is known as antipsychotic and antihypertensive (Bunkar 2017). **Ergotamine** and **ergometrine** are obtained from the rye fungus *Claviceps purpurea*. It can be used for uterine contraction, uterine bleeding, and postpartum hemorrhage after delivery, incomplete recovery of uterus, retrogression, etc. It causes constriction of peripheral and cranial blood vessels to control extra blood flow and produces depression of central vasomotor centers (Ma et al. 2018). **Yohimbine** is isolated from the bark of *Pausinystalia yohimbe* belonging to the family Rubiaceae. It is chemically identical to reserpine. It increases parasympathetic (cholinergic) activity and decreases sympathetic (adrenergic) activity by acting on peripheral autonomic nervous system. It has a mild anti-diuretic action and has effect on blood pressure. Headache and excessive sweating are common side effects of yohimbine (Cohen et al. 2016). **Vinblastine** and **vincristine** are extracted from the pink periwinkle plant, *Catharanthus roseus*, belonging to the family Apocynaceae (Das and Sharangi 2017). Vinblastine is an antineoplastic agent, and it inhibits mitosis at metaphase by interacting with tubulin (Alam et al. 2017). It also has immunosuppressant effect. Major side effects of vinblastine are cough, fever, and painful

urination. Vincristine is employed for the treatment of some types of cancer like breast cancer, Hodgkin's disease, Kaposi's sarcoma, and testicular cancer. The antitumor activity of vincristine is same to vinblastine. Most common side effects of vincristine are blurred or double vision, constipation, difficulty in walking, drooping eyelids, headache, jaw pain, joint pain, lower back or side pain, and stomach cramps (Alam et al. 2017). **Ergine** is a D-lysergic acid amide (LSA) found in various species of vines belonging to the family Convolvulaceae and *Argyreia nervosa*. It is also isolated from rye fungus *Claviceps purpurea*. Ergine has psychedelic effects (Paulke et al. 2013).

6.6.8 Biological Activities of Purine Alkaloids Group

Caffeine is a purine alkaloid. It is found naturally in the seeds and leaves of the plants *Theobroma cacao* (Malvaceae) and *Thea sinensis* (Theaceae), respectively (Rusconi and Conti 2010). Caffeine is the most widely consumed stimulant drug in the world. It is also consumed in cold medications, analgesics, and anorectants and in CNS stimulant. CNS stimulation is the main pharmacological action of caffeine because it can also act on the peripheral adenosine receptor (A1) on adipocyte that suppresses lipolysis by inhibition of adenylate cyclase activity (Cappelletti et al. 2015).

6.6.9 Biological Activities of Imidazole Alkaloids Group

Pilocarpine is the main alkaloid of imidazole group, and L-histidine is the biosynthetic precursor of the imidazole moiety. Pilocarpine is isolated from the leaves of *Pilocarpus microphyllus* that belongs to the family Rutaceae. It has cholinergic properties to stimulate the parasympathetic system (bladder, tear ducts, sudoriferous, and salivary glands). This alkaloid is an elected drug for glaucoma treatment. It has been exploited to treat the xerostomy (dry mouth) of throat cancer caused by the chemotherapy. Small doses of pilocarpine generally cause fall in blood pressure but in higher doses elicit rise in blood pressure (Santos and Moreno 2004).

6.6.10 Biological Activities of Terpenoid Alkaloids Group

Capsaicin is a unique alkaloid found primarily in the fruit of the *Capsicum* genus like *Capsicum annum* and *Capsicum frutescens* belonging to the family Solanaceae. Capsaicin can be bonded to TRPV1, which is mainly expressed in the sensory neurons. It also acts in the gastrointestinal tract, for weight loss and as an analgesic. The common side effects of capsaicin are burning, itching, dryness, pain, redness, swelling, or soreness (Reyes-Escogido et al. 2011). **Choline** is found in diverse plant foods in small amounts. It is a constituent of cell and mitochondrial membranes and of the synaptical neurotransmitter acetylcholine. Hence, this supplement impacts different cycles, for example, lipid metabolism, signaling through secondary

messengers, and methylation-dependent biosynthesis of molecules. Major side effects of choline are constipation, diarrhea, dizziness, drowsiness, and migraine (Corbin and Zeisel 2012).

6.7 Commercial Utilization and Prospects

Plant biotechnology techniques provide valuable tools to synthesize a wide range of alkaloids as obtained from plants, as well as novel compounds are also synthesized via biotransformation and genetic engineering tools. These alkaloids have been used in the various commercial products. The in vitro cultures (shoot, callus, suspension, and hairy root cultures) are found to be used as sustainable system for the production of various secondary metabolites. Over the past twenty years, the concept of plant-based production of high-quality pharmaceutical alkaloids has increased the research interest and offered critical advantages over traditional extraction systems. In this chapter, some approaches (techniques) discussed have proven that medicinal plants can be used efficiently to produce various pharmaceutical alkaloids for remedial applications.

6.8 Conclusions and Recommendations

This chapter gives an insight to the different aspects of tissue culture for the production of alkaloids under in vitro conditions and their biosynthesis scenario. The extended use of plant cell culture systems in recent years is probably due to a benignant understanding of the alkaloid pathway in economically important plants. Advancement in plant cell culture system could provide the cost-effective, commercial production of rare, endangered, or even exotic plants, their cells, and the bioactive molecules that they will produce. These discussed alkaloids are found beneficial for certain life-threatening disease and will serve to extend and enhance the continued usefulness of higher plants as renewable sources of chemicals, especially alkaloids.

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