Biotechnological approaches to the production of promising plant-derived anticancer agents: An update and overview

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Abstract

The plant kingdom is a rich source of bioactive compounds, many of which have used since pre-history for their therapeutic properties to treat a range of illnesses. More recently, some of these metabolites have attracted attention to their antineoplastic activities to treat various cancers relying on different mechanisms to kill. Some of these molecules are glycosides, which have proven useful as anti-cancer agents, namely podophyllotoxin (PPT) anaryltetralin lignan or alkaloids. There are three primary forms of alkaloids, such as indole alkaloids (vincristine and vinblastine from Catharanthus roseus) quinoline alkaloid (camptothecin from Camptotheca acuminata) and diterpenoid alkaloid (taxol and it's analogous from Taxus and Corylus species). This review considers a variety of plant biotechnology approaches used to enhance the production of these anticancer molecules in different species. In this regard, many in vitro culture techniques such as stimulation of suspension culture and hairy roots are being used to investigate the effects of plant growth regulators and elicitors on various explants.

1. Introduction

Depending on the type and condition of cancer, conventional treatments may involve chemotherapy in combination with surgery, radiation therapy, hormone therapy, and/or highly targeted therapies such as immunotherapy and monoclonal antibody therapy. Chemotherapeutic drugs (synthetic, semi-synthetic, and naturally occurring compounds) are cytotoxic and often kill both cancerous and healthy cells, which are dividing rapidly. Their main mode of action involves signaling through both death receptors (extrinsic pathways) and mitochondrial pathways (intrinsic pathways) and by inducing one or more apoptotic pathways. Chemotherapeutic drugs can be classified by their mode of action into alkylating antineoplastic agents, kinase inhibitors, aromatase inhibitors, and topoisomerase inhibitors. Nature is an excellent and useful source of anticancer agents, about 60% of which are extracted from microorganisms, marine organisms, or plants (Iqbal et al., 2017). Important anti-cancer phytochemicals include vinca alkaloids, podophyllotoxin (PPT), taxol, camptothecin, and their derivatives.

These phyto-compounds are being used in various fields due to their active role against different types of cancers and are preferred over conventional chemotherapy due to their lesser. It is reported that 80% of the population worldwide traditionally uses natural compounds contained in medicinal plants, and more than 1800 experimentally authenticated anti-cancer compounds-target interactions have been documented at Naturally occurring Plant-based Anti-cancer Compound-Activity-Target Database (NPACT) (Aung et al., 2017).

There is a need for the continuous production of these compounds, which may be achieved using a range of biotechnological approaches involving the use of axenic plant cell and organ cultures. Moreover, bioengineering and metabolic engineering approaches can be used to study their biosynthetic pathways (Nobili et al., 2009). This review focuses on state-of-the-art biotechnological approaches for the production of effective

anticancer compounds with a short description of their chemical structure, mode of action, and biological activities.

2. Podophyllotoxin aryltetralin lignan

Podophyllotoxin (PPT) (**Fig.1A**), an aryltetralin lignan, found naturally in rhizomes and roots of many herbs such as *Podophyllum peltatum, P. hexandrum, Linum album, L. narbonense,* (**Fig. 1B**) with supplies of PPT from natural sources possibly limited due to the endangered status of plants in the wild and/or environmental stresses due to overharvesting and environmental threats (Chaudhari et al., 2014: Chaurasia et al., 2012). This compound shows different biological activities, such as vigorous antimitotic and antiviral activities (Ayres and Loike, 1990; Gross, 2001). In addition to being important anticancer agent in its own right, it is also a starting material for the production of different semisynthetic derivatives such as teniposide, and etoposide which have huge commercial importance due to their potent effects in Hodgkin's and non-Hodgkin's lymphoma, testicular/small cell lung cancers and acute leukemia (Gordaliza et al., 2004; Malik et al., 2014). The main mode of action of PPT is via inhibition of Topo II, thereby arresting the cell cycle at the G2 phase and causing DNA breakage and apoptosis via inhibition of protein tyrosine kinase (Dholwani et al., 2008). The organic synthesis of this compound is difficult due to the complicated chemical structure involving high-cost procedures (Satake et al., 2013; Umezawa, 2003).

2.1. Podophyllotoxin biosynthesis

The first step in the production of PPT is the deamination of phenylalanine by phenylalanine ammonia-lyase to cinnamic acid, which is converted into feruloyl-CoA by caffeoyl-CoA O-methyl transferase. Feruloyl-CoA further undergoes a series of formation of intermediates such as coniferyl alcohol to produce pinoresinol and lariciresinol. These compounds lead to the production of secoisolariciresinol in the presence of pinoresinol-lariciresinol reductase leading to the synthesis of matairesinol, which eventually yields PPT (**Fig. 2**) (Bahabadi et al., 2012; Javadian et al., 2017).

2.2. Pharmacokinetics

The pharmacokinetics of semisynthetic derivatives of PPT depends on the hydrophobicity of the molecule. PPT has reportedly been given to the patient as a 30 minutes infusion in skin cancer treatment or via direct application of the cream/solution to the lesion (Ardalani et al., 2017; Lacey et al., 2003). The maximum tolerated dose (MTD) is 120 mg/m^2 in human beings, and elimination half-life is up to 5 hours depending on the derivative (Frazier and Price, 2010; Smyth et al., 1985). Clinical studies showed that dermal absorption of this drug is limited, and its metabolism takes place in the liver and kidneys.

2.3. Mode of action

The mode of action of PPT is to polymerize tubulin, thus destabilize mitotic-spindles microtubules and prevent cell division. Various semi-synthetic products of PPT, such as etoposide and, teniposide act directly on the enzyme topoisomerase II and form an irreversible enzyme-DNA complex resulting n DNA double-strand breaks (Hartmann and Lipp, 2006). Both inhibitory effects of PPT on microtubulin formation and topoisomerase II are depicted in **Fig. 3** .

2.4. Techniques involved in a biotechnological production

2.4.1. In vitro production

Various alternative techniques for the commercial production of PPT depend on the availability of cost-effective raw materials. In this regard, the callus cultures of *P. peltatum* shows a higher accumulation of PPT in Murashige and Skoog's (MS) medium supplemented with kinetin and 2, 4-dichloro phenoxy acetic acid. Chromatographic analysis of PPT by thin-layer chromatography (TLC) identified two factors, age of callus and concentration of the hormone in medium, respectively, which control the *in vitro* production of PPT (Kadkade, 1981). Analysis of the metabolites by HPLC coupled to tandem mass spectrometry analysis showed incorporation of 3,4-methylenedioxy cinnamic acid (MDCA) in feeding experiments in cell culture of *L. album* , and DOP appeared to be a rate-limiting point in PPT biosynthesis (Seidel et al., 2002).

An alternative source of PPT from *P. peltatum* can be achieved by the use of embryogenic callus and adventitious root culture techniques (Anbazhagan et al., 2008). The addition of 2,4-dichloro phenoxy acetic acid (2,4-D) in embryogenic callus and indole-3-butyric acid in adventitious root callus trigger the PPT production in MS medium. High-performance liquid chromatography (HPLC) analysis showed that a higher amount of PPT was obtained from adventitious roots than embryogenic cell clumps of *P. peltatum* . Still, potentially, both approaches can efficiently be used to produce PPT on a large-scale via the use of bioreactors (Anbazhagan et al., 2008). Podophyllotoxin production from *P. hexandrum* adventitious root can also be optimized by varying concentrations of sucrose, ammonium, phosphate, and pH of the nutrient medium. Podophyllotoxin accumulation was reported to be highest (6.4 mg/g dry weight, DW) at 6% sucrose, 10mM ammonium, 2.25mM phosphate, and pH 6.0 in the optimized MS medium (Rajesh et al., 2014).

An alternative hairy root culture of *L. narbonense* for the production of PPT yielded five-fold higher levels of justicidin B (7.78mg/g, DW) compared to callus. The content of justicidin B in the*L. narbonense* intact roots is about 0.5mg/g DW, in callus cultures is 1.57mg/g DW. To meet the industrial requirement and large-scale production of plant anticancer agent justicidin B from*L. narbonense,* hairy roots bioreactor system "BIOSTAT B plus" can be a feasible alternative. Compared to callus, fivefold higher yields of justiidin B (7.78 mg/g DW) can be produced from hairy roots by this technique (Ionkova et al., 2013). A cross-species co-culture technique for the production of PPT by *P. hexandrum* using hairy roots of *L. flavum* showed that PPT concentration was increased to 240% and 72% in dual shake flasks and dual bioreactors respectively (Lin et al., 2003). Transgenic hairy root cultures from *L. mucronatum* can also be used for scaling-up production of PPT. The maximum PPT amount obtained by transgenic hairy root was 5.78 mg/g DW (Samadi et al., 2014).

2.4.2. Elicitation

Methyl jasmonate (MeJA), a plant-specific endogenous phytohormone, regulates the biosynthesis of specialized plant metabolites (Rahnamaie-Tajadod et al., 2017).

MeJA (100-200µM) not only triggers the biosynthetic pathway but also, stimulates specific gene expression that leads to a higher yield of many anticancer compounds such as by elicitation of cell suspension cultures of *L. album* cell line 2-5aH with MeJA, accumulation of PPT reached 7 mg/g, DW (Wasternack and Hause, 2013; van Fürden et al., 2005). Salicylic acid is a potent signaling molecule present in plants that are actively involved in providing resistance against pathogens and growth responses. Salicylic acid has a profound role in plant defense systems via the production of plant defense compounds. It has also been used in the production of various biologically active compounds in plant cell culture systems (Nadeem et al., 2019). Salicylic acid in *the L. album* produces 333µg/g PPT; it also increases the expression of three genes involving in the first step of the PPT biosynthetic pathway. However, genes involved in the last biosynthetic steps were not affected by salicylic acid (Yousefzadi et al., 2010). Biotechnological production of anticancer compound PPT in*L. album* cell cultures shows a seven-fold increase in compound production than control by fungal elicitor *Fusarium graminearum* . Extracts of *Fusarium graminearum* in cell cultures of *L. album* produce 140µg/g DW of PPT. Besides, the up-regulation of lignan biosynthesis pathway genes was also observed in fungal elicitor cultures (Bahabadi et al., 2012).

2.4.3. Metabolic engineering

Another efficient, stable, and sustainable approach for lignans production in metabolic engineering of enzymes involved in the biosynthesis of lignans in *Linum* , *Forsythia* , and*Podophyllum* species (Ionkova, 2011; Malik et al., 2014). Silencing of*Forsythia* cell cultures by pinoresinol-lariciresinol reductase-RNA interference (PLR-RNAi) triggered an approximately 20-fold increase in total pinoresinol (pinoresinol aglycone and glucoside), compared with levels observed in the control wild-type cells (Kim et al., 2009). Stable transfection of PLR-RNAi and the *Sesamum CYP81Q1* gene in *Forsythia* cell cultures triggered an approximately 20-fold increase in pinoresinol (pinoresinol aglycone and glucoside) (Kim et al., 2009) along with sesamin (0.01 mg/g DW of the cell) (Satake et al., 2015). The reaction catalyzed by PLR is crucial in plant defense mechanisms against pathogens. In the presence of a fungal elicitor, the lignan deficient roots usually divert

the phenylpropanoid synthesis pathway to the production of defense metabolites to enhance the expression of genes involved in lignan synthesis to cope pathogen attack. Hence, enhanced synthesis of lignan has been noticed in silenced PLR plants compared to controls, and no other pathway seems able to bypass downregulated PLR (Tashackori et al., 2019).

Similarly, transcriptomic analyses of *P. hexandrum* Royle (having anticancer lignan PPT) were used to investigate the expression of key genes involved in the production of this compound. Quantitative expression analysis of pathway genes exhibited ˜23-fold increase in transcript abundance of nine genes namely secoisolariciresinol dehydrogenase (SD), prephenate dehydrogenase (PD), p-coumarate 3-hydroxylase (PCH), chorismate mutase (CM), caffeic acid 3-O-methyltransferase (CMT), cinnamyl alcohol dehydrogenase (CAD), cinnamoyl-CoA reductase (CCR), cinnamate 4-hydroxylase (C4H), and arogenate dehydratase (ADH). This higher expression of genes also correlated with a higher yield of PPT in the root and rhizomes of*P. hexandrum* (Kumar et al., 2015) (**Table1**).

3. Taxol

Taxus species belong to the family Taxaceae (Yew) and produce the natural anticancer diterpenoid taxol (Paclitaxel) (**Fig. 4A**). Taxol is highly effective in the treatment of various types of cancers and diseases that require microtubule stabilization, including cardiac functional recovery and psoriasis (Ehrlich et al., 2004; Xiao et al., 2012). Taxol was firstly isolated from the bark of the Pacific yew tree (*Taxus brevifolia*). Still, later, it has been produced from various other natural plant sources, although the yield is meager when extracted traditionally (Yamgar and Sawant, 2015) (**Fig. 4B**).

3.1. Taxol biosynthesis

Taxol is an expensive anti-cancer drug due to its low availability from a natural source (about 0.02% of DW) and a high cost of extraction (Ajikumar et al., 2010). Biosynthesis of taxol takes place in plants through 19 enzyme-catalyzed steps, starting from the cyclization of geranylgeranyl diphosphate. Universal diterpenoid precursor, geranylgeranyl diphosphate, is produced by condensation of three units of isopentenyl diphosphate (IPP) with dimethylallyl diphosphate (DMAPP) (**Fig.5**). In plants, these compounds are produced either by the mevalonic pathway (MVA) in the cytosol or via the methylerythritol phosphate (MEP) pathway in plastids. Over the years, the MVA pathway considered the sole source of IPP in all living organisms. However, inconsistencies in this pathway lead to the discovery of the MEP pathway in bacteria and plants. The actual origin of a precursor in the taxol biosynthetic pathway remained unclear. In the taxol pathway, IPP is synthesized through two distinct routes. In MVA, IPP is synthesized from acetyl-CoA and an MEP from D-glyceraldehyde 3-phosphate pyruvate from glycolysis. The pathway bifurcates after the formation of isoprenoid precursor IPP and DMAPP, which then leads to the formation of geranylgeranyl diphosphate. Subsequently, taxa-4(5), 11(12)-diene, also known as taxadiene, from geranylgeranyl diphosphate is produced, which is catalyzed by taxadiene synthase (TS) (**Fig. 5**). In the next step, CYP450-dependent hydroxylation at the C-5 position of the taxane ring resulted in the allylic rearrangement of the 4(5)-double bond to the 4(20) positions to yield taxa-4(20), 11-diene-5-ol. Further, hydroxylation of this intermediate resulted in the production of 10-hydroxytaxa-4(20), 11-diene-5á-yl-acetate, which upon subsequently acetylation by 10-deacetylbaccatin III-10-o-acetyltransferase formed Baccatin III. Consequently, this compound along with side chains of phenylalanine attached to the taxane core after several steps of hydroxylation resulted in the formation of final product taxol (Engels et al., 2008; Guo et al., 2006; Howat et al., 2014; Jennewein and Croteau, 2001).

3.2. Pharmacokinetics

The pharmacokinetics of taxol has been studied extensively against lung, breast, and ovarian cancers. Taxol is administered in patients through the intravenous route with the use of a formulation vehicle (mainly Cremophor® EL) for drug entrapment in micelles (Alex et al., 2005; Alves et al., 2018; Shin et al., 2018). The dose of taxol stated differently in various studies ranging from 105-270 mg/m^2 in humans. The formulation vehicle not only affects the disposition of taxol but also influences the non-linear pharmacokinetics of taxol, specifically at a higher dosage. Plasma and urine samples were collected for estimation of taxol concentration

through HPLC (Ishikawa et al., 2018). Analysis of blood samples from patients depicts clearance of this drug in approximately 3 hours, depending on the dose and vehicle used.

3.3. Mode of action

Taxol and its derivatives exert anticancer activity through various mechanisms. Taxanes stabilize the microtubule assembly and prevent it from de-assembling, thus preventing normal metaphase spindle configuration of chromosomes. As a result, the progression of mitosis blocks and prolonged activation of mitosis checkpoint leads to apoptosis or reversion of the cancer cell to the GO phase. It is also involved in the stimulation of apoptotic cascades by upregulating Fas/FasL and Bax, and downregulating Bcl-2 leading to the activation of caspase-3, -8, and -9 in MG-63, U-2OS, Saos-2, and MNNG/HOS human osteosarcoma cells (Liang et al., 2012). The reduction of Bcl-2/Bax protein and mRNA levels leads to cell cycle arrest in cancer cells (**Fig. 6**).

3.4. Techniques involved in a biotechnological production

3.4.1. Metabolic engineering

For industrial scale and sustainable production of taxol and related taxanes, numerous studies have focused on the synthesis of precursors involved in the synthesis of taxol from plant cell cultures. Cloning of taxadiene synthase (*TXS*) and 10-deacetylbaccatin III-10-O-acetyltransferase (*DBAT*) genes is an important biotechnological approach to increase the production of two precursor taxoids: 10-deactylbaccatin III (DB) and bacctin III (BC) to synthesize taxol. *Taxus mairei* cells transformed with *TXS* and*DBAT* genes with the addition of an elicitor MeJA produces 2.5 times higher taxoids than non-MeJA treated control cells (Ho et al., 2005). Similarly, transformed roots of *T. baccata* shows 265% greater taxane production after MeJA elicitor treatment, with over-expression of the *TXS* gene also being identified as an important contributory factor (Wilson and Roberts, 2014; Expósito et al., 2009; Engels et al., 2008). Exogenous feeding of taxol is another phenomenon to produce taxol and related taxane products in *Taxus baccata*suspension cultures. DNA laddering analysis reveals that the addition of taxol caused a considerable increase in taxadiene synthase activity (Expósito et al., 2009).

3.4.2. Heterologous expression

E. coli also serves as a heterologous host to produce taxadiene, the key intermediate of taxol. *In vitro* synthesis of taxadiene in genetically engineered *E. coli* with deoxyxylulose-5-phosphate (DXP*)* overexpression produces 1.3 mg/l of taxadiene. This is an alternative scheme to produce taxoids by non-taxol producing organisms (Huang et al., 2001). Taxadiene synthesis is divided into two pathways; precursor metabolism (upstream MEP pathway) and taxadiene synthesis (downstream pathway). Induction of pathway genes in K and B-derived *E. coli* strains triggered a higher yield of taxadiene roughly 2.5-fold was obtained in K-derivative as compared to B-derivative *E. coli.* It has also noted that environmental conditions (temperature and varying exogenous concentration of indole) influence taxadiene production in strains of*E. coli* (Boghigian et al., 2012).

Greater knowledge of the importance of biosynthetic pathway regulatory genes can also be achieved by employing eukaryotic microbial heterologous host. For example, the production of taxol biosynthetic pathway precursor baccatin III in *Saccharomyces cerevisiae* using episomal vectors resulted in increased taxol production by 100-fold compared to *Arabidopsis* (Besumbes et al., 2004; DeJong et al., 2006). Co-overexpression of taxadiene synthase gene from *T. chinensis*and geranylgeranyl diphosphate synthase from *Sulfolobus acidocaldarius* along with, mutant regulatory protein UPC2-1, resulting in a 40-fold increase in taxadiene to 8.7 mg/l in the yeast cell (Engels et al., 2008). Genetic engineering of the taxol biosynthetic pathway in plant heterologous hosts may also augment the accumulation of desired anticancer compounds. For example, the insertion of the taxadiene synthase gene from the*Taxus* into *Artemisia annua* L. produced a high yield of taxadiene in *A. annua* (129.7µg/g DW). It also resulted in the accumulation of antimalarial compound artemisinin in the leaves of Artemisia (Li et al., 2015). Likewise, transformed roots of ginseng (*Panax ginseng* C.A. Meyer) harboring a taxadiene synthase (*TS*) gene from *Taxus brevifolia*produces 1 µg taxadiene per

gram of dry weight. Furthermore, successful accumulation of taxadiene in transgenic ginseng root is irrespective of any change in phenotype and growth differences as compared to wild type (Cha et al., 2012). De novo synthesis of taxadiene also reported by using *Nicotiana benthamiana* as a heterologous host. *Agrobacterium*-mediated transformation of*Nicotiana benthamiana* containing the taxadiene synthase gene from the *Taxus* produced 11µg taxadiene/g of dry weight (Hasan et al., 2014).

3.4.3. Elicitation

Numerous studies reported enhanced taxol production using a wide range of elicitors such as salicylic acids, MeJA, coronatine. Salicylic acid (SA) has a vital role in the plant defense regulatory system. The interaction of plant to pathogen resulted in the accumulation of salicylic acid at the infection site, which triggers signals to the defense system (Pieterse and van Loon, 1999). Salicylic acid response leads to the production of the secondary metabolite, acting as a secondary metabolite elicitor (Dučaiová et al., 2013). Salicylic acid elicitation on the *Taxus* genus resulted in improved diterpene alkaloid production. In suspension cultures of *Taxus chinensis*induction of 20mg/l, salicylic acid resulted in 1.5 mg/g DW taxol. Salicylic acid, in combination with mevastatin, an inhibitor of HMG-CoA, and increased intensified taxol production up to 1.6 mg/g DW (Wang et al., 2007). A low level of taxol production was also reported in suspension culture cells of hazelnut plant *Corylus avellana* L. with the use of salicylic acid as an elicitor enhancing taxol production. The amount of taxol produced is 50 mg/l in salicylic acid-treated cultures. Moreover, salicylic acid (50 mg l^{-1}) and ultrasound treatment (40 kHz for 2, 3, 5, and 10 min) synergistically increased taxol production (0.7 mg l^{-1}) 14 times higher than control cultures (0.05 mg l^{-1}) (Rezaei et al., 2011). It has been reported that the inclusion of 200 µm of MeJA alone in a cell suspension culture of *Taxus* increased the taxane yield to 23.4 mg/l 20% of total taxol production using this plant (McElroy and Jennewein, 2018) (**Table 2**). The synergistic effect of MeJA with fructose at 100µm concentration resulted in 17.07 mg/l taxol production. While elicitation with MeJA along with another cyclodextrin increases 55 times higher taxol production in *Taxus*(Singh and Dwivedi, 2018) (**Table 2**).

4. Terpenoid indole alkaloids

Alkaloids, flavonoids, saponins, terpenes, polyphenols, fatty acids, and essential oils are of traditional Chinese medicine (TCM). Vinblastine, vinorelbine, vincristine, and vindesine are active ingredients of anticancer drugs, derived from alkaloid (**Fig. 7A**).

Catharanthus belongs to the family Apocynaceae, producing terpenoid indole alkaloids (TIAs). It has eight species and had enormous pharmaceutical potential as only *Catharanthus Roseus* produces more than 130 different TIAs. Among them, vinblastine, vincristine, vinorelbine, vindesine, anhydrovinblastine, vinblastine, and vincristine are reported as effective anti-cancer agents (Jacobs et al., 2004). Other compounds, such as ajmalicine and serpentine, both monoterpene indole alkaloids are used as antihypertensive and anti-neuro-inflammatory, as well as vindolicine is used as an antidiabetic. Vinblastine and vincristine as powerful anticancer drugs, commonly known as vinca alkaloids, function as spindle inhibitors (Barrales-Cureño et al., 2019) (**Fig. 7B**).

4.1. Terpene indole alkaloids biosynthesis

The chief precursor in the synthesis of expensive anticancer drugs vinblastine and vincristine are strictosidine. This compound undergoes a series of reactions and forms compounds of diverse structure and biological function. Strictosidine is formed from the combination of tryptamine and secologanin, which are synthesized through the indole and mevalonate pathway, respectively. Firstly, in the TIA synthesis pathway, geraniol is formed from geranyl diphosphate (GDP) in the presence of geraniol synthase (GES). Later, geraniol is hydroxylated to 10-hydroxy-geraniol catalyzed by geraniol 10-hydroxylase, which has a regulatory role in the TIAs synthesis pathway. Following a series of steps, loganin is formed. In the last stage of the iridoid pathway, in the presence of secologanin synthase, loganin is converted into secologanin. On the other hand, a single enzymatic reaction comprises of conversion of L-tryptophan catalyzed by tryptophan decarboxylase yields tryptamine. Condensation of secologanin and tryptamine lead to the formation of strictosidine, a central intermediate of the TIAs pathway, by an enzyme strictosidine synthase. In the vindoline pathway, this

compound undergoes a series of enzymatic reactions to produce catharanthine and vindoline, which includes two recently discovered enzymes dihydroprecondylocarpine synthase (PAS) and dehydrogenase dihydroprecondylocarpine synthase (DPAS) (Caputi et al., 2018) that converts an immediate stemmadenine acetate to tabsersonine and catharanthine. This reaction is catalyzed by vacuolar class III peroxidase to produce α3', 4"anhydrovinblastine, which after several steps, converted into vinblastine and vincristine (Dutta et al., 2005; Zhou et al., 2010; Zhu et al., 2014) (**Fig. 8**).

4.2. Pharmacokinetics

Pharmacokinetics of terpene indole alkaloids includes intravenous injection and continuous infusion showing triphasic clearance from plasma. For all indole alkaloids, in alpha and beta phases, distribution is similar, involving uptake from peripheral tissues and formed blood elements (Emanuela et al., 2018). In the later phase, distribution differs among indole alkaloids depending upon drug release from different tissues. The mitotic agents are metabolized in the liver. The plasma half-life of various indole alkaloids is vincristine (23-85 h), vinblastine (20-64 h), vindesine (20-24 h), and vinorelbine (18-49 h). Furthermore, all terpene indole alkaloids follow the clearance route of biliary elimination through feces with negligible renal clearance (Nelson, 2010).

4.3. Mode of action

Vinca alkaloids bind to tubulin and cause polymerization, which inhibits microtubule assembly leading to the disruption in the mitotic spindle eventually metaphase-arrested (**Fig. 9**). Mode of action of most of the alkaloid derivatives is similar to taxanes by stimulation of apoptotic cascades and reduction of Bcl-2/Bax protein and mRNA levels, which leads to cell cycle arrest in cancer cells. In SW1116 human colon cancer cells, alkaloids derivatives downregulate human telomerase reverse transcriptase (hTERT) and upregulate p53 as well as mad1 in a concentration-dependent manner and cause cytotoxicity (Barrales-Cureño et al., 2019).

4.4. Techniques involved in a biotechnological production

4.4.1. In vitro **production**

Compact callus cells feeding with tryptamine, succinic acid, and tryptophan increased the terpene indole alkaloids production 3-5 fold. Hairy root culture of *C. roseus* fed with precursors (geraniol and nitroprusside) (Li et al., 2011; Morgan and Shanks, 2000) and immobilized cells techniques with high cell density did not increase terpene indole alkaloids (TIA) production in shoot and callus cultures as compared to control (Zhao et al., 2001). Suspension culture elicited with malate and sodium alginate proved to increase three-fold production of terpene indole alkaloids (ajmalicine and catharanthine) (Almagro et al., 2011).

In suspension culture of *C. roseus* , overexpression of strictosidine synthases (*STR)* and tryptophan decarboxylase (*TDC)* gene with the feeding of loganin and secologanin showed remarkable production by 24 fold (Whitmer et al., 2002). Elicitation studies also demonstrated that methyl jasmonate (MeJA) alone (Zhou et al., 2010) and/or in combination with another cyclodextrin could improve the output of catharanthine, one of the two precursors of the anticancer compound vinblastine, in *C. roseus* hairy roots through the enhanced expression of pathway genes (Almagro et al., 2014). UV-B light stress has been used as an elicitor to enhance TIAs, including vincristine and vinblastine production in *C. roseus* hairy roots (Lalaleo et al., 2016).

4.4.2. Metabolic engineering

Metabolic engineering is another strategy for enhancing the biosynthesis of secondary metabolites of economic importance by *C. roseus* . TIA biosynthetic pathway can be accelerated at any stage of synthesis, tryptamine synthesis (Shikimate pathway), secologanin synthesis (terpenoid pathway), and terpenoid synthesis. Several cloned genes have been used to enhance the production of TIAs including Anthranilate synthase α *(AΣa),* Tryptophan decarboxylase *(TDC),*1-deoxy-D-xylulose 5-phosphate synthase *(DXS),* 1-deoxy-D-xylulose 5-phosphate reductoisomerase *(DXR),* Strictosidine synthase*(STR),* Strictosidine β-D-glucosidase *(SGD),*Octadecanoid-responsive *Catharanthus (ORCA1,ORCA2,ORCA3)* and zinc finger protein

family from *C. roseus* (ZCT1, ZCT2, ZCT3) (Liu et al., 2007). The *STR*promoter in *C. roseus* contains three cis-regulatory sequences. The BA element (BA region) in the *STR* gene promoter also acts as a functional unit in elicitor and jasmonate-responsive gene expression (Hughes et al., 2004). By interacting with *TDC* and *STR* promoters, the ORCA proteins consequently activate gene expression. ORCA1 contains a single AP2-domain on the N-terminal part of the protein, whereas the *ORCA2 cDNA* encoded an AP2-domain protein on the C-terminal part of an ORCA2 protein (Pauw et al., 2004).

Genetic manipulation of genes involved in the metabolic pathways of these compounds can lead to an increase in the yield of TIAs in *C. roseus* . Overexpression of 1-deoxy-D-xylulosesynthase (*DXS*) or geraniol-10-hydroxylase (*G10H*) in a hairy root line of *C. roseus produced* a significant increase in metabolite production (Peebles et al., 2011). Terpenoid biosynthesis involves transactivation of several genes by specific transcription factors such as jasmonate-regulated basic helix-loop-helix (*bHLH*) iridoid synthesis 1 (*BIS1*) transcription factor controls the expression of genes lead to the synthesis of iridoid organic acid. Overexpression of *BIS1* in hairy root lines of*C. roseus* upregulated the tabersonine (0.18 mg/ gm DW), which is an essential precursor in the vindoline synthetic pathway for the production of vincristine and vinblastine (Van Moerkercke et al., 2015). Expression studies of ORCA3 showed no change in TIAs synthesis. However, co-overexpression of pathway regulator ORCA3 and pathway gene strictosidine glucosidase (*SGD*) remarkably increased alkaloid pool by 47% in *C. roseus* hairy root lines (Sun and Peebles, 2016). Genetic transformation experiments on *C. roseus* using hypocotyls as explants effectively increases anticancer compound synthesis.

A 2.4-fold increase in vindoline accumulation in transgenic *C. roseus* compared to control was evident by the overexpression of deacetylvindoline-4-O-acetyltransferase (*DAT*) in the TIA pathway (Wang et al., 2012). Transcriptomic studies of the TIA pathway in *C. roseus* indicated that anthranilate synthase (AS), a rate-limiting enzyme, regulates the transcription of various genes involved in the TIA pathway (Sun et al., 2016) (**Table 3**).

4.4.3. Elicitation

Abiotic elicitors MeJA and β-cyclodextrin (β-CD) in *Taraxacum officinale* Weber *in vitro* culture techniques can increase the production of taraxerol (TA) and taraxasterol (TX) that has antitumor activity against breast cancer (Bishayee et al., 2011). Quantification of TA and TX by high-performance liquid chromatography (HPLC) analysis showed an increase in TA and TX content at various concentrations of MeJA and β-CD in the elicited root suspension callus culture of *T. officinale* Weber as compared to plant root (Sharma and Zafar, 2016). Similarly, cyclodextrin and MeJa also increases terpene indole alkaloid production (ajmalicine, tabersonine, and catharanthine) in*Catharanthus roseus* as compare to non-elicited plants.

5. Camptothecin

Camptothecin (CPT), an anticancer drug produced from the roots of*Ophiorrhiza prostrate* (**Fig. 10**), is a member of the monoterpenoid family. Its synthesis involves some crucial steps, which are not yet fully elucidated. The main precursors for its synthesis are tryptamine and secologanin, which are synthesized from the shikimate pathway and MEP pathway, respectively. The intermediate strictosidine is produced from these two precursors in the presence of strictosidine synthase. After a series of enzymatic reactions involving the formation of multiple intermediates, camptothecin is formed from strictosamide in the plant (Cui et al., 2015; Yamazaki et al., 2004) (**Fig. 8)** . Camptothecin inhibits the action of DNA topoisomerase I by binding to it and block DNA re-ligation, which results in DNA damage. Three analogs of CPT, topotecan, irinotecan, and belotecan have received approval from the FDA for clinical treatment of several types of cancer (Liu et al., 2015b).

5.1 Pharmacokinetics

Pharmacokinetics of CPT varies among individuals (Rivory and Robert, 1995; Rubin et al., 1995). When CPT is administered via intravenous drip infusion, the peak plasma concentration is attained just after the end of drug infusion and declines in a biexponential manner with a half-life of ˜7.2 h (Venditto and Simanek, 2010). It is metabolized by carboxylesterase in the liver, and major excretion route is thought to

be non-renal. Patients who received CPT experienced diarrhea in most cases (Rivory et al., 1996).

5.2. Mode of action

The main mode of action of camptothecin against cancer cells includes binding with topoisomerase I to form a cleavable complex in-between DNA strands. Topoisomerase I involve in the unwinding of DNA strands during replication and ligation of DNA after replication. CPT inhibits the re-ligation function of topoisomerase I by forming a stable complex with it (Liu et al., 2015a) (**Fig. 11**).

5.3. Techniques involved in a biotechnological production

5.3.1 In vitro **production**

C. roseus has become endangered because of excessive harvesting for the extraction of anti-cancer compounds from this plant. For this reason, plant *in vitro* culture techniques to produce adventitious root from explants of shoots, leaves, and internodes has been used to cultivate this plant in farms because it is cost-effective and less-laborious. Adventitious roots induced from leaves and internodes yields a higher amount of CPT production compare to *ex-vitro*rooted stem cutting (Martin et al., 2008). UV-treatment as elicitor has improved CPT yields up to 11-fold in *Camptotheca acuminate* plant cell cultures (Kai et al., 2015), whereas salicylic acid-induced a 25-fold increase in 10-hydroxycamptothecin yields (Pi et al., 2010).

5.3.2. Heterologous expression

As noted above, *C. acuminate is* a camptothecin-producing plant in which geraniol is the biosynthetic precursor of secologanin and camptothecin. A heterologous host strategy may be useful to gain a more detailed understanding of camptothecin biosynthesis to enable improved production to improve its production by employing genetic engineering approaches. In this regard, genes from *C. roseus* encoding strictosidine synthase and geraniol 10-hydroxylase (*G10H*), were transformed into *Ophiorrhiza pumila* hairy roots. Co-overexpression of both genes enhances camptothecin production by 56% in comparison to non-transgenic lines and expression of the single gene(s) in the heterologous host (Cui et al., 2015). Microbial organisms are multipurpose heterologous hosts for pharmaceutically important natural products through metabolic engineering of their biosynthetic pathways (Morrone et al., 2010). Expression of the geraniol synthase gene from *C. acuminate* for geraniol production has successfully been achieved. Heterologous expression of geraniol synthase gene with optimization condition in recombinant *E. coli*produces 48.5 mg/l geraniol. This yield was higher than using transgenic tobacco and yeasts (Chen et al., 2016). Likewise, co-overexpression of strictosidine synthases (*STR*) and geraniol 10-hydroxylase (*G10H*) genes from *C. roseus*induced a 56% increase in camptothecin (CPT) production (Cui et al., 2015) (**Table 4**).

6. Miscellaneous specialized metabolites

Cancer suppression through the use of natural plant specialized metabolites, such as flavonoids, phenolic acids, carotenoids have been shown more effective and beneficial (Russo et al., 2005). The main mode of action of flavonoids and its derivatives is to inhibit cell proliferation and angiogenesis (development of abnormal blood cells), cause cell cycle arrest, induce cell apoptosis, and reverse multidrug resistance. Some derivatives such as quercetin targets the cell cycle at G1/S and G2/M checkpoints by inducing the p21 CDK inhibitor while decreasing pRb phosphorylation, thereby blocking an important transcription factor of DNA synthesis proteins which is E2F1.

In vitro studies of *Fagonia indica* revealed its potential to produce important specialized metabolites such as phenolic compounds (gallic acid, caffeic acid, catechin, epigenin, myricetin) with anticancer activity by the use of plant growth regulator thidiazuron (TDZ) in callus culture of stem and leaf of this species. TZD acts as an elicitor in the callus culture of this plant (Khan et al., 2016). There is also improved production of anticancer compound naphthodianthrones from*Hypericum perforatum* L. when elicitor salicylic acid is incorporated in the shoot and callus cultures of this plant (Gadzovska et al., 2013). Secondary compounds, β-glucan, and galactomannan, polysaccharides extracted from lichens, are also active against several cancer cell lines (Shrestha and Clair, 2013; Watanabe et al., 1986). Another class of secondary metabolites, rosmarinic

acid (RA) and salvianolic acid B (Sal. B) obtained by *in vitro* techniques such as callus cultures of stems and leaves of *Salvia miltiorrhiza* (Danshen) is yet another anticancer compound known to produce cytotoxicity against leukemia cell lines. Here, callus stem extracts proved to show more cytotoxicity towards leukemia cell lines than callus leaf extracts. Thus, callus culture techniques from Danshen for producing RA and Sal. B can be promising for anticancer therapy (Wu et al., 2016).

7. Conclusion and perspectives

Biotechnological tools like *in vitro* propagation and cell culture are attractive and cost-effective interventions for the synthesis and production of effective anticancer compounds at an industrial scale. A plethora of literature has been published on optimization of biomass growth using these techniques to increase the production of podophyllotoxin (from *Linum, Podophyllum),* taxanes (from *Taxus),* camptothecin (from *C. acuminate)* and terpene indole alkaloids (from *C. roseus)* . Studies have revealed the importance of different elicitors (both abiotic and biotic) for the activation of genes involved in the metabolic pathways to enhance the biotechnological production of anticancer compounds. Moreover, understanding and elucidation of highly complex biosynthetic pathways involving transcription factors and master regulators are inevitable for the successful application of these techniques. Recent developments in "Omics" technologies, especially proteomics and metabolomics, will help to improve the elicitation of metabolic pathways of secondary plant compounds. Such mechanistic insights provided by transcriptomic profiles and analysis of differential expression networks after elicitation will facilitate the identification of limiting steps while revealing prospective targets for metabolic engineering.

Moreover, these technologies also offer exciting opportunities to manipulate these metabolic pathways by controlling the expression of genes encoding transcription factors or master regulators. Advances in metabolomics will also facilitate to harvest elicitor-driven effects to develop highly productive cell cultures. Recent developments in synthetic biology techniques would also pave the way for the production of high added value secondary metabolites in heterologous systems. These technological breakthroughs will lead to cost-effective and sustainable commercial production of plant bioactive compounds with potent anticancer activity

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Table 1: Enhanced production of PPT compounds using different techniques

Species	**Compound**	**Techniques**	**Compound**	**References**
			content	
Ophiorrhiza prostrata	Camptothecin	Callus culture	0.16%	(Martin et al. 2008)
Fusarium solani	Camptothecin	Suspension culture	12.3 µg/l	(Venugopalan et al. 2016)
Ophiorrhiza pumila	Camptothecin	Heterologous host	1.64 mg/g DWT	(Cui et al. 2015)

Table 2: Enhanced production of taxol compounds using different techniques

Species	**Compound**	**Techniques**	**Compound**	**References**
			content	
Ophiorrhiza prostrata	Camptothecin	Callus culture	0.16%	(Martin et al. 2008)
Fusarium solani	Camptothecin	Suspension culture	12.3 µg/l	(Venugopalan et al. 2016)
Ophiorrhiza pumila	Camptothecin	Heterologous host	1.64 mg/g DWT	(Cui et al. 2015)

Table 3: Enhanced production of TIAs compounds using different techniques

Species	**Compound**	**Techniques**	**Compound**	**References**
			content	
Ophiorrhiza prostrata	Camptothecin	Callus culture	0.16%	(Martin et al. 2008)
Fusarium solani	Camptothecin	Suspension culture	12.3 µg/l	(Venugopalan et al. 2016)
Ophiorrhiza pumila	Camptothecin	Heterologous host	1.64 mg/g DWT	(Cui et al. 2015)

Table 4: Enhanced production of camptothecin using different techniques**.**

Species	**Compound**	**Techniques**	**Compound**	**References**
			content	
Ophiorrhiza prostrata	Camptothecin	Callus culture	0.16%	(Martin et al. 2008)
Fusarium solani	Camptothecin	Suspension culture	12.3 µg/l	(Venugopalan et al. 2016)
Ophiorrhiza pumila	Camptothecin	Heterologous host	1.64 mg/g DWT	(Cui et al. 2015)

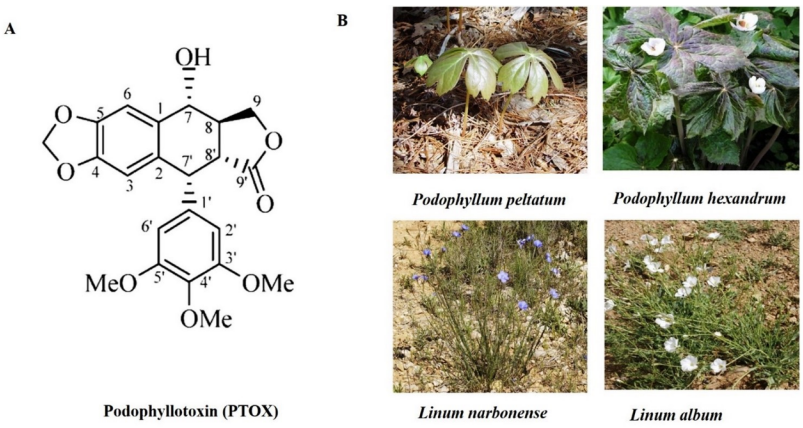

Figure 1. (A) Structure of PPT (B) Images of plant species producing PPT.

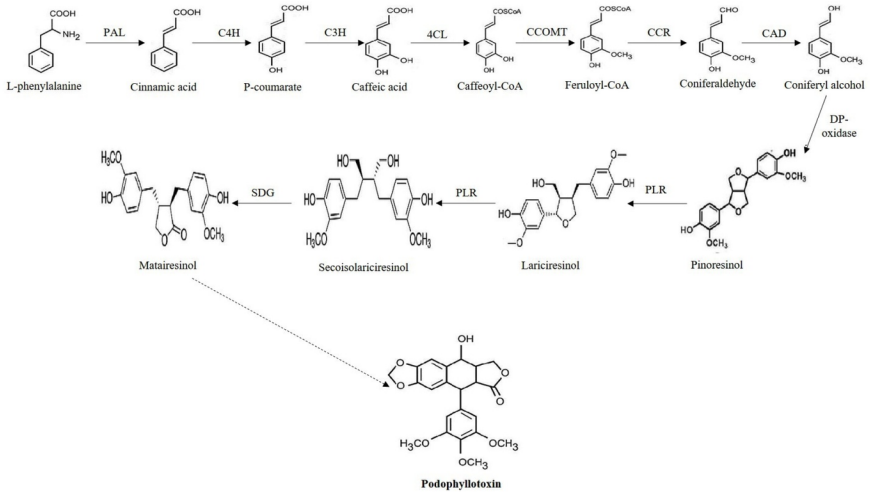

Figure 2. Podophyllotoxin biosynthetic pathway. PAL: Phenylalanine ammonia lyase; C4H: Cinnamate 4-hydroxylase; C3H: p-coumarate 3-hydroxylase: 4CL: 4coumarate: CoA ligase; CCOMT: caffeoyl CoA o-methyltransferase; CCR: cinnamoyl CoA reductase; CAD: cinnamyl alcohol dehydrogenase, DP oxidase: Dirigent protein oxidase; PLR: pinoresinol lariciresinol reductase, SDG: Secoisolariciresinol dehydrogenase. Dashed arrows represent multiple unknown reactions.

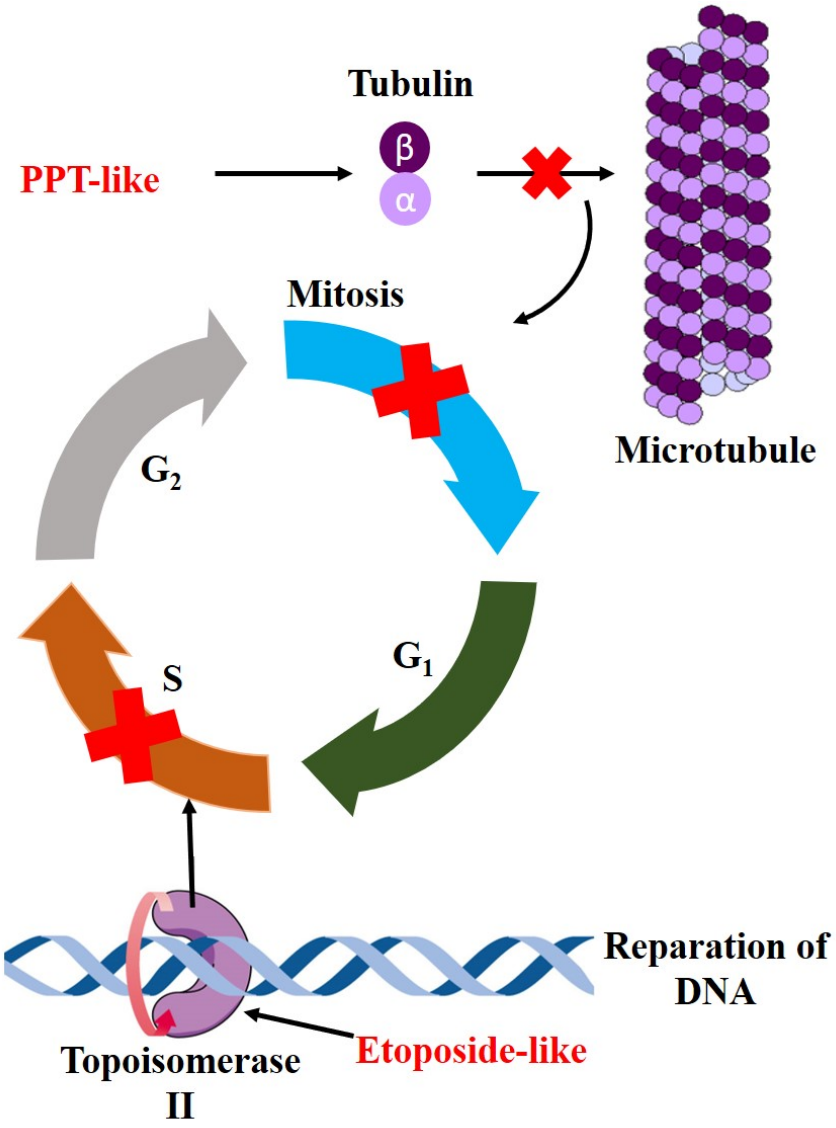

Figure 3: Mode of action of PPT and derivatives

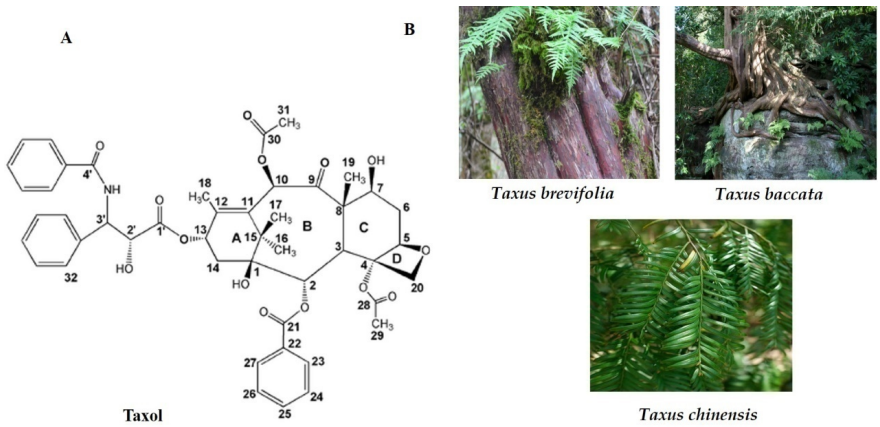

Figure 2. (A) Structure of Taxol. (B) Images of plant species producing Paclitaxel.

Figure 4. (A) Structure of taxol (B) Images of plant species producing taxol.

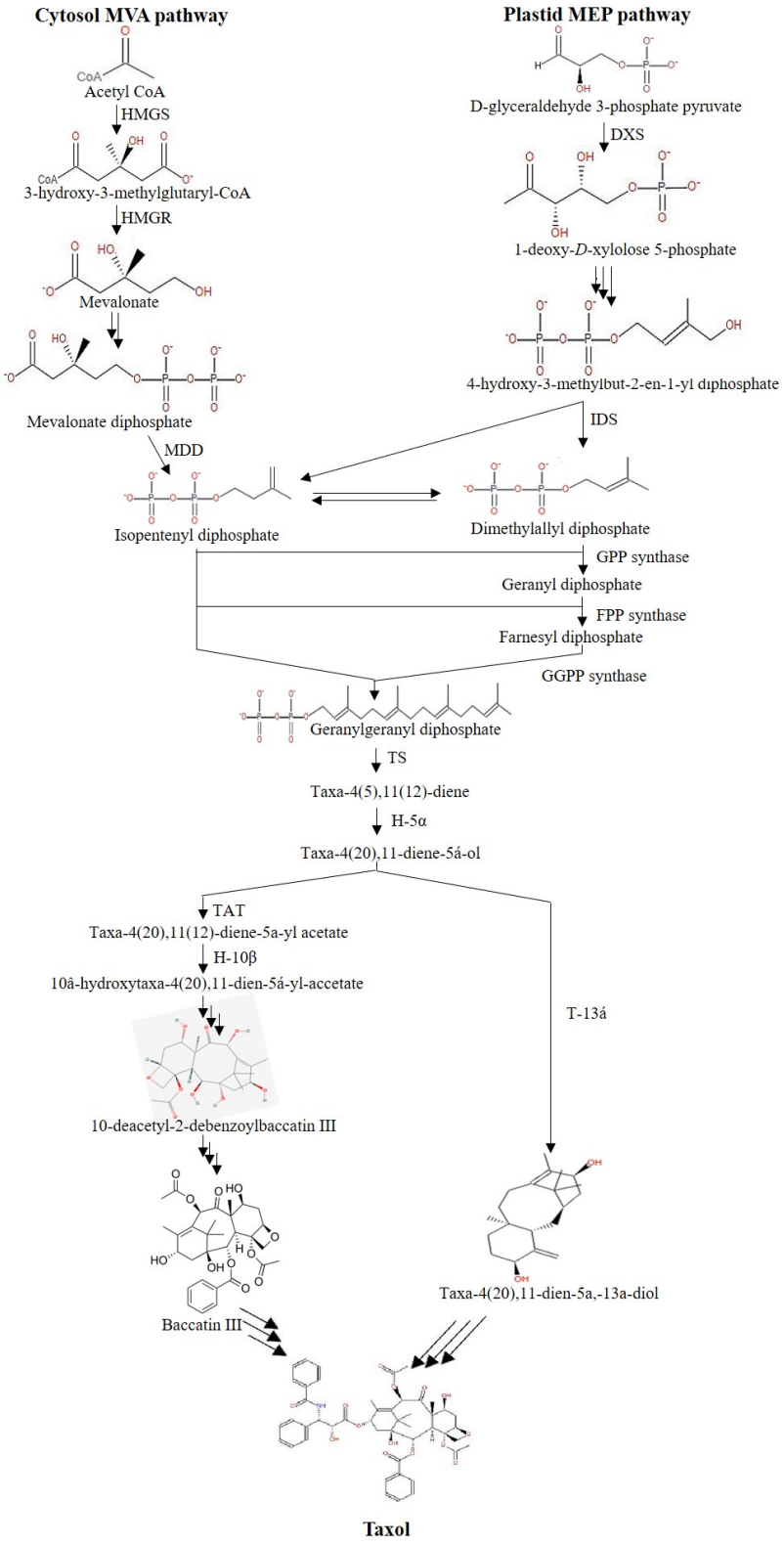

Figure 5. Taxol biosynthetic pathway. HMGS: 3-hydroxy-3-methylglutaryl-CoA synthase; HMGR: 3-hydroxy-3-methylglutaryl CoA reductases; MDD: MVA 5-diphosphate decarboxylase; DXS: 1-deoxyxylulose-5-phosphate; IDS: diphosphate synthase; IDI: isopentenyl-diphosphate isomerase; GPP synthase: geranyldiphosphate synthase; FPP synthase: farnesyl diphosphate synthase; GGPP synthase: geranylgeranyl pyrophosphate synthase; TS: taxadiene synthase; H-5α: cytochrome P450 taxadiene 5α-hydroxylase; TAT: taxa-4(20),11(12)-dien-5α-ol-O-acetyltransferase; H-10β: CYP450 taxane 10β-hydroxylase; T-13á: Taxane 13á-hydroxylase Multiple arrows indicate several steps.

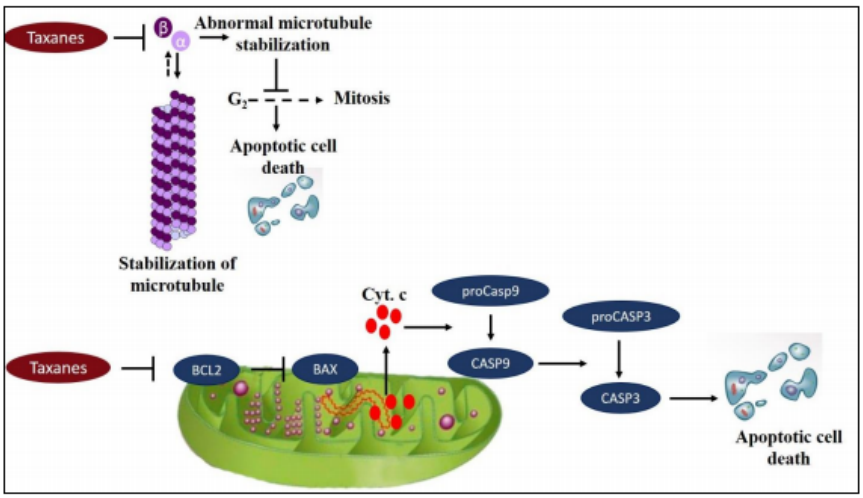

Figure 6: Mode of action of taxol and its derivatives.

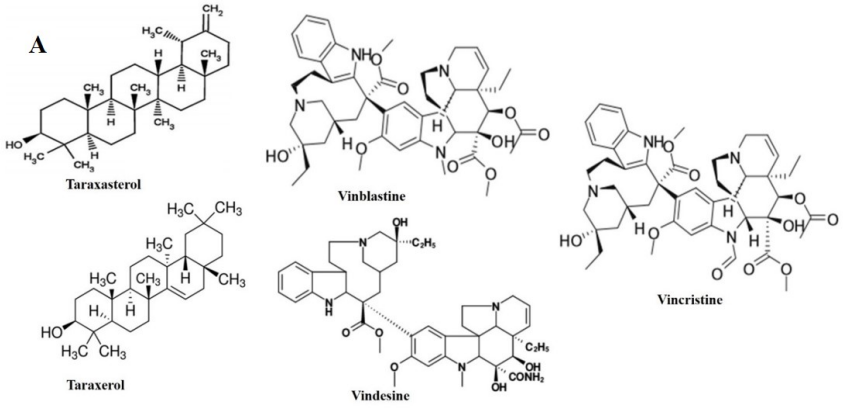

Figure 7. (A) Structure of terpene indole alkaloids. (B) Images of plant species producing TIAs.

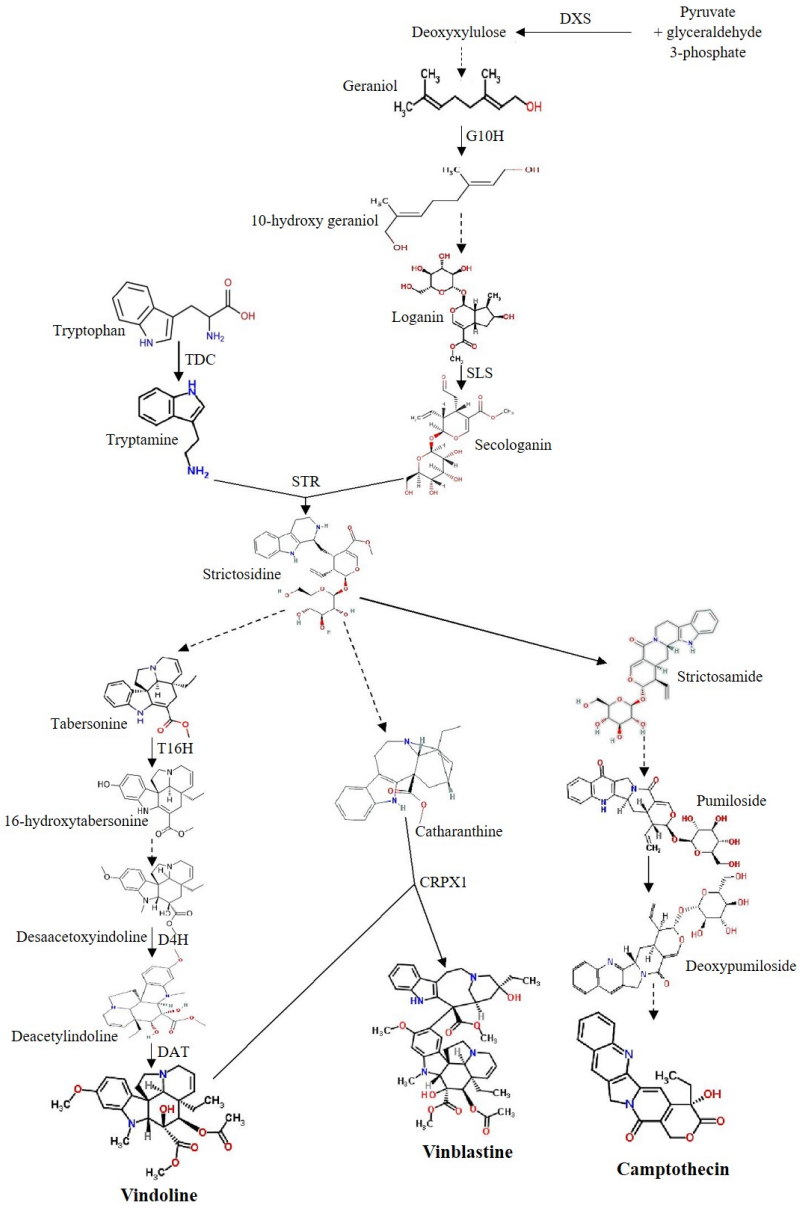

Figure 8. Biosynthesis of TIAs and Camptothecin: DXS: 1-deoxyxylulose-5-phosphate; G10H: geraniol 10-hydroxylase; SLS: secologanin synthase; TDC: tryptophan decarboxylase; STR: strictosidine synthases; T16H, tabersonine 16-hydroxylase; D4H: desacetoxyvindoline 4-hydroxylase; DAT: deacetylvindoline 4-O-acetyl transferase; CRPX1: vacuolar class III peroxidase. Dashed lines indicate multiple enzyme conversions.

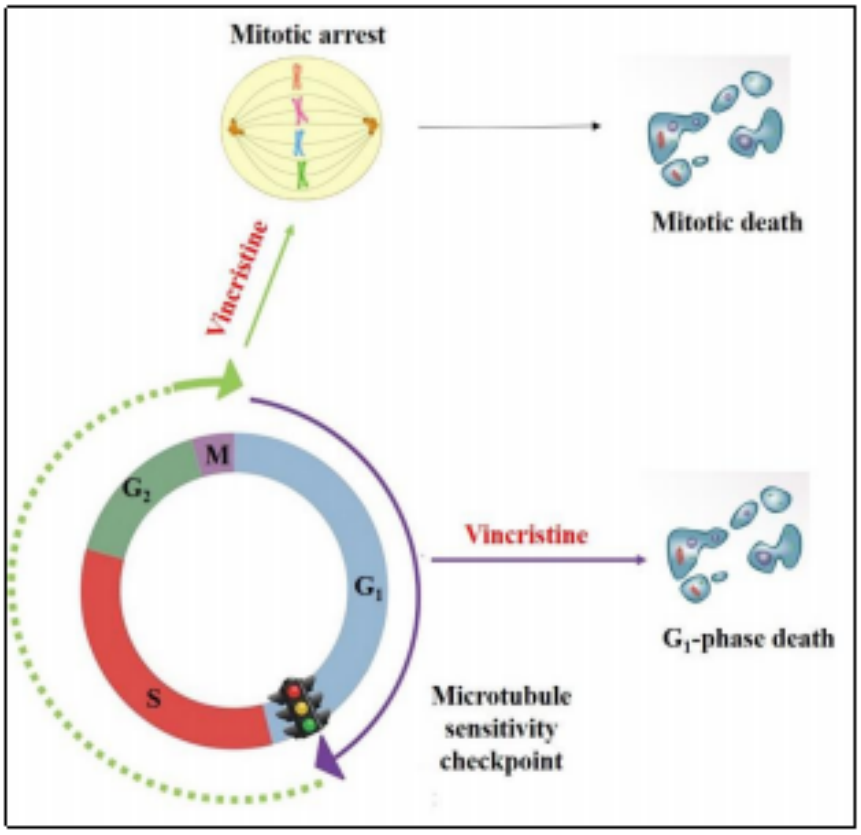

Figure 9: Mode of action of alkaloids.

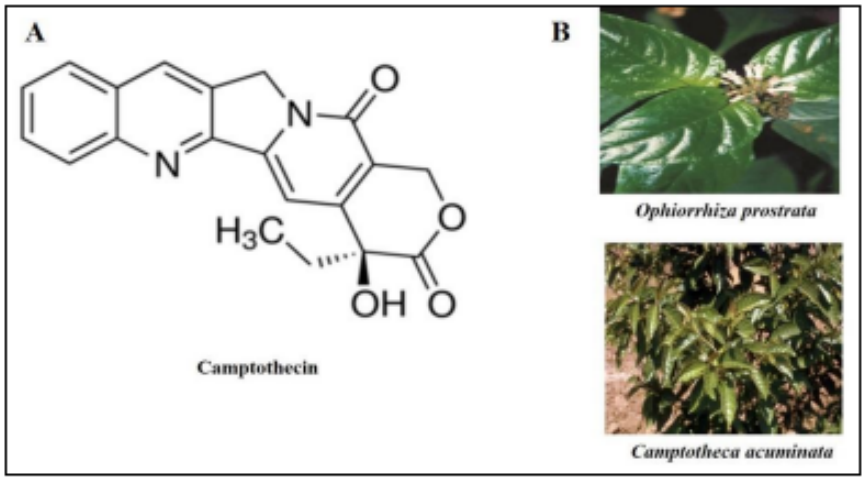

Figure 10. (A) Structure of camptothecin. (B) Images of plant species producing camptothecin.

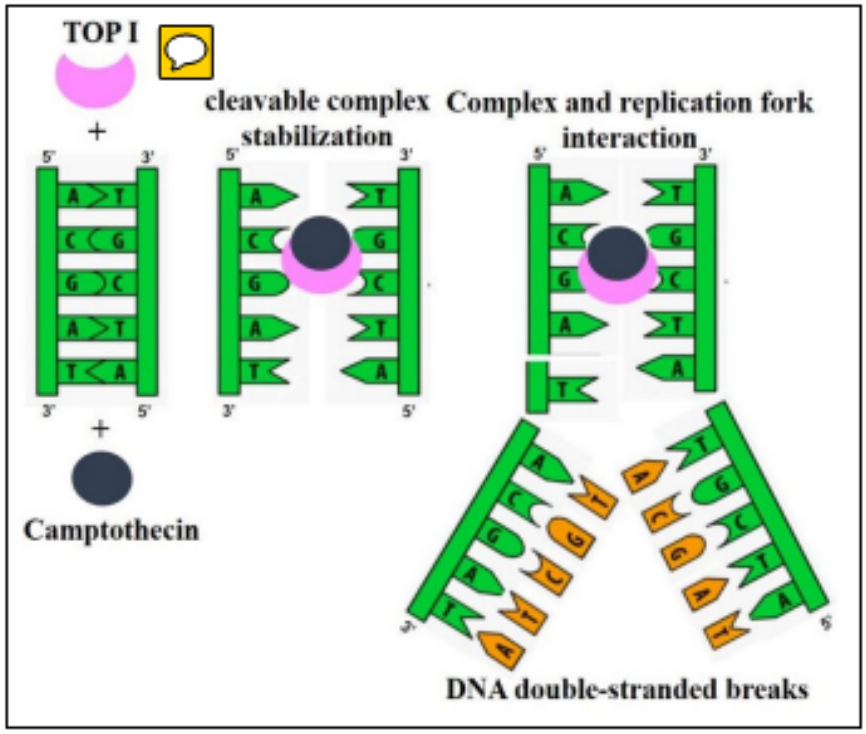

Figure 11: Mode of action of camptothecin.