Microbiological Advances in Bioactives from High Altitude

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Abstract

Owing to high altitude and extreme environmental conditions, Himalayas represents one of the biodiversity hotspot in India and home to several plants and microbial species. Thus, the plants and microbes present in these regions exhibit characteristic adaptations. The plants of high altitude Himalayan region are rich in varied secondary metabolites possessing several pharmacological activities. In this chapter, we focused on the antimicrobial and anticancer activities of the secondary metabolites present in the high-altitude plants and microbes, respectively, from the Himalayas. The ethnopharmacology of several plant species have been discussed which have been reported for antimicrobial activity by their essential oils. The major constituents of the essential oils that are responsible for such properties have also been explored. Later, the specific classes of secondary metabolites have been examined for their anticancer potential. However, the recognition of tremendous medicinal applications of Himalayan plants has resulted into their heavy exploitation in the past few decades. Due to this, several plant species like the Himalayan Yew have become endangered. To reduce their over exploitation and to obtain high-altitude bioactives in a sustainable manner, microbial production of such compounds have been investigated in past two decades. In the last section, we discuss about the biosynthesis of one of the largest class of bioactives i.e. terpenoids from microbial sources. Overall, the present

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chapter boasts the antimicrobial and anticancer potential of the bioactives from high-altitude and their sustainable production approach through microbial system.

Keywords
Antimicrobials · Higher altitude bioactives · Ethnopharmacology · Himalaya · Biodiversity

17.1 Introduction

The Himalayas are a mountain range in Asia that separates Indian subcontinent from Tibetan plateau and extend across 3500 km from Afghanistan to China. It comprises of many of the Earth's highest peaks. Owing to its high altitudes, extremely low temperature with high snow storms, wind velocity, scanty rainfall and blizzards and high ultraviolet (UV) radiation are common in Himalayan regions. Because of such extreme environmental conditions, the organisms that thrive in Himalayas possess characteristic survival adaptations. The principal component of such huge habitat multiplicity is the compression of thermal life zones and the fragmentation of the landscape into a various microhabitats. These microhabitats represent archipelagoes of peculiar life forms of therapeutic biodiversity. Even though Himalayas offer greater possibilities of having novel molecules and even largest quantities of active compounds (Dhawan 1997; Hazlett and Sawyer 1998), the information on medicinal biodiversity of high altitude regions in Indian Himalayan Region (IHR) is fragmentary. The chapter will discuss the antimicrobial and anticancer activities of some of the bioactivities from high altitudes and their sustainable production from microbial sources.

17.2 Major Families and Their Members Reported for Antimicrobial Essential Oils from Himalayas

Many microbial diseases are wreaking havoc on the world population. The greatest challenge is identifying potent novel entities which can be developed into novel antimicrobial agents. The section describes the major plant families and their aromatic plant species from Himalayas, with emphasis on Indian regions, known to be in used traditionally for their pharmacological applications, which are reported for antimicrobial potential of the essential oils extracted from them.

17.2.1 Asteraceae or Compositae Family

The family of flowering plants which is commonly referred as sunflower family, daisy family, or thistle family composed of almost 24,000–30,000 species and 1600–1700 genera distributed globally (Funk et al. 2005). The expression 'Aster'

which means composite, refers to the typical inflorescence pattern—flower heads comprising many small flowers, encompassed by bracts gives the family its name. Almost 900 species under 167 genera represents the family in India. Forms such as annual, biennial or perennial herbs, shrubs, undershrubs, a few trees, aquatics and some scramblers can be observed in the taxa. Succulents, few spiny, and some plants possessing milky sap are a part of this family. The perennial species sustain the harsh winter season by the means of underground storage organs and production of annual stems in spring (Moreira-Muñoz and Muñoz-Schick 2007). The family is found globally, but usually distributed in the tropical mountains and temperate regions (Bisht and Purohit 2010). Different phytochemical entities such as polyphenols, flavonoids and diterpenoids have been extracted from many members of the family which have displayed diverse pharmacological activities viz. the antibacterial, antifungal, anti-inflammatory, insecticide, antitumor and wound healing (Ertürk and Demirbag 2003; Singh et al. 2002; Suntar 2014).

The species are applied in traditional system of medicine, Unani-tibb and Ayurveda and ethnobotanically used as incense owing to their sweet aromatic odour or as an offering to local deities. Numerous members of the genus exhibit varied medicinal properties like antibacterial, antifungal, antihepatotoxic, antioxidant and antimalarial. Out of about 500 species reported, 45 are found in India (Shah 2014). About 19 species of the family are known for their pharmacological applications in the Himalayas (Sah et al. 2010; Semwal et al. 2015). The following artemisia species have been reported for antimicrobial essential oil.

***A. dubia*:** The plant known to be used in Ayurvedic and folk medicine is commonly known as 'dau'un' or 'dawn' like, 'nagdaun', 'nag damni' and more precisely, in Marathi- 'davan', Gujratati- 'damro'. 'Dauna' (Shah 2014). The leaf juice acts as an antiseptic and is used on cuts and bruises by the inhabitants of the Dolpa district (Kunwar and Adhikari 2005) and the Newar group of Kathmandu, Nepal (Balami 2004). Chrysanthenone (29.0%), coumarins (18.3%), and camphor (16.4%) are the chief elements of the essential oil (Satyal et al. 2012a). The leaf oil displayed cytotoxicity against human breast tumour cells and anti-fungal potential against *Aspergillus niger*. However the oil was inert against *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia* coli and *Pseudomonas aeruginosa* (Satyal et al. 2012a, b). The non-volatile elements impart the antiseptic properties to the plant.

***A. indica*:** The aerial parts of the plant sample from Kashmir was used to extract the essential oil which displayed an minimum inhibitory concentration (MIC) of 16 μg/mL against *S. aureus* and *Penicillium chrysogenum* consisted of artemesia ketone (42.1%), germacrene B (8.6%) and borneol (6.1%) (Rashid et al. 2013). However, it exhibited cytotoxicity against many human tumour cell lines viz. THP-1 (leukaemia), A-549 (lung), HEP-2 (liver) and Caco-2 (colon) cells. Whereas the leaf oil from Nepal constituted of ascaridole (15.4%), isoascaridole (9.9%), trans-*p*-mentha-2,8-dien-1-ol (9.7%) and trans-verbenol (8.4%) did not display any antibacterial, antifungal or cytotoxic activities (Satyal et al. 2012a, b).

***A. nilagirica*:** The tall aromatic shrub locally known as 'Indian wormwood' is a medicinal plant well known to be used for treating many microbial diseases, malaria, inflammation, diabetes and depression. The plant is applied in traditional along with Ayurvedic and Homeopathic applications (Mohanty et al. 2018). The paste derived from the plant leaves is traditionally used to control the bleeding through wounds by the residents of the Parvati valley, Himachal Pradesh, India (Stappen et al. 2014). With camphor (12.6%), artemisia ketone (10.2%), caryophyllene oxide (7.4%) and borneol (5.3%) as its major components, it demonstrated antifungal property against *Colletotrichum acutatum*, *Colletotrichum fragariae*, and *Colletotrichum gloeosporioides*, the plant fungal pathogens; but no antibacterial effect on *S. aureus*, *E.* coli*, Salmonella abony*, *P. aeruginosa* or *Candida albicans* (Singh et al. 2012). The essential oil sample from the plants collected from Uttarakhand, India primarily contained α-thujone, which exhibited activity against plant pathogenic fungi *Rhizoctonia solani*, *Sclerotium rolfsii* and *Macrophomina phaseolina* (Jaitak et al. 2008). Another oil sample from Uttarakhand which displayed decent MIC values of 6.25 and 12.5 μg/mL on *S. aureus* and *P. aeruginosa,* respectively, suggesting strong antibacterial potential contained linalool (16.3%), α-thujone (13.9%), β-caryophyllene (7.5%), germacrene D (7.1%) (Semwal et al. 2015).

***A. scoparia*:** The bitter aromatic herb commonly known as as 'Chauri Saroj' and 'Danti' in Bombay, 'Dona' and 'Jhan' in Punjab in India is known for various medicinal properties including antibacterial, insecticidal, antioxidant, anticholesterolemic, antipyretic, cholagogue, diueretic, purgative and vasodilatory effects (Shome et al. 1984; Bora and Sharma 2011). The paste obtained from the plant leaves are applied on cuts and wounds by the local population inhabiting in Nanada Devi, Uttarakhand (Rana et al. 2010). The leaf oil extracted from the sample collected from the Milam glacier, Uttarakhand, contained capillene (60.2%), γ-terpinene (11.1%), and 1-phenyl-2,4-pentadiyne (1.0%), while the root essential oil was rich in capillene (82.9%) and 1-phenyl-2,4-pentadiyne (2.6%) (Joshi et al. 2010a). The capillene (known for antibacterial and antifungal activities) rich oil is customary known to be used on cuts and wound in Uttarakhand as well as to relieve from colic, abdominal, cough and cold related issues (Rana et al. 2010; Yashina and Vereshchagin 1978; Christensen 2010). The essential oil displayed a MIC value of 12.5 μg/mL against *S. aureus* and *Bacillus subtilis*, demonstrating its significant antibacterial potential (Semwal et al. 2015).

***Ageratum houstonianum* Mill:** The plant commonly referred to as floss flower, distributed globally in tropical and subtropical regions is applied in traditional medicine for skin diseases and wound healing. Bioactives such as alkaloids, steroids, flavones, pyrrolizidine, precocenes and benzofuran have been extracted from this plant. Antifungal, antibacterial and antimicrobial properties are well reported in the literature for this plant (Shin et al. 2017). The essential oil derived from the samples collected from Himachal Pradesh was rich in chromenes, precocene-I (22.45%) and precocene-II (52.64%) along with two other chromene derivatives, desmethoxyencecalin (0.78%) and androencecalinol (0.3%). The oil lacked

monoterpene hydrocarbons but sesquiterpene hydrocarbons (15.97%) were abundantly present, with β-caryophyllene among the significant contributor. The chromene dominated essential oil earlier reported to possess acaricidal activity exhibited antibacterial effect on *Micrococcus luteus* and *Rhodococcus rhodochrous* (Kurade et al. 2010).

***Eupatorium adenophorum* Spreng.:** The plant commonly called as crofton weed is applied in folk medicines as, antiseptic, antimicrobial, antipyretic, analgesic, blood coagulant and enhancer of phenobarbitone induced sleep (Rai and Sharma 1994; Ansari et al. 1983; Mandal et al. 1981). The inhabitants of hilly areas of Kurseong and Darjeeling in Eastern Himalayas use leaves of the plant found at an altitude of 800–2050 m, for remedial purposes to treat oral and skin sores. The plant is reported for analgesic and anti-inflammatory properties (Chakravarty et al. 2011). The leaf juice of the plant in Nepal is believed to be antiseptic and is applied to treat injuries (Uprety et al. 2010; Uprety et al. 2011). The GC/MS investigation uncovered the major constituents to be 1-napthalenol (17.50%), α-bisabolol (9.53%), bornyl acetate (8.98%), β-bisabolene (6.16%), germacrene-D (5.74%) and α-phellandrene (3.85%). The essential oil samples of areal parts of the plant from Kangra valley, Himachal Pradesh exhibited moderate to high anti-bacterial activity against *Arthrobacter protophormiae*, *E.* coli, *M. luteus*, *S. aureus* and *R. rhodococcus,* respectively. The major constituents from the areal parts comprised of *p*-cymene (11.6%), α-phellandrene (5.7%), γ-curcumene (5.0%), δ-2-carene (5.0%), camphene (4.8%) and endo-bornyl acetate (4.4%) (Kurade et al. 2010).

***Blumea lacera*:** An annual herb called as kakronda in Hindi, bears a strong odour of turpentine is a common rabi weed which is described as antipyretic, anti-inflammatory, anthelminthic, astringent, acrid, thermogenic, errhine, styptic, ophthalmic, expectorant, liver tonic, febrifuge, deobstruant, diuretic and stimulant in ayurveda (Warrier and Nambiar 1993). The essential oil from *the plant* exhibits analgesic, hypothermic and tranquilizing properties (Pal et al. 1972). The plant also finds application as an important homoeopathic drug applied to treat enuresis, neuralgia, headache and cold borne cough (Oudhia et al. 1998). The plant is known to be traditionally used as antipyretic, antihelmintic, febrifuge and diuretic in the Sewa river region of Jammu and Kashmir, India (Khan et al. 2009). The oil derived from the areal parts of the plant collected from Baratnagar, Nepal majorly constituted (*Z*)-lachnophyllum ester (25.5%), (*Z*)-lachnophyllic acid (17.0%), germacrene D (11.0%), (*E*)-β-farnesene (10.1%), bicyclogermacrene (5.2%), (*E*)-caryophyllene (4.8%), and (*E*)-nerolidol (4.2%). The (*Z*)-Lachnophyllum ester has been reported to be antibacterial as well as anti-fungal (active against *S. aureus, C. albicans* and *A. niger*) and cytotoxic against MCF-7, MDA-MD-231 and 5637 human tumour cells (Satyal et al. 2015a).

***Inula cappa*:** Several reports in the literature exist for varied bioactivities for the genus Inula such as the reports against bronchitis and intestinal diseases; antipyretic, antihelmintic and antiseptic properties; or its combinatorial application with other

plants for nausea, excessive sputum and its description in traditional Chinese medicine against tumour (Song et al. 2002; Bai et al. 2006; Kim et al. 2002; Kobayashi et al. 2002). The major chemical entities identified in the oil derived from areal parts of the plant (commonly known as sheep's ear) samples from Okhalkanda in Nainital district at an altitude of 1400 m (Uttarakhand, India) are sesquiterpene hydrocarbons (50.6%), oxygenated sesquiterpenoids (20.7%), oxygenated monoterpenoids (12.6%) and monoterpene hydrocarbons (4.3%). The oxygenated diterpenoids comprised of only 3.0% of the oil composition. Constituents such as β-Caryophyllene (27.3%), (*E*)-β-farnesene (5.6%), β-bisabolene (6.5%) and cis-dihydro-mayurone (6.7%) were the major components observed. The oil inhibited the growth of both Gram positive and Gram negative bacteria. The maximum zone of inhibition was observed against Gram-positive *Enterococcus faecalis* (16 ± 1.00 mm), *B. subtilis* (14 ± 1.00 mm) followed by Gram negative *Xanthomonas phaseoli* (15 ± 1.73 mm), *Klebsiella pneumoniae* (14 ± 2.00 mm), *Agrobacterium tumefaciens* (13 ± 1.73 mm), *E. coli* (13 ± 1.00 mm) and lowest (9 ± 1.00 mm) against *Erwinia chrysanthem*. The oil exhibited a MIC of 2 μL/mL against *K. pneumoniae*, and 125 μL/mL for *E. coli*, *X. phaseoli*, *Salmonella enterica*, *Pasteurella multocida* and *B. subtilis* (Priydarshi et al. 2016).

***Matricaria recutita* L.:** The annual plant commonly called as chamomile possesses thin spindle-shaped roots only penetrating flatly into the soil (Singh et al. 2011). In ancient Egypt, Greece and Rome, it is known for herbal applications for many years (Issac 1989). It is well known as 'star among medicinal species'. for its various pharmacological properties such as antimicrobial, antiviral, antioxidant, anti-inflammatory, antispasmodic and sedatory effects. It is used tropically to treat skin and mucous membrane inflammation, bacterial infections; anal and genital disorders and respiratory irritations (Bruneton 1999; Satyal et al. 2015b). Terpenoids, flavanoids, coumarins and spiroethers are the major constituents responsible for the varied pharmacological activities. The GC/MS results of the oil extracted from the areal parts of the plant samples from Nepal revealed the composition to be (*E*)-β-farnesene (42.2%) and α-bisabolol oxide A (22.3%), (*E*,*E*)-α-farnesene (8.3%), cisbicycloether (5.0%), α-bisabolol oxide B (4.5%), and α-bisabolone oxide A (4.0%). The oil exhibited no notable toxic effects for larvicidal activity against glassworm (*Chaoborus plumicornis*), brine shrimp (*Artemia salina*) lethality, insecticidal activity against fruit fly (*Drosophila melanogaster*) and nematicidal activity against *Caenorhabditis elegans*, however displayed only moderate activity against *S. aureus, P. aeruginosa* and *C. Albicans* with a MIC of 313 μg/mL (Satyal et al. 2015b).

***Tanacetum longifolium* Wall. Ex DC:** The genus Tanacetum, commonly referred to as tansy comprises of six species viz. *T. nubigenum, T. tibeticum, T. longifolium, T. arteminiodes, T. gracile* and *T. senecionis* found in Kumaun and Garhwal regions (India) at an altitude of 3600–4300 m (Strachey and Duthie 1974; Polunin and Stainton 1984). These plants are reported for anthematic, carminative, stimulant,

antispasmodic and anti-migrant properties (Joshi and Bisht 2012). The Gaddi shepherd community of Kashmir use the root powder of the plant for stomach pain and the leaves are also applied for stomach ache and indigestion by residents of Kedarnath Wildlife Sanctuary, Uttarakhand (Ballabh and Chaurasia 2007; Singh and Rawat 2011). The essential oil derived from areal parts from Milam glacier, Uttarakhand is dominated by trans-sabinyl acetate (43.2%) and trans-sabinol (12.7%) was found to be antifungal (*C. Albicans* and *C. glabrata)*. The oil also displayed good antibacterial effect against *E. coli* with 22 mm zone of inhibition and low to moderate killing potential against *S. aureus* (9 mm), *B. subtilis* (9 mm), *K. pneumonia* (11 mm) and *Streptococcus mutans* (14 mm) when compared with standard antibiotic chloramphenicol (21–30 mm) (Joshi 2013).

17.2.2 Lamiaceae

The mint family of flowering plants mostly shrubs and herbs represents around 236 genera and 6900–7200 species is known to be distributed in temperate forests of India and elsewhere (Tamokou et al. 2017; Gairola et al. 2010). The square and opposite leaves are typical characteristics of the species of this family. Most species are aromatic and are known to possess essential oils (Lawrence 1992). The oils find their applications in cosmetic, flavouring, fragrance, perfumery, pesticide and pharmaceutical industries (Özkan 2008). Due to ease of cultivation and propagation through stem cuttings, many species of the family are widely cultivated (Raja 2012). Many phenolic classes of compounds such as simple phenols (e.g. eugenol), tannins, quinones, flavonoids, lignans and some terpenoids are observed in the family possessing diverse biological activities such as antimicrobial, anti-inflammatory, analgesic, anti-tumour, antioxidant, immunostimulant, antitussive, expectorant and cytotoxic. Owing to the existence of thymol and carvacol which interfere with the cellular metabolism after penetrating the cell wall in many members of the family are reported to possess antimicrobial properties (Carović-StanKo et al. 2016).

***Anisomeles indica* (L) Kuntze:** Commonly called as Kala bhangra in Hindi is a camphor-scented large perennial woody shrubby herb, prevalent in tropical and subtropical regions of India. It possesses aromatic astringent, carminative and tonic characteristics and is known to be applied in traditional medicine as an antidote to gastric catarrh and fever and essential oil found in the herb is employed in urine infection (Patel and Patel 2013; Murthy et al. 2015). The plant is reported to be applied for various disorders such as colic dyspepsia in children, liver issues, rheumatism, stomach ache, fever, abdominal pain, psoriasis and many others. The plant extract is reported to be antibacterial and antifungal (Patel and Patel 2013; Kundu et al. 2013). The oil form Toranmal forest, Satpuda valley in Maharashtra, India at an elevation of 1800 m is reported to be antibacterial. The GC/MS investigation of the oil extracted from aerial parts and roots of the plant revealed the presence of monoterpenes and sesquiterpenes compounds such as isobornyl acetate (64.6% and 55.36%), isothujone (6.01% and 12.37%), nerolidol (3.17% and 7.19%),

camphene (3.54% and 5.52%), decanal (2.29% and 1.61%) and eugenol (3.25% and 4.15%). The root oil in general was more potent than the oil extracted from the aerial parts. The oils displayed antibacterial effects with decent MIC values against *E. coli* (125–250 μg/mL), *S. aureus* (125–250 μg/mL), *P. aeruginosa* (62.5–125 μg/mL) and *Bacillus pumilus* (31.25–62.5 μg/mL). The oils were also found to exhibit bactericidal effect against these pathogens (Ushir et al. 2010).

***Leucas aspera* (Wild) Link:** Commonly referred as 'Thumbai', is a species of annual branched herb spread throughout South Asia (India, Bangladesh and Nepal), Malaysia and Mauritius. In India the plant is applied traditionally to cure headache, asthma and bronchitis. The plant is also useful against scabies psoriasis, snake bite, toothache and is applied tropically as insect repellent and the leaf extract is employed to soothe toothache (Das et al. 2012; Rajakumar and Shivanna 2010). The plant is known for its various pharmacological activities viz. antimicrobial, antioxidant, antinociceptive and cytotoxic activity (Prajapati et al. 2010). The essential oil of *L. aspera* collected from Biratnagar, Nepal analyzed by GC-MS contained 1-octen-3-ol (30.6%), β-caryophyllene (23.4%) and caryophyllene oxide (24.4%). The oil was tested for antimicrobial effect against *B. cereus*, *S. aureus*, *P. aeruginosa*, *E. coli*, *C. albicans*, and *A. niger*. It was moderately hostile to *S. aureus* (MIC = 625 μg/mL), B. *cereus* (MIC = 313 μg/mL), and *A. niger* (MIC = 313 μg/mL), most probably the sesquiterpenes present in the oil accounts for the effect (Satyal et al. 2015b).

Nepeta genus: The genus containing around 280 annual and perennial species, mostly aromatic plants is also called as Glechoma and Cataria. The genus is widely spread in temperate Asia, Europe, North Africa and North America and in the Mediterranean region. Almost 30 species are reported in India, mainly recorded in the temperate Himalaya. The members are characterized by the presence of terpenoids, flavonoids and phenolic constituents which are responsible for various biological properties such as antimicrobial, antispasmodic, diuretic, febrifuge, diaphoretic, insecticidal, larvicidal, cytotoxic, anticancer, analgesic, anti-inflammatory, anticonvulsant and antiseptic (Süntar et al. 2018; Bisht et al. 2010). Besides these activities various species are also employed for tooth troubles; as laxatives to treat dysentery, and for liver and kidney ailments. Various Nepeta species are applied in traditional medicines for digestive disorders, cold, influenza, diarrhoea, fever and malaria (Dutt et al. 2015; Bano et al. 2014; Sharma et al. 2015; Bisht et al. 2012). Ethnobotanical investigations reveal the use of many species such as *N. Indica* L. infusion as tonic and for treating bronchitis in Turkey; *N. betonicifolia* C.A. Mey application against cough, wound healing, cancers and rheumatism; *N. Cataria* L. use for relieving menstrual problems in Serbia; *N. lagopis* Benth. extract application on wounds and the decoction obtained from the aerial parts of *N. praetervisa* Rech. f. is utilized to treat helminth infections in Pakistan (Süntar et al. 2018). The existence of pharmacologically potent iridoids, monoterpene neptalactones in Nepeta species accounts for the diverse pharmacological effects. The essential oil from various *Nepta* species viz. *Nepeta leucophylla, Nepeta discolour, Nepeta govaniana, Nepeta*

clarkei, Nepeta elliptica and *Nepeta erecta* collected from regions of Uttarakhand are reported to be antimicrobial with moderate to high effect against five Gram negative bacteria viz. *P. aeruginosa*, *E. coli*, *P. multocida*, *Proteus vulgaris* and *Serratia marcescens*; one Gram positive bacterium *S. aureus* and fungal pathogens *C. albicans* and *Trichophyton rubrum*. The essential oils of the *Nepeta* species notably exhibited the high inhibition zones varying from 18.2 to 28.4 mm against *P. aeruginosa* with the observation that oils of *N. elliptica* and *N. Erecta* were most potent (28.4 mm, MIC = 0.31 μL/mL and 28.0 mm, MIC = 0.62 μL/mL) followed by *N. leucophylla* (27.4 mm, MIC = 0.42 μL/mL) and N. clarkei (22.0 mm, MIC = 0.15 μL/mL). The essential oils from *N. elliptica* and *N. erecta* were very active against *S. marcescens* (20.2 mm, MIC = 0.43 μL/mL and 18.3 mm, MIC = 1.59 μL/mL) as well. The oil from *N. Leucophylla* also showed notable hostility to *P. vulgaris* and *S. aureus* (21.2 mm, MIC = 3.21 μL/mL; 16.4 mm and MIC = 1.78 μL/mL). The oils also displayed antifungal activities with *N. leucophylla* displaying killing potential against both *C. ablicans* (20.0 mm, MIC = 0.78 μL/mL) and *T. rubrum* (19.2 mm, MIC = 0.19 μL/mL). The oils from *N. elliptica, N. erecta* and *N. govaniana* also exhibited noteworthy activity against both the fungal strains; however, *N. clarkei* and *N. discolor* demonstrated poor activity against both the strains (Bisht et al. 2010). The oils extracted from above mentioned Nepeta species majorly contained iridodial derivatives (*N. Leucophylla*), viz. iridodial β-monoenol acetate, dihydroiridodial diacetate and iridodial dienol diacetate; β-caryophyllene, 1,8-cineolep-cymene (*N. discolor*); isoiridomyrmecin and pregeijerene (*N. govaniana*); germacrene D, β-sesquiphellandrene, α-guaiene and diastereomeric iridodial esters (*N. clarkei*); and 7(R)-trans,trans-nepetalactone and isoiridomyrmecin (*N. elliptica* and *N. erecta*) (Bisht et al. 2010).

***Origannum vulgare* L.:** The plant commonly known as 'oregano' and 'Himalayan Marjoram' in India is an aromatic perennial herb which is vastly applied in traditional medicine for treating ulcers, bronchitis, diarrhoea, dysentery, wounds, weeping eczema, insect bites, cough and cold, stomach ache and many others (Sharma et al. 2004; Rana et al. 2010; Uniyal and Shiva 2005; Pezzani et al. 2017). Various medicinal bioactives, such as phenolic glycosides, sterols, flavonoids, tannins, and large amounts of terpenoids are reported from its aerial parts which accounts for its varied biological properties like antimicrobial, anti-inflammatory, anticancer and antioxidant (Pezzani et al. 2017). Monoterpenes (contributing 90.5% of the total oil constituents) forms the chief component of *Origanum vulgare* essential oil. The main fraction of the monoterpene hydrocarbon component was *p*-Cymene (10.3%), whereas thymol (53.2%) and carvacrol (3.9%) served as the most abundant oxygenated monoterpenes. The thymol rich oil from Uttarakhand was found to be antifungal against *Aspergillus flavus* and *A. niger*. The huge amount of oxygenated compounds recovered may explain for the antifungal activity observed (Bisht et al. 2011).

***Thymus serppyllum* L:** The plant commonly called as the wild thyme is extensively distributed in Jammu and Kashmir. The aromatic plant is widely applied in

traditional folk medicine for years as antiseptic, diaphoretic, analgesic and diuretic (Aziz et al. 2010). The plant juice is taken to treat cough and asthma in Almora district of Uttarakhand (Kumari et al. 2012). It is also an emmanagogue, carminative and stimulant. The plant is helpful in digestive, genito-urinary system and respiratory problems. The major fractions of the essential oil extracted from the plant in regions of Muzaffarabad, Pakistan at an elevation of 1200 ft are thymol (30%), carvacrol (20%), *p*-cymol, linalool and other terpenes. The oil displayed significant activity (inhibition 60%) against *Trichophyton longifusus* and *Fusarium solani* (inhibition 70%), but was inactive against *C. albicans, A. flavus* and *C. glaberata.* The presence of thymol and carvacrol are thought to account for the activity (Aziz et al. 2010).

17.2.3 Lauraceae

The woody plants family (barring Cassytha, the herbaceous parasite) representing 50 genera and about 2500–3000 species is spread around the tropical to subtropical latitude are deciduous or evergreen shrubs or trees often with aromatic bark and leaves (Oliveira-Filho et al. 2015). Globally, most of its members bear great economic value because they not only provide timber but are extensively used in the pharma and food industries, with prominent applications of the genera Aniba, Nectandra, Licaria and Ocotea (Silva et al. 2009). The members are reported to display varied biological properties such as antibacterial, fungicidal, antiviral, antipyretic, antispasmodic, antitumour, anticonvulsant, cytotoxic, cruzain inhibitory activities and antioxidant effects (Oliveira-Filho et al. 2015). Bioactives like monoterpenes and sesquiterpenes, triterpenes, alkaloids, and sterols, 2-pyrones, benzophenones, flavonoids and arylpropanoids are known to be a part of various members of the family (Chin et al. 2010). On analyzing the terpenoid diversity in the family through biochemical investigations, the Himalayan Lauraceae species are categorized into two groups, viz., furan-carrying genera (Neolitsea, Lindera, and Dodecadenia), mono- and sesquiterpenoid-rich genera (Persea and Phoebe); and oxygenated monoterpenoids-dominating genus (Cinnamomum) (Joshi et al. 2009).

***Cinnamomum* species:** The genus Cinnamomum contains over 250 aromatic evergreen trees and shrubs, primarily spread in Asia and Australia. The cinnamon bark is known to be applied in Ayurvedic medicine as an antiemetic, antidiarrheal, antiflatulent and general stimulant (Barceloux 2009). Different plant species are employed as antiviral, antiseptic, bactericidal, anti-inflammatory, diuretic, counterirritant, expectorant, stimulant, vermifuge, rubefacient, decongestant, analgesic and cough suppressant (Agarwal et al. 2012; Hamidpour et al. 2013). Many traditional uses of the species are observed such as *Cinnamomum glanduliferum*is is used to treat toothache and wounds in Nepal; *Cinnamomum glaucescens* is applied for kidney problems in Manipur, India and *Cinnamomum tamala* is utilized for gastric issues in far western Nepal. Cinnamomum species are commercially valuable source of camphor, cinnamaldehyde and safrole oil in the world (Kumar and Kumari 2019). The

essential oil extracted from the *Cinnamomum camphora* collected from various places viz. Pantnagar and Naukuchiatal, Uttarakhand and Mekwanpur, Nepal majorly contained camphor along with linalool, 1,8-cineole, nerolidol, borneol, camphene, limonene, sabinene, and β-pinene. The leaf oil from Uttarakhand displayed antibacterial effects against *P. multocida* whereas the sample from Nepal was observed to be antifungal against *A. niger* (Agarwal et al. 2012; Satyal et al. 2013). The Nepal sample also exhibited cytotoxic activity against MCF-7 human breast tumour cells, allopathic properties and insecticidal activity as well (Satyal et al. 2013). The leaf oil extracted from *C. glanduliferum* from northern India contained 1,8-cineole (41.4%), α-pinene (20.3%), α-terpineol (9.4%), germacrene D-4-ol (6.1%) and α-thujene (5.10%) (Singh et al. 2013). The plant leaves are traditionally used in northern India against cough and cold. The high content of 1,8-cineole in the oil is responsible for its potency against coughs and cold (Kumar and Kumari 2019). The oil showed antibacterial activity against Gram-positive bacteria and Gram-negative bacteria viz. *Micrococcus luteus*, and *E. coli, P. aeruginosa* and *Aeromonas salmonicida* with MIC values of 6.86 and 3.40, 3.43 and 1.72 μg/mL, respectively (Singh et al. 2013). The leaf oil extracted from *C. tamala* collected from Munsyari and Lohaghat Uttarakhand displayed antibacterial activity against *S. enteric*, *E. coli*, *P. multocida.* The oil constituted of linalool, (*E*)-innamaldehyde, 1,8-cineol and (*E*)-cinnamaldehyde (Agarwal et al. 2012).

Dodecadenia grandiflora **Nees:** The plant commonly referred as Tailiya is dispersed in India, Myanmar, Bhutan and Nepal. Traditionally, it is known to be applied for diabetes. The compounds phenylpropanoyl esters of catechol glycosides and two lignane bis esters account for the antihyperglycemic effect (Kumar et al. 2009). The aqueous decoction of the leaves is used by the traditional practitioners in the parts of Uttaranchal, India to check the blood sugar level in humans (Kumar et al. 2010). The chief constituents of the leaf essential oil from the plant sample collected from Uttarakhand were furanosesquiterpenoid furanodiene (13.7%) and germacrene D (26.0%). The oil displayed antibacterial property against *S. aureus* and *P. multocida* along with potent free radical scavenging and impediment of lipid peroxidation activities (Joshi et al. 2010b).

Lindera neesiana **(Wall. Ex Nees) and** ***Linedera pulcherrima*****:** *Lindera neesiana* is commonly called as 'Siltimur' in Nepal. It is a medium-sized tree known to grow in temperate regions of Himalayas. The leaves of this aromatic and spicy plant are applied as a carminative. In Nepalese traditional medicine, the fruit is usually chewed to treat diarrhoea, tooth pain, gastric disorders and headache; while its paste is tropically applied as a remedy to boils and scabies (Uprety et al. 2010; Rokaya et al. 2010; Comai et al. 2010). The plant is also used to obliterate intestinal parasites, and to medicate against plant poisoning in cattle (Comai et al. 2010). The essential oil proposed to be used tropically displayed antimicrobial activities against *S. aureus* and *C. albicans* with no cytotoxic properties. *Linedera pulcherrima*, an evergreen shrub is spread in temperate Himalayan regions. The bark and leaves are applied as spice to treat cold, cough and fever. The essential oil majorly contained

furanodienone (46.6%), curzerenone (17.6%) which are reported to possess antimicrobial, insecticidal, analgesic and anti-inflammatory properties (Joshi et al. 2009; Joshi et al. 2010b). The essential oil manifested activity against *S. aureus and S. enteric*. It also displayed potent free radical scavenging and retardation of lipid peroxidation activities (Joshi et al. 2010b).

***Persea duthiei* (King) Kosterm. and *Persea gamblei* (King ex Hook. F.) Kosterm.:** In India the leaves are utilized for fodder and the fruits are consumable, however the plants are not reported to for any medicinal applications (Negi 2005; Rijal 2011). The essential oil extracted from leaf sample *of P. duthiei* and *P. gamblei* collected from Uttarakhand contained (*E*)-nerolidol (13.2%), limonene (10.1%), α-pinene (10.0%), β-pinene (10.0%), epi-cubebol (5.8%), b-caryophyllene (5.8%) and b-eudesmol (4.0%); and sesquiterpene hydrocarbons (62.8%) viz. b-caryophyllene (22.1%), c-gurjunene (16.8%) and b-cubebene (7.2%), respectively ([b]Joshi et al. 2009). The essential oil of *P. duthiei* was effective against Gram negative bacteria viz. *E. coli* and *P. multocida*. On the other hand, *P. gamblei* essential oil demonstrated potency against Gram positive bacteria *S. aureus* only (Joshi et al. 2010b).

17.2.4 Cupressaceae

The well-known gymnosperm family that produces allergenic pollen is most widely distributed worldwide. The family represents approximately 30 genera, and about 160 species of monoeciuos, subdioeceous (rarely) or dioecious trees and shrubs (Bartel 1994). Many members of the family are known for their antimicrobial, anti-inflammatory, antifungal, analgesic, hepatoprotective, antidiabetic and antihyperlipidemic activity, antioxidant activity, antihypercholesterolemic, anticataleptic and cytotoxic activities, and are also reported to stimulate cancer cells towards apoptosis (Bais et al. 2014). Traditionally different members are applied to relieve pain, cough and cold, digestive issues, haemorrhoid, varicose veins and venous circulation disorders, and urinary problems (Pirani et al. 2011; Al-Snafi 2016).

***Thuja orientalis* L.:** The traditional medicines and homeopathy use of the evergreen, monoecious plant commonly referred as morpankhi in many ways is well known. Traditionally it finds its application to treat cystitis, bronchial catarrh, enuresis, psoriasis, amenorrhea, uterine carcinomas and rheumatism (Srivastava et al. 2012). The boiled decoction of the bark of the evergreen species which is extensively cultivated ornamental plant is consumed orally to treat leucorrhea in the Garhwal belt of India (Ghildiyal et al. 2014). The powdered seeds are known to be applied for tooth ache in the Khyber Pakhtunkhwa, Pakistan (Khan et al. 2015). The GC and GC/MS results of the essential oil extracted from leaf sample of the plant collected from Kangra, Himachal Pradesh revealed α-Pinene (29.2%), Δ-3-carene (20.1%), α-cedrol (9.8%), caryophyllene (7.5%), α-humulene (5.6%), limonene

(5.4%), α-terpinolene (3.8%) and α-terpinyl acetate (3.5%) as its chief constituents. The sample exhibited antifungal potential against *Alternaria alternata*. One of the bioactive compounds in the oil was identified as α-cedrol (Guleria et al. 2008).

***Juniperus macropoda* Boiss.:** The evergreen tree commonly called as the Indian juniper or Himalayan pencil cedar with its origins in the Indian subcontinent (India, Pakistan) along with western and middle Asia is spread in Himalayas at an elevation up to 4500 m above sea level. The plant berries are applied to treat diarrhoea, skin diseases, cough, colic, indigestion, while the resin is applied on ulcers in Himachal Pradesh and the leaf paste works as incense in Kashmir (Bhattacharyya 1991; Rather et al. 2012) and Tibet (Choedon and Kumar 2012). The berries along with other fruits are consumed for kidney ailments (Ballabh et al. 2008). Variation in oil constituents was observed in samples collected from various regions of Himalayas. The leaf samples from Himachal Pradesh contained mainly sabinene (27.5%), Cedrol (14.1%), terpinen-4-ol (9.4%) and pcymene (4.2%) (Stappen et al. 2015). The leaf oil derived from plants grown in Garhwal areas of Uttaranchal, India contained β-elemene (42.5%), trans-sabinene hydrate (8.8%) and α-cubebene (7.9%). Whereas α-thujone (22.6%), biformene (7.7%) and sabinene (5.8%) was prominently found in the leaf sample from Mussorie, Uttarakhand (Srivastava et al. 2005). The oil exhibited moderate activity against *C. albicans* (MIC 250 μg/mL) but a low antibacterial potential against *S. aureus*, *E. coli* and *S. abony* (MICs of 1000 μg/mL, each). Previous reports of weak antifungal activity by other species containing sabinene as major constituent indicates that the effect displayed by *J. macropoda* is due to terpinen-4-ol (Stappen et al. 2015).

17.2.5 Caprifoliaceae

The family is cosmopolitan in distribution, primarily found in temperate regions The members of the family mostly shrubs and vines, rarely herb reside at cold and inaccessible high altitudinal realms of lofty mountain ranges of the Himalaya and the Western Ghats (Clarke 1880). The family is known to include 260 species of 12 genera. The members are known to be used to treat fevers, bacterial dysentery, stomach disorder, enteritis, conjunctivitis, laryngitis, inflammations of the urinary tract and reproductive organs, flu, cardiac disorders, rheumatism and pain (Acharya 2016).

***Morina longifolia* Wall. Ex DC:** The plant commonly known as Whorlflower, Biskandra or Somrus spread over the temperate and alpine areas of the Himalayas from Kashmir to Bhutan at an elevation of 2400–4200 m is a perennial aromatic herb of medicinal value. There are several reports of the plant's medicinal applications in traditional system of Indian and Tibetan medicines. The root powder is used on boils and wounds in Parvati valley of Himachal Pradesh; fresh leaves are applied to treat boils and injuries in Chamoli district of Uttarakhand and the root juice is utilized for dysentery and diarrhoea by the local population of Kaverpalanchowk district of

central Nepal (Sharma et al. 2004; Phondani et al. 2010; Malla and Chhetri 2009). Morinoursolic acids A and B, *n*-triacont-3-one, 8-methylditriacont-7-ol and β-sitosterol 41, 2,6-dihydroxy-5-methoxy-(3-C-glucopyranosyl) benzoic acid, β-sitosterol, *p*-hydroxybenzoic acid, caffeic acid and oleanolic acid have been reported in the plant. The leaf essential oil demonstrated higher antibacterial potential against Gram positive *S. subtilis* and *S. aureus*, than Gram negative *E. coli*, *P. aeruginosa* and *P. vulgaris*. The leaf oil exhibited antifungal activities against *F. solani*, *A. alternata*, *A. flavus* and *A. fumigates* as well (Kumar et al. 2013).

***Nardostachys grandiflora* DC.:** The plant is widely applied in folk medicine system in Nepal. Some of its medicinal applications are, the use of rhizome and its oil is for headaches and epilepsy, respectively in far western Nepal (Kunwar et al. 2009); the consumption of juice from whole plant during high altitude sickness and headache in central region of Nepal (Uprety et al. 2010); utilizing the root powder for treating food poisoning, cough, cold, fever, intestinal worms, stomach disorder, headache due to high altitude sickness in north western Nepal; taking the root decoction early morning is supposed to be a tonic; the plant is also applied as an incense and few others (Rokaya et al. 2010). The dried rhizome from sample collected from Jaljale, Nepal was used to extract the essential oil which constituted of calarene (9.4%), valerena-4,7(11)-diene (7.1%), nardol A (6.0%), 1(10)-aristolen-9β-ol (11.6%), jatamansone (7.9%), valeranal (5.6%), and cis-valerinic acid (5.7%). The oil exhibited antimicrobial potential against *B. cereus*, *E. coli* and *C. albicans* (MIC = 156 μg/mL), but displayed cytotoxicity against MCF-7 cells (Satyal et al. 2015c).

17.3 Anticancer Bioactives from High Altitude

Cancer is one of the leading causes of deaths worldwide. One in eight people die because of cancer (WHO 2005; Mathers and Loncar 2006; Lopez et al. 2006). And researchers claim that by 2030, the number of people dying from cancer will escalate from 7.1 million to 11.5 million (Mathers and Loncar 2006). Most preferred treatment of cancer is chemotherapy but it has its own intrinsic problems. It results in a variety of toxicities e.g. chemotherapeutic agent 5-fluorouracil cause myelotoxicity, cardiotoxicity and in rare cases may also act as a vasospastic agent (Rastogi et al. 1993). Therefore, most of the times use of chemotherapeutic drugs lead to serious problems for the patient. Various microbial/ plant-derived products based alternate therapies have also been proposed. Among these, utilising microbes for cancer treatment appears to be the most basic one. Even though it is not used commonly anymore, but it may assist our fight against cancer. Some of the anticancer agents obtained from Himalayan microbes are mentioned in Table 17.1.

Table 17.1 Anticancer agents from microorganisms that are found in Himalayan region

Anticancer agents	Organism	Type	Mode of action	Target	References
Staphylococcal superantigens-like (SSL)	*Staphylococcus aureus*	Proteins	Binds overexpressed eukaryotic receptors in cancer cells	Lymphoma and cervical carcinoma cells.	Walenkamp et al. (2009); Mokta et al. (2015)
Amino acid-degrading enzyme arginine deiminase (Ma-ADI)	*Pseudomonas* sp.	Enzyme	Deplete arginine	Hepatocellular carcinoma, melanoma, leukemia, renal cell carcinoma and prostate cancer, fibrosarcoma, breast cancer and leukemia cells.	Lind (2004)
Exotoxin A (PE) fused immunotoxins	*Pseudomonas* sp.	Protein	Binds overexpressed cell-surface receptors, arrest protein synthesis and induce apoptosis	Leukemia and bladder cancer	Frankel et al. (2000); Michl and Gress (2004)
Manumycin A	*Streptomyces* sp.	Metabolite	Farnesyltransferase inhibitors	Human pancreatic tumor, thyroid carcinoma, leukemias, myeloma and hepatocellular carcinoma	Ito et al. (1996); Pan et al. (2001); ENVIS (2015)
Prodiginines	*Serratia marcescens*	Secondary metabolite	Induce or correct DNA damage; arrest cell cycle	Liver, spleen, blood, colon, gastric, lung, breast and chronic myeloid leukemia	Manderville (2001); ENVIS (2015)

(continued)

Table 17.1 (continued)

Anticancer agents	Organism	Type	Mode of action	Target	References
Antibiotics (Doxorubicin, Bleomycin)	*Streptomyces peucetiusvar.*	Proteins/ peptides	Intercalation between the base pairs of the DNA strands and inhibition of the synthesis of DNA and RNA, generation of iron-mediated free radicals, causing oxidative damage to cellular membranes, proteins and DNA	Neuroblastoma, soft tissue and bone sarcomas, breast carcinoma, ovarian carcinoma, transitional cell bladder carcinoma, thyroid carcinoma, gastric carcinoma	Botlagunta et al. (2016); Vittorio et al. (2018)
Bacteriocins. (Laterosporulin 10, Nisin A, Bovicin HC5)	*Streptococcus* sp., *Lactococcus* sp., *Brevibacillus* sp.	Proteins/ peptides	Induction of apoptosis, stopping of cell cycle and reduction of HNSCC cell proliferation	Embryonic kidney cancer (HEK293T), fibrosarcoma (HT1080), lung carcinoma (H1299) breast cancer (MCF-7	Paiva et al. (2012); Baindara et al. (2017); ENVIS (2015)
Toxicins (Diphtheria toxin, Diphtheria toxin)	*Pseudomonas* sp.	Proteins/ peptides	Induces caspase-3 and -7 dependent apoptotic processes in the breast cancer cell line	Glioblastomas (U118MG, U373MG, U87MG), cutaneous T cell lymphomas (CTCL), breast carcinoma (MCF 7), cervical adenocarcinoma (HeLa)	Vallera et al. (2002); Lutz et al. (2014)

17.4 Other Himalayan Compounds Having Potent Anticancer Activity

17.4.1 Polyphenols

Polyphenols are secondary metabolites that play crucial roles in the growth and metabolism of organisms. Apart from these, polyphenols have also been shown to possess various biological activities that make them a target of a number of research studies to investigate its health benefits; like protection against neurodegenerative disease, diabetes, cardiovascular disease and even aging (Scalbert et al. 2005). They are also shown to possess anticancer properties (Ramos 2008).

Polyphenols are divided into phenolic acids, stilbenes, lignans and flavonoids; depending on the number of phenol rings and other structural elements (Table 17.2).

Polyphenols affect carcinogenesis by modifying various biochemical pathways and processes involved in its progression, by strengthening immune system, and also by safeguarding cells against oxidative shock. The anti-proliferative effect of polyphenols is dose and time-dependent. Polyphenols exert their anti-carcinogenic effect by preventing oxidation, detoxifying xenobiotic compounds, inducing apoptosis, affecting immune system, affecting nuclear factors. These processes in turn modulate cell signaling cascades, DNA transcription, gene expression, cell proliferation and survival (Shen et al. 2007; Chen et al. 2000).

17.4.2 Flavonoids

Flavonoids are secondary metabolites produced by plants. They are safe to be consumed by human beings and do not cause any toxicity to living organisms. Flavonoids have shown to have remarkable anticancer properties. They are classified

Table 17.2 Classification of polyphenols

Class	Source	Target	References
Lignans	Algae, and certain vegetables	Carcinogenic tumors, in particular hormone-sensitive ones such as breast, endometrium and prostate tumors	Buck et al. (2010)
Phenolic acids	Blueberries, kiwis, plums, apples, and cherries	Murine leukemia cell line (l1210), human promylolytic cell line (hl-60), human breast cancer cell line (mcf-7), parenteral human acute lymphoblastic cells (ccrf-cem)	Nandi et al. (2007)
Flavonoids	Milk thistle, acai palm, grape juice, kale, cherries	Lung cancer, leukemia, thyroid, stomach, laryngeal, colon	Batra and Sharma (2013)
Stilbenes	Mulberries, peanuts, grapes, red wine	Breast, lung, colon, skin (nonmelanoma skin cancer and melanoma), prostate, ovarian, liver, oral cavities, thyroid, and leukemia	Sirerol et al. (2016)

into various sub classes depending on their structure and functions. Subclasses of flavonoids along with their specific compounds, their sources and their target cancer types are listed (Table 17.3). Fruits and vegetables have enough flavonoid content to fight cancers in human beings. Some flavonoids are able to fight against breast cancer (Jahanafrooz et al. 2017) e.g. compounds under the subclass flavones have the ability to regulate macrophage function in cancer cell elimination and act as a potential inhibitor of cell proliferation resulting in anti-proliferative activity.

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Many *in vitro* and *in vivo* studies and some intervention trials have confirmed that flavonoids have good activity against various cancer cell lines (Rossi et al. 2010; Batra and Sharma 2013). They cause apoptosis after blocking the cell cycle in cancer cells (Chahar et al. 2011). They have been utilized for the remedy of various cancers

Table 17.3 Classification and anti cancer properties of Flavanoids

S. No.	Subclasses	Compounds	Sources	Target	References
1.	Flavanols	Catechin, Gallocatechin, Epicatechin, Epigallocatechin	Strawberries, apple, chocolate, cocoa, beans, cherry, green, and black tea	Human oral, rectal, and prostate cancer	Rossi et al. (2010); Batra and Sharma (2013)
2.	Anthocyanidins	Cyanidin, Malvidin, Petunidin	Blueberries, blackberries, blackcurrant and aubergine	Colorectal cancer	Lagiou et al. (2008)
3.	Flavones	Apigenin, Chrysin, Luteolin	Siberian larch tree, onion, milk thistle, acai palm, lemon juice, pepper, broccoli, capsicum, parsley, and celery	Breast cancer, lung cancer, leukemia, thyroid, stomach, laryngeal, colon, and oral cancer	Batra and Sharma (2013)
4.	Isoflavonoids	Daidzein, Genistein, Glycitein, Equol	Soybeans, soy flour, soy milk, beer, and tempeh	Prostate cancer, breast cancer, and thyroid cancer	Batra and Sharma (2013)

including ovarian, cervical, prostrate, breast and pancreatic cancers. Cancer cells have been shown to cease or at least downgrade variety of cancer pathological factors such as cyclin dependent kinases (CDKs) (Bueno et al. 2012), protein kinases (Kupcewicz et al. 2013), epidermal growth factor receptors (EGFRs), COX (cyclo-oxygenase), LOX (lipoxygenase), (Kim et al. 2017) etc.

Flavonoids affect cancer cells only and not normal cell; therefore can prove to be useful in finding newer and better drugs for treatment of cancer (Chahar et al. 2011).

17.4.2.1 Flavonoid Dual Modes Against Cancer

17.4.2.1.1 Anti-proliferation

In cancer cells, the mechanism of proliferation, among other things, involves the retardation of pro-oxidant processes which results in tumor advancement. The reactive oxygen species (ROS) and growth promoting oxidants like xanthine oxidase (Chang et al. 1993), COX or LOX55 are the major catalysts that assist cancer cells to proliferate. Flavonoids are known to inhibit them and hence retard tumor cell proliferation (Kim et al. 2017). Also, the inhibition process of polyamine biosynthesis mechanism may assist the anticancer activities of flavonoids. An enzyme in the biosynthesis of polyamines (Ornithine decarboxylase) is associated with the rate of DNA synthesis in several tissues. It has been shown that flavonoids inhibit ornithine decarboxylase resulting in corresponding decrease in polyamine and inhibition of DNA and protein synthesis (Ünlü et al. 2017; Kumar et al. 2017; Radan et al. 2017).

17.4.2.1.2 Induction of Apoptosis

Apoptosis is a tightly regulated form of cell death. It removes damaged and unwanted cells and thus has a crucial role in their development and survival. It is under the control of a number of genes and a network of interacting protease and their inhibitors. Dysregulation of apoptosis plays a critical role in oncogenesis. Flavonoids induce apoptosis only in certain cancer cell lines and not in normal cells. Even though exact mechanism is not known, certain processes like inhibition of DNA topoisomerase I/II activity, (Okoye et al. 2016; Kaleem et al. 2016) decrease of ROS, (Aslan et al. 2016) regulation of heat shock protein expression, (Toton et al. 2016) modulation of signaling pathways (Mansour et al. 2016) down-regulation of nuclear transcription factor kappa B (NF-κB), activation of endonuclease and suppression of Mcl-1 protein (Panat et al. 2016; Venkatesan et al. 2016) are involved.

17.4.2.1.3 Alkaloids

Alkaloids refer to the class of compounds that consists of a ring structure along with a nitrogen atom. Most of them possess cytotoxic activity and some of them have been commercially developed as chemotherapeutic drugs like camptothecin (CPT) and vinblastine, which interacts with tubulin (Morin 2003). As the structure and the activities of alkaloids are quite varied and complex, it is a challenge to conventionally classify alkaloids. Therefore, a classification of alkaloids is attempted here depending on the strategies (mode) employed by them to terminate cancer progression (Table 17.4).

Table 17.4 Mode and mechanism of anticancer properties in alkaloids

Mode	Alkaloid	Mechanism	References
DNA damage	Hirsutine	PI3k/Akt signal transduction cascade	Yang et al. (2014)
Apoptosis	Subditine and scutebarbatine A	Cleavage of caspases-3,8 and -9 as well as the down-regulation of Bcl-2 protein expression	Liew et al. (2014); Yang et al. (2014)
Cell-cycle arrest	Noscapine	Induces G2/M arrest in breast cancer lung cancer, and colorectal cancer	DeBono et al. (2015)
Alteration of the MAPK pathway	Rohitukine, beta caboline and hirsutine	Activate p38 MAPKs leading to a dose-dependent cytotoxicity against breast cancer, ovarian, and lung cancer	Safia et al. (2015); Lou et al. (2015); Fan et al. (2015)
Suppression of the NF-κB pathway	α-tomatine, taxol, Hirsutine	Inhibit NF-κB pathway activation thereby abolishing cancer progression	Lee et al. (2011); Lou et al. (2015); Kampan et al. (2015)
Formation of G-Quadruplexes	β-carboline	Regulate genes on oncogenes, act as antitumor agents against human promyelocytic leukemia, prostate cancer, and gastric cancer	Okamoto and Okamoto (2010); Neidle (2016)
HER2 targeting	Hirsutine	Impede growth signals leading to cell death in cancer cells	Lou et al. (2015)
Inhibition of the p-Glycoprotein ABCB1	Pretazettine	Inhibit p-glycoprotein (P-gp) (ABCB1-member of the ABC proteins) in breast cancer, cervical cancer, and skin epidermoid carcinoma	Zupkó et al. (2009)

17.4.2.1.4 Terpenoids

Terpenoids are the largest class of natural products comprising of 25,000 compounds that are mainly used in flavour, pharmaceutical and chemical industries (Gershenzon et al. 2007). Depending on their structures, they can be divided into multiple subclasses i.e. monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids and tetraterpenoids. Some derivatives with prominent anti-cancer activity are discussed below (Table 17.5).

17.5 Microbial Advances in Production of Bioactives from High-Altitude

In the previous sections, we have discussed the antimicrobial and anticancer potential of the several classes of bioactives including polyphenols, alkaloids, flavonoids and terpenoids. Among these terpenoids are the largest sources of bioactives having vast array of application. To date, plants are the major source of terpenoid and due to this, their exploitation has been increased since ever. To overcome such challenges,

Table 17.5 Different subclasses of terpenoids with mode of action against cancer cells

Subclass	Compounds	Target	Mode of action	References
Monoterpenoids	D-limonene	Pancreas, stomach, colon, skin, and liver cancers in animal models and human gastric cancer	Inhibit HMGCoA reductase resulting in inhibition of protein isoprenylation of small G proteins, including p21, and its membrane localization	Clegg et al. (1982)
	Cantharidin	Broad spectrum of cancer cells, including leukemia, colorectal carcinoma	Target serine/threonine protein phosphatase 1 (PP1) and 2A (PP2A)	Chen et al. (2002); Huan et al. (2006); Huh et al. (2004)
Sesquiterpenoids	Artemisinin and its derivetives	Leukemia, breast cancer, ovarian cancer, prostate cancer, colon cancer, hepatoma, gastric cancer, melanoma, and lung cancer	Mediate G1 cell cycle arrest by affecting cyclin D, cyclin E, CDK2, CDK4, p21, etc., induce apoptosis in various cancer cell types via activation of p38 MAPK, enhancement of Fas expression and activation of caspases	Hou et al. (2006)
Diterpenoids	Tanshinone IIA	Leukemia, breast cancer, colon cancer, and hepatocellular Carcinoma	Binds to DNA minor groove resulting in DNA structure damage	Sung et al. (1999); Liu (2006); Wang et al. (2005); Su et al. (2008); Wu et al. (1991); Tang et al. (2003); Yuan et al. (2004)
	Triptolide	60 US National Cancer Institute cancer cell lines	Affect transcriptional machinery of cancer cells	Liu (2011)
	Pseudolaric acid B	lung, colon, breast, brain, and renal cell lines	Destabilize microtubules	Pan et al. (1990)
	Andrographolide	Colon cancer	NF-kB signaling blockage, inhibition of JAK-STAT and PI3K, suppression of HSP90, cyclins, and cyclin-dependent kinases, metalloproteinases and growth factors, and induction of tumor suppressor proteins p53 and p21	Lim et al. (2011)

(continued)

Table 17.5 (continued)

Subclass	Compounds	Target	Mode of action	References
	Oridonin	Liver cancer, skin carcinoma, osteoma, and colorectal cancers	Inhibit DNA binding activity of NF-kB thereby blocking the NF-kB signal pathways	Ikezoe et al. (2005)
Triterpenoids	Celastrol	Breast cancer	Directly inhibits the IKKA, b kinases and proteasome function	Pang et al. (2010); Yang et al. (2006); Sethi et al. (2007); Salminen et al. (2010)
	Cucurbitacins	Lung, breast, pancreatic cancer lines	Induce cell cycle arrest,mainly G2/M, S phase arrest	Chen et al. (2010a); Chen, et al. (2010b); Lui et al. (2009); Rivat et al. (2005); Tang et al. (2010)
	Alisol	Human epithelial colorectal adenocarcinoma cells, (Caco-2)	Induces endoplasmic reticulum stress, autophagy, and apoptosis in several cancer cell lines by targeting sarcoplasmic/endoplasmic reticulum Ca2 + ATPase	Chou et al. (2003); Huang et al. (2006)
	Pachymic Acid	Lung cancer A549 cells, prostate cancer DU145 cells and colon carcinoma HT29 cells	Activates PARP, caspases-9, and caspases-3. It also shows inhibitory activities on both DNA topoisomerase I and II	Gapter et al. (2005)
Tetraterpenoids	Lycopene	Lung cancer	Modulates the expression of a broad range of proteins, including cell cycle proteins and heat shock proteins	Vaishampayan et al. (2007)

microbial production of terpenoid has been investigated in past two decades (Phulara et al. 2016). In the later sections, we will discuss about the biosynthesis of terpenoids, challenges in their production from natural sources and advances in the microbial production of terpenoids.

17.5.1 Terpenoids: Current Status and Future Opportunities

Terpenes or terpenoids, or isoprenoids form the largest class of secondary metabolites existing in almost all living organisms. It is the highly diverse group of natural products available on the earth and comprised of over 50,000 known compounds (Phulara et al. 2016). Terpenoids are used as a source of fragrances, flavours, and medicines in several traditional systems due to their tremendous structural and functional diversity (Breitmaier 2006; George et al. 2015a). To date, plants are the major resources of isoprenoids. Several plant varieties including the high altitude plants such as, conifers, balm trees, citrus species, eucalyptus, lemon grass, peppermint species, thyme, or plant parts of those have characteristic smell, taste and pharmacological activities (Breitmaier 2006). This is due to the presence of terpenoids. In addition to their role as secondary metabolites, terpenoids also play vital role in plants such as, quinones ubiquinone and plastoquinone as electron transport components; carotenoids and side chains of chlorophyll as pigments; gibberellins, and abscisic acid as hormones; cholesterol and ergosterol as sterols; and limonene, pinene and menthol, as other characteristic plant compounds (Ajikumar et al. 2008).

Due to their diverse nature, this tremendous class of biomolecules possesses several other biological activities, such as regulation of cation channels (Roullet et al. 1997), suppression of tumor proliferation (Yu et al. 1995; Burke et al. 1997; He et al. 1997), clampdown free radical generation (Ludwiczuk et al. 2017), protective effects against several cancers (Huang et al. 2012; Ludwiczuk et al. 2017) etc. Using animal model *C. elegans* we have also found that isoprenoids such as iridoids and isopentenol can be helpful to improve longevity, health-span and stress tolerance (Shukla et al. 2012; Pandey et al. 2019). In addition, to their remarkable activities against life-threatening disorders, isoprenoids have also been foreseen as potential alternate to diesel and gasoline fuel (Phulara et al. 2016). This is due to their poor hygroscopic nature, higher energy density and good fluidity at low temperatures (Gupta and Phulara 2015).

17.5.1.1 Biosynthesis

The wide range of terpenoids, which includes branched-chain/cyclic alkanes, alkenes and alcohols, are produced from two common five-carbon common precursor, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Gupta and Phulara 2015) (Figs. 17.1 and 17.2). The first discovered pathway for the biosynthesis of isoprenoids was the mevalonate (MVA) pathway (Katsuki and Bloch 1967; Lynen 1967) (Fig. 17.1). It is present in almost all eukaryotes and in few prokaryotes. The MVA pathway synthesizes isoprenoids from acetyl-CoA in six

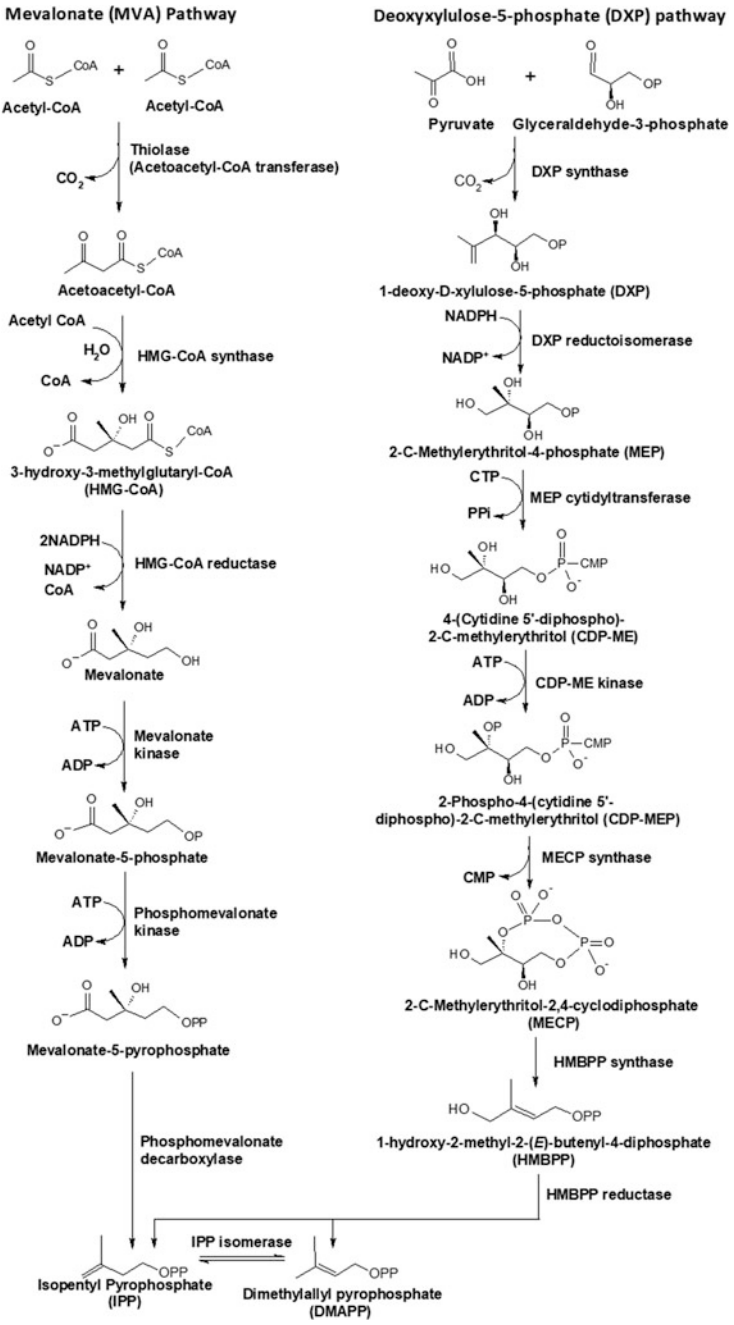

Fig. 17.1 Terpenoid biosynthesis pathway (upper/upstream module)

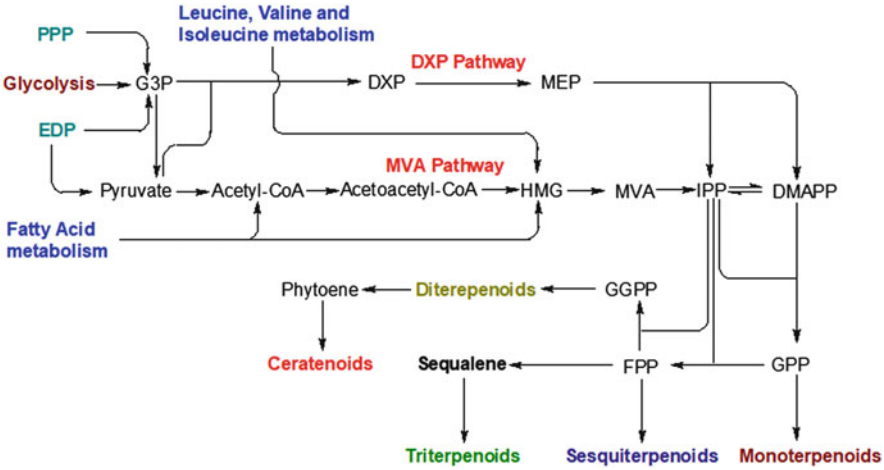

Fig. 17.2 Biosyntheic routes for isoprenoids production. *PPP* pentose phosphate pathway, *EDP* Entner–Doudoroff pathway, *G3P* glyceraldehyde-3-phosphate, *DXP* deoxyxylulose-5-phosphate, *MEP* 2*C*-methyl-D-erythritol-4-phosphate, *HMG* 3-hydroxy-3-methylglutaryl-CoA, *MVA* mevalonate, *IPP* isopentenyl pyrophosphate, *DMAPP* dimethylallyl pyrophosphate, *GPP* geranyl pyrophosphate, *FPP* farnesyl pyrophosphate, *GGPP* geranylgeranyl pyrophosphate. The PPP and EDP along with glycolysis supply precursors (G3P and pyruvate) to DXP pathway for the biosynthesis of isoprenoids. Whereas, fatty acid metabolism and amino acid metabolism provide precursors (acetyl-CoA and HMG) to MVA pathway

enzymatic steps (Miziorko 2011). The first three steps of the MVA pathway are committed to transform three molecules of acetyl-CoA into MVA. First, two molecules of acetyl-CoA are condensed to acetoacetyl-CoA via a raction catalyzed by the acetoacetyl-CoA thiolase (atoB). The HMG-CoA synthase (HMGS) then convert acetoacetyl-CoA into hydroxymethylglutaryl-CoA (HMG-CoA) (Ferguson and Rudney 1959), which is further converted to MVA by HMG-CoA reductase (HMGR) (Durr and Rudney 1960). The later three steps transform MVA to IPP through successive phosphorylation and decarboxylation steps. First, the mevalonate kinase (MK) phosphorylates MVA into mevalonate-5-phosphate (MVAP) by utilizing ATP (Tchen 1958). The MVAP is then again undergone phosphorylation by phosphomevalonate kinase (PMK) to from mevalonate-5-diphosphate (MVAPP) (Helling and Popjak 1961). In the last step, MVAPP is decarboxylated to IPP by phosphomevalonate decarboxylase (PMD) in an ATP dependent manner (Bloch et al. 1959). The conversion of IPP to its isomer DMAPP is catalyzed by IPP isomerase (IDI) through a stereospecific isomerization reaction (Wilding et al. 2000) (Fig. 17.1).

For several decades, MVA pathway was believed to be responsible for the biosynthesis of terpenoids. However, in past two decades, existence of an MVA independent pathway has been elucidated in several eubacteria and plant organelles such as, chloroplast etc. due to extensive genomics studies (Rohmer et al. 1993; Eisenreich et al. 1996). This pathway was recognized as 1-deoxy-D-xylulose-5-

phosphate (DXP) pathway that recruits seven enzymatic steps to convert glyceraldehyde-3-phosphate (G3P) and pyruvate into IPP and DMAPP (Rohdich et al. 2002b; Hunter 2007) (Fig. 17.1). The DXP pathway starts with the condensation of G3P & pyruvate into DXP that is catalyzed by DXP synthase (DXS) enzyme (Lange et al. 1998), followed by reduction to 2*C*-methyl-D-erythritol-4-phosphate (MEP) by DXP reductoisomerase (DXR or IspC) (Lange and Croteau 1999). MEP is then cytidilyzed to 4-diphosphocytidyl-2*C*-methyl-D-erythritol (CDP-ME) by MEP-cytidyltransferase (IspD) (Rohdich et al. 1999), which is further phosphorylated to 4-diphosphocytidyl-2*C*-methyl-D-erythritol-2-phosphate (CDP-MEP) by CDP-ME kinase (IspE) (Lüttgen et al. 2000). Conversion of CDP-MEPto 2-*C*-methyl-D-erythritol-2,4-cyclopyrophosphate (MEcP) is catalyzed MEcP synthase (IspF) (Herz et al. 2000). Finally, MEcP is converted to IPP and DMAPP by two successive reduction and elimination steps catalyzed by 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase (HMBPP synthase or IspG) and HMBPP reductase (IspH), respectively (Rohdich et al. 2002a) (Fig. 17.1).

Several downstream enzymes such as, geranyl pyrophosphate synthase (GPPS, C_{10}), fernesyl pyrophosphate synthase (FPPS or IspA, C_{15}), geranyl-geranyl pyrophosphate synthase (GGPPS, C_{20}), etc. utilize IPP and DMAPP to from the precursors for the synthesis of the largest class of secondary metabolites i.e. terpenoids (bisabolene, pinene, limonene etc.) by enzymes such as bisabolene synthase (BS), pinene synthase (PS), limonene synthase (LS) etc. Based on carbon atoms present, terpenoids can be further divided into following sub-categories like, hemiterpens (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}) and carotenoids (C40) (Fig. 17.2).

17.5.1.2 Concerns in the Production of Commercially Important Terpenoids from High-Altitude

Conifers, the most abundant higher plants of high-altitudes, are the rich source of oleoresin (a mixture of different classes of terpenoids and phenolics). Monoterpenes and diterpenes compose about almost all of the resin produced by conifer species, while sesquiterpenes occur in small amounts (Michelozzi 1999). Pinene and paclitaxel (Taxol) are amongst the commercially important isoprenoid present in high amounts in various Himalayan *Pinus* sp. and *Taxux* sp. plants respectively. Pinene, a major component of resin, is produced by conifers and is a potential antimicrobial agent (da Silva et al. 2012). On the other hand Taxol is extensively utilized as a chemotherapeutic agent for the treatment of various cancers (Ajikumar et al. 2010).

International Union for conservation of Nature (IUCN) has reported a 90% decline in *Taxus wallichiana* (Himalayan yew) population across most of its range through the Indo-Nepal Himalayan region (Thomas and Farjon 2011). Due to the heavy exploitation of Himalayan Yew for its leaves and bark (to produce paclitaxel or similar chemicals), the IUCN has classified it as endangered (Thomas and Farjon 2011). Though, the *Pinus roxburghii* or chir pine that is exploited for its resin, has been classified as least concern by IUCN (Farjon 2013); however, the resin extraction process sometime cause other serious issues such as forest fire, which is

damaging to high-altitude ecosystem and biodiversity. There are several other concerns, which limit the natural production of these commercially important terpenoid from high-altitude plants, such as (1) slow growth rates of plants, (2) low-level and tissue-specific synthesis of the terpenoids, (3) soil and land requirements for the propagation of such plants and (4) difficulties in harvesting of plants and extraction of such terpenoid (Tippmann et al. 2013; Phulara et al. 2016).

17.5.1.3 Alternative Strategies for the Production of Terpenoids

Because of the concerns related with plant based extraction of pinene and Taxol, researchers have explored other ways for the production of these chemicals, such as chemical route and/or microbial route. Though, there are some stereochemical complexities in the synthesis of Taxol by chemical route; however, the present process for the commercial production of Taxol relies on plant-based semi-synthetic routes (Chandran et al. 2011). Despite of success, the semi-synthetic route has some limitation of scale and cost as the process reliant on plants (Chandran et al. 2011). Use of hazardous solvents in chemical synthesis is another environmental and health concern that restrains the production of Taxol and pinene in large amounts from this route (Tippmann et al. 2013; Gupta and Phulara 2015). These barriers have led researchers' to investigate the alternative strategies for the production of Taxol and pinene through biotechnological approaches. To achieve this goal, several metabolically engineered microbial cell factories have been explored yet for the large-scale production of taxadiene, taxadiene-5α-ol (both are Taxol precursors) and pinene (Ajikumar et al. 2010; Sarria et al. 2014; Gupta and Phulara 2015) (Fig. 17.3).

17.5.1.3.1 Microbial Production of Terpenoids

It is well known that microbes have several advantages over plants such, (1) it is easy to culture and handle microbe in less space, (2) their growth rates are far higher than plants, (3) growth medium requirements are also low and (4) their genetic traceability and tractability (Keasling 2008). In addition, the success rates of genetic modifications in microbes are greater. The high-throughput synthetic biology tools and techniques make it more easier either to introduce an entire novel pathway or to silence the existing pathway in microbial hosts (Li and Pfeifer 2014). Recent years have seen nonnatural production of terpenoids from genetically modified microorganisms either by tuning endogenous pathways or by engineering heterologous pathways/genes in suitable hosts (Tippmann et al. 2013; George et al. 2015a; Wong et al. 2017). To bypass the endogenous regulations of native pathways, components of the non-native pathways have been incorporated in microbial host for increasing metabolite titres (Carlsen et al. 2013; Sarria et al. 2014). Most of the studies, which are concentrated on the heterologous production of higher terpenoids such as pinene and Taxol have been conducted on genetically tractable hosts like *E. coli* and *S. cerevisiae* (Engels et al. 2008; Ajikumar et al. 2010; Sarria et al. 2014).

Manipulation of a host's genetic code always comes up with several challenges, which includes competition between the host and foreign enzymes for substrate, feedback inhibition by product or intermediates, and accumulation of undesired or

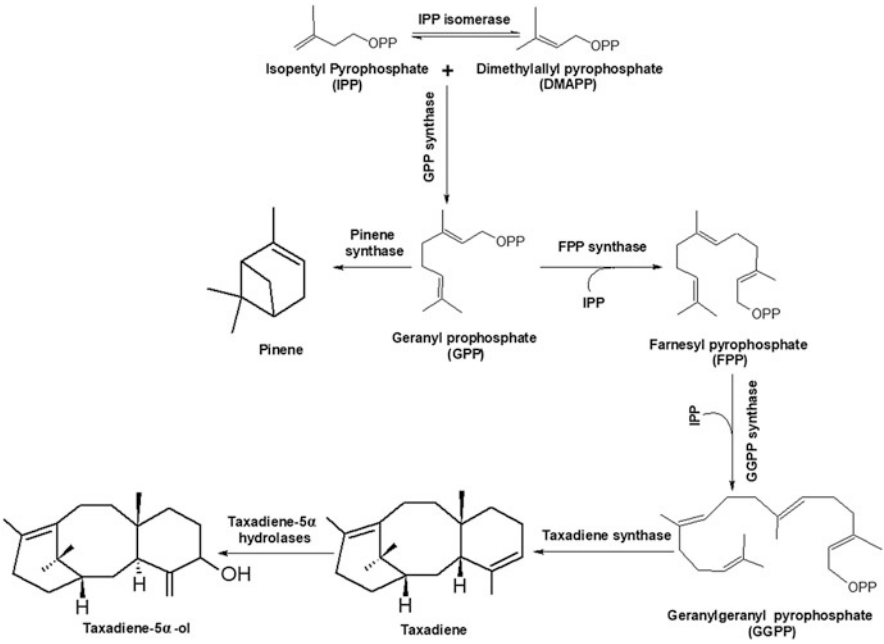

Fig. 17.3 Pinene, taxadiene and taxadiene-5α-ol biosynthesis

toxic metabolites (Gupta and Phulara 2015). However, microbial system provides more opportunity to overcome such bottlenecks over the plants system. Therefore, past decade has seen several advances in terms of computational, rational and combinatorial approaches to boost up the nonnatural production of higher terpenoids in microbial system (Tippmann et al. 2013; George et al. 2015a). To enhance the precursor or cofactor supply for terpenoid production, alternate pathways of a microbial host can also be tuned using such approaches (Zhao et al. 2013; Liu et al. 2014). It was reported that activating Entner-Doudoroff (ED) pathway and silencing Embden-Meyerhof-Parnas (EMP) pathway in *E. coli* could increase G3P and NADPH supply towards DXP pathway (Liu et al. 2014). Construction of fusion proteins by linking two successive enzymes of a pathway by a linker has been utilized to overcome the feedback regulation of isoprenoid pathway and to enhance terpenoid production in microbes (Wang et al. 2011; Sarria et al. 2014). Several other approaches, such as localization of intermediates into micro-compartments, harnessing efflux pump and protein tagging have been utilized to improve terpenoid production (Martin 2010; Chen and Silver 2012; Foo and Leong 2013; Niu et al. 2018).

17.5.1.3.2 Cyanobacteria as a Microbial Host for the Production of Terpenoid

Metabolic engineering together with microbial technology have provided the opportunity to grasp advantages of the less commonly utilized microorganisms for

producing economically valuable metabolites (Phelan et al. 2014; Davies et al. 2014). Cyanobacteria or blue-green algae are amongst such microbial hosts that have gained a considerable interest in past decade. This is because they can produce metabolites directly utilizing CO_2 (the least complex carbon source on the plant) and solar energy. These cyanobacteria are also an integral part of high-altitude ecosystems. Several studies have found them as a dominant microbial community in extreme environments of high-altitude, such as hot springs, bare rocks, cold-desert environments (Čapková et al. 2016; Singh et al. 2018). Due to their ability to withstand with extreme environments, cyanobacteria have established themselves as one of the most widely utilized alternate microbial hosts to develop microbial cell factories for the large-scale production of terpenoids (Nozzi et al. 2013; Halfmann et al. 2014a). In addition, other advantages such as fast growth, genetic traceability and tractability, high photosynthetic rates, easy genetic manipulations compared to plants and sequenced genome enable cyanobacteria to compete with heterotrophic microbes (capable of metabolizing lingo-cellulosic biomass) in the field of microbial metabolite production.

Despite their respective advantages, a microbial host also have some limitations, which need to be rectified prior their commercial utilization. To achieve maximum photosynthetic efficiency light exposure is a prerequisite in case of cyanobacteria. This limitation can be overcome by providing saturating amount of light (Iwaki et al. 2006) and by avoiding shelf shading via continuous monitoring of cell mixing rate and depth of the culture (Qiang et al. 1998). Another limitation associated with use of cyanobacteria is the generation of heat as large amount of sunlight is not utilized during photosynthesis. Use of hyperthermophilic cyanobacteria from hot springs on high-altitudes could effectively solve this problem. Alternatively, the non-photosynthetically active radiations could be converted into usable wavelengths (Wondraczek et al. 2013), that is a tedious and energy consuming task. Diurnal running condition is also a constraint that is faced while developing a photo-bioreactor. Metabolic engineering efforts have given a platform to overcome such limitation by incorporating sugar transporter system in cyanobacteria (McEwen et al. 2013). It allows cyanobacteria to grow in the absence of sunlight and one could obtain chemical production throughout a day i.e. for 24 h from cyanobacterial species (McEwen et al. 2013).

Apart from these technical issues, one major issue that could put an additional strain on fertilizer industry while using cyanobacteria, is their competition with plants for similar nutrients (nitrogen and phosphorous). These nutrients are essential for the survival of both and the world-wide fertilizer industry is facing a heavy demand due to the current agricultural practices. (Nozzi et al. 2013). Despite the discussed advantages and disadvantages, utilization of cyanobacteria for the production of high-altitude terpenoids (Halfmann et al. 2014b; Davies et al. 2014) is still in the primary phase of research and there is a long way to go.

17.5.2 Microbial Advances for the Nonnatural Production of Commercially Important High-Altitude Terpenoids: Pinene and Taxol

As discussed earlier, metabolic engineering efforts have enhanced the nonnatural production of a wide-range of terpenes from microbes by either endogenous or heterologous genes/pathway expression. Both the DXP and MVA pathway have been engineered in microbes to achieve this goal (Zheng et al. 2013; Liu et al. 2014). Despite the stoichiometrical superiority of DXP pathway over MVA pathway (Dugar and Stephanopoulos 2011), the terpenoid titers achieved to date by the optimizing DXP pathway (Liu et al. 2014; Kang et al. 2014) are far lesser than the titers obtained by the expression of heterologous MVA pathway (Sarria et al. 2014; George et al. 2015b). Among the terpenoid molecules that are abundant in high-altitude plants, pinene and Taxol have gained a considerable interest in recent years due to their commercial potential.

17.5.2.1 Advances in Microbial Production of Pinene

Pinene is a monoterpenoid compound that possesses antimicrobial activity (da Silva et al. 2012) and widely used as flavouring and fragrance agent (Sarria et al. 2014; Kang et al. 2014). Recently, it is projected as a potential jet-fuel (Yang et al. 2013; Kang et al. 2014) due to the similar physico-chemical properties of pinene dimers to that of missile fuel J-10 (Harvey et al. 2010; Meylemans et al. 2012). Bokinsky et al. (2011) have achieved the first microbial production of pinene from an engineered *E. coli*. To enhance precursors (IPP and DMAPP) supply, the heterologous MVA pathway was incorporated in *E. coli.* Since pinene is a monoterpene and *E. coli* is lacking GPPS enzyme responsible of the conversion of IPP and DMAPP into monoterpene precursor GPP, a GPPS enzyme from *Abies grandis* was also incorporated. Finally, to convert GPPS into pinene, a downstream pinene-specific enzyme pinene synthase (PS) from *Pinus taeda* was incorporated to convert GPP into pinene (Bokinsky et al. 2011). This study did not focus upon improvement of pinene production, as its primary aim was to engineer *E. coli* for the conversion of biomass-derived sugars into advanced biofuels. However, it unlocked the opportunities to take advantages of the metabolic engineering approaches for the enhanced production of pinene from microbial hosts. It also provided the opportunity to explore alternate microbial hosts capable of digesting biomass-derived sugars for the low-cost production pinene.

Culture condition modulations together with metabolic engineering have shown improved terpenoid production productions in microbes (Zhou et al. 2013; Phulara et al. 2018). Utilizing similar strategy, Yang et al. (2013), improved pinene production from engineered *E. coli*. For metabolic engineering of the host (*E. coli*), *A. grandis* GPPS and *P. taeda* PS genes were co-expressed with heterologous MVA pathway. To optimize culture conditions temperature, IPTG and nitrogen source were taken as parameters in a shake flask experiment. It was found that the engineered strain produced highest titre in a shake flask supplemented with 0.25 mM IPTG and MD beef extract (organic nitrogen source) at 30 °C (Yang et al. 2013),

Table 17.6 Summary of titer, engineered microbe, and production time of high-altitude terpenoid production from engineered microbes.

Isoprenoid	Engineered microbes	Titer	Time	References
Pinine	E. coli	1.7 mg/L	N.A.	Bokinsky et al. (2011)
		0.97 mg/L	32 h	Yang et al. (2013)
		32.4 mg/L	N.A.	Sarria et al. (2014)
		140 mg/L	24 h	Tashiro et al. (2016)
		166.5 mg/L	N.A.	Niu et al. (2018)
	C. glutamicum	~27 μg/g DCW	48 h	Kang et al. (2014)
	Synechocystis spp.	~80 μg/L	N.A.	Tashiro et al. (2016)
Taxadiene	E. coli	1.3 mg/L	N.A.	Huang et al. (2001)
		1 g/L	120 h	Ajikumar et al. (2010)
		26.77 mg/L	120 h	Boghigian et al. (2012)
	S. cerevisiae	1 mg/L	65 h	DeJong et al. (2006)
		8.7 mg/L	N.A.	Engels et al. (2008)
taxadiene-5α-ol	E. coli	58 mg/L	100 h	Ajikumar et al. (2010)
	S. cerevisiae	~25 μg/L	65 h	DeJong et al. (2006)

which was ~threefold higher than the previous report (Bokinsky et al. 2011) (Table 17.6).

To date, the pinene titers (Tashiro et al. 2016; Niu et al. 2018) from engineered microbes do not surpass the levels that have been achieved for hemiterpenes (George et al. 2015b) and sesquiterpenes (Peralta-Yahya et al. 2011; Wang et al. 2011). This might be due to the (1) toxicity of pinene, (2) competition for the GPP between PS and IspA, (3) inhibition of GPPS by GPP or high Mg^{2+}, (4) inhibition of PS by GPP and pinene or lower enzymatic activity due to the use of Mg^{2+} as a cofactor rather than Mn^{2+} and (5) low-level expression of PS in engineered hosts (Sarria et al. 2014; Niu et al. 2018).

Fusion protein product of two consecutive enzymes of terpenoid pathway has shown improved production of terpenoids from engineered microbes (Wang et al. 2011; George et al. 2015b). Utilizing similar strategy Sarria et al. (2014) expressed various fusion combinations of GPPS/PS to improve GPP availability for PS. It was found that expression of fusion product significantly increased the pinene titers over co-expression. Highest pinene production was achieved from the fusion product of *A. grandis* GPPS/PS (Sarria et al. 2014) that boosted pinene production by ~sixfolds than the previously reported by Yang et al. (2013). Later, Tashiro et al. (2016) screened different mutant variants of PS after a single round of mutagenesis and then utilizing a high-throughput screening approach to elevate the consumption of GPP. Expression of PS_{mut}, a mutant variant of PS (PS_{mut}), in engineered *E. coli* not only outperformed the wild-type PS (PS_{wt}) enzyme, but also significantly altered its metal dependency. A typically conifer monoterpene synthase requires Mn^{2+} as a cofactor (Savage et al. 1994) and the activity of wild type PS drops by 1/25 to 1/20 in the absence of Mn^{2+} (Tashiro et al. 2016). Interestingly, the screened mutant PS_{mut} demonstrated 60% activity in buffer deficient in Mn^{2+} ions (supplemented only with Mg^{2+}), which enables it to retain its activity in Mg^{2+}-rich and Mn^{2+}-deficient cytosol

of *E. coli* (Outten and Halloran 2001; Tashiro et al. 2016). Co-expression of PS_{mut} with heterologous MVA pathway enzymes, IDI, and GPPS yielded 140 mg/L pinene in a shake flask (Tashiro et al. 2016) that is fourfolds over the previous report of Sarria et al. (2014).

In a recent study, improved nonnatural production of pinene has been achieved by improving pinene tolerance and utilizing a modular coculture system of the whole-cell biocatalysis (Niu et al. 2018). Overexpressing efflux pump in *E. coli* improved its pinene tolerance to 2%. Further, utilizing the error-prone PCR and DNA shuffling approach, a more active variant of GPPS ($GPPS^{D90G/L175P}$) was obtained to improve GPP flux by competing with endogenous IspA enzyme for DMAPP. The previously evolved PS_{mut} ($Pt1^{Q457L}$) (Tashiro et al. 2016) was used to efficiently convert GPP into pinene. Incorporation of a tunable intergenic region (TIGR) between $GPPS^{D90G/L175P}$ and $Pt1^{Q457L}$ stabilized the expression of multiple genes. Using chemically induced chromosomal evolution, the TIGR-mediated gene cluster was incorporated along with the MVA pathway into the genome of the pinene tolerance *E. coli* strain. Finally, a 166 mg/L pinene titer was achieved via *E. coli-E. coli* modular coculture system of whole-cell biocatalysis (Niu et al. 2018).

As discussed earlier, less commonly used microbial host can also be employed for the production of valuable chemicals by utilizing metabolic engineering efforts. Recent years have also seen such advancements in the production of pinene from alternate microorganisms, such as *Corynebacterium glutamicum* (Kang et al. 2014) and cyanobacteria *Synechocystis* spp. (Tashiro et al. 2016). To enhance precursor flux towards pinene production in *C. glutamicum*, the host's DXP pathway was altered by overexpressing endogenous DXS and IDI enzymes along with *P. taeda* GPPS and *A. grandis* PS (Kang et al. 2014). On the other hand, to obtain pinene form *Synechocystis* spp., Tashiro et al. (2016) separately introduced PSs (PS_{wt} or PS_{mut}) into *Synechocystis* at a silent locus on its chromosome. The pinene production from alternate microbes is far less than that obtained from engineered *E. coli*; however, it provides an opportunity for the low-cost production of such chemicals from variety of carbon sources.

17.5.2.2 Advances in Microbial Production of Taxol

Taxol is a complex, diterpene-based, highly effective, less toxic, and broad-spectrum natural antineolastic drug that has been used against a wide range of cancers, such as breast, uterine, colon, ovarian and other cancers (Li et al. 2009; Zhou et al. 2010). The international market for Taxol is fast paced and rapidly growing (~12.3% average growth rate) with a global revenue of ~$80 that establishes Taxol at the forefront of worldwide best-selling anticancer drug (https://www.reportsweb.com/reports/global-paclitaxel-market-growth-2019-2024). The bark of Yew (*Taxus* spp.) is the major natural source of Taxol, where it exists in small quantities ranging from 0.01–0.05% (Zhou et al. 2010). The *Taxus* species are very slowly growing and the present extraction techniques for Taxol are less efficient. To extract 1 kg of taxol that is sufficient to treat just few hundred patients requires 10 tons of bark or 300 trees (Zhou et al. 2010). Thus, the current extraction process of Taxol from its natural source is environmentally and economically costly due to the heavy exploitation of

Yew (which is now endangered). In addition, like several other natural products, the structural complexity of taxol limits its synthetic production due to the requirement of multiple steps. This also complicates its economic production through chemical routes as there is a subsequent loss in yield at every step (Li et al. 2009). Cell cultures of yew however have come up with some hope and contributed notably to manage the Taxol supply. Yet, the lengthy culture duration, sensitivity to shear stress poor yield and high cost involved restrict its production and extraction via cell culture (Kusari et al. 2014). Therefore, in an immense need to seek new ways for obtaining Taxol to protect its natural reserves (for maintaining ecological balance and saving high-altitude biodiversity) and to reduce the cost of drug therapy, microbial production of Taxol has been explored in past two decade (Li et al. 2009; Ajikumar et al. 2010; Kusari et al. 2014).

As discussed earlier, microbes can be a potential alternate to obtain plant-based terpenoids due to their ease of culture, fastidious growth, easy genetic manipulations and less costly media requirement. The current research for Taxol production is based on either exploring Taxol producing entophytic fungi (Li et al. 2009; Kusari et al. 2014) or engineering microbial hosts like *E. coli* or *S. cerevisiae* (Engels et al. 2008; Ajikumar et al. 2010). It is well established that endophytic fungi have fast growth, can be isolated easily from the ex-plants and can be cultured with a comparative ease. The Taxol producing endophytic fungi (TPEF) have gained considerable interest in the past few years. These TPEF can be isolated from several tissues and organs of yew trees, namely, leaves, stems, roots, and fruits (Zhou et al. 2010). Majority of the researches that have been carried out to obtain Taxol from endophytic fungi includes screening of endophytic fungi with high primeval taxol yield, strain improvement by mutation and/or modern biotechnological methods, and advanced fermentation methods (Zhou et al. 2010).

Mutations are known to induce novel genetic characteristics in microorganisms and commonly used to screen superior microbial strains. Both chemical, such as ethyl methyl sulfomar (EMS), nitrosoguanidine (NTG), etc. and physical mutagens (ultraviolet, χ-ray, γ-rays, fast neutron, laser, microwave, etc.) can induce genetic variation in a host. In fungi, the production of bioactives can be enhanced by altering their mycelium via mutations. It has been reported that induction of mutations in TPEF from *Taxus cuspidate* improved Taxol production over 2.5 folds in mutant strain compared to wild-type (Zhou et al. 2001). In another study, it was found that among treatments UV, NTG, and UV + NTG, the treatment of UV + NTG to Taxol-producing endophytic fungi increased taxol yield ~1.4 folds over the wild type.

Advanced molecular biology techniques, such as gene manipulation, genetic recombination, protein engineering, and modulation of the inherent metabolic pathways have provided important breakthrough and direction to improve final titers of terpenoids from microbial sources. The biosynthesis of Taxol is a complex process and yet to determine fully. It consists of 19 enzymatic steps that include 8 cytochrome P450-mediated oxygenations (Croteau et al. 2006). Using recombinant technology Huang et al. (2001) laid the foundation for further development in the area of microbial Taxol production. Taxadiene, a key intermediate of Taxol biosynthesis has been produced enzymatically in engineered *E. coli* by

overexpressing IDI, GGPPS, and taxadiene synthase (TS) genes (Huang et al. 2001). Similarly, in *S. cerevisiae*, a eukaryotic host, eight taxoid biosynthetic genes were expressed to produce Taxol precursors and related taxoids (DeJong et al. 2006).

Presently, researchers primarily focusing on three main aspects of microbial Taxol production: (1) enhanced supply of GGPP; (2) overexpression of TS for the efficient conversion of GGPP into taxadiene (Engels et al. 2008); and engineering cytochrome P450-mediated oxygenations for the conversion of taxadiene to taxadien-5α-ol (Ajikumar et al. 2010). In an attempt to obtain Taxol precursor, taxadiene, from engineered *S. cerevisiae*, GGPP synthase from *Sulfolobus acidocaldarius* and a codon optimized taxadiene synthase from *Taxus chinensis* were overexpressed (Engels et al. 2008). To overcome sterol steroid-based negative feedback a truncated HMG-CoA reductase (tHmg1) was overexpressed. In addition, upc2-1, a mutant allele of the transcriptional sterol regulator was expressed to allow steroid uptake under aerobic conditions. The resultant strain was able to produce 8.7 mg/L taxadine after 48 h incubation (Engels et al. 2008) (Table 17.6). Ajikumar et al. (2010) raised up taxadiene levels in engineered *E. coli* utilizing an approach termed as 'multivariate-modular pathway engineering (MMPE)'. They have demonstrated that splitting DXP pathway into two modules and fine-tuning each module distinctly could improve the titers of desired metabolites. By utilizing this approach, they were able to identify a correct balance between upper and lower DXP pathway modules and obtained a combination that produced 1.0 g/L taxadiene (Ajikumar et al. 2010) (Table 17.6). In the next step, to convert taxadiene into Taxol, a chimeric protein was expressed in taxadiene producing strains that was obtained by fusion of the CYP450, taxadiene-5α-hydroxylase from *Taxus cuspidate* with its CYP450 reductase (CPR) counterpart. The resultant strain produced 58 mg/L taxadiene-5α-ol (Ajikumar et al. 2010), which was ~2400-fold higher than the previously reported titers in S. cerevisiae (DeJong et al. 2006) (Table 17.6). Later, a computational approach was also applied to enhance taxadiene production in *E. coli* (Boghigian et al. 2012). A variation of the minimization of metabolic adjustment (MoMA) algorithm was utilized to identify gene targets for the improved production of taxadiene. Though the study could not surpass the previously achieved taxadiene levels (Ajikumar et al. 2010); however, it was able to identify four genetic engineering targets outside of the native DXP pathway, which could be utilized further with MMPE to enhance cofactor supply for increasing taxadiene accumulation.

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