Bioactive metabolite profiling for identification of elite germplasms: a conservation strategy for threatened medicinal plants

Padma Venkatasubramanian[1,*], S. P. Balasubramani[1], S. K. Nandi[2] and Mohd Tariq[2]

[1]TransDisciplinary University, Foundation for Revitalisation of Local Health Traditions, 74/2, Jarakabhande Kaval, Attur Post, via Yelahanka, Bengaluru 560 106, India
[2] G.B. Pant National Institute of Himalayan Environment and Sustainable Development, Kosi-Katarmal, Almora 263 643, India

Medicinal plants are used as a source of raw drugs, chemical compounds or bioactive metabolites. Many of the medicinal plant species are facing threat of extinction due to indiscriminate harvesting by humans. Conservation of such species is no longer an altruistic choice but a necessity to ensure sustainable supply of bioactive compounds to the drug industry. This article demonstrates that conservation of threatened species is possible through large-scale cultivation of elite germplasm identified using biochemical markers. Six species, viz. *Aconitum balfourii* Stapf, *Aconitum heterophyllum* Wall. ex Royle, *Podophyllum hexandrum* Royle (syn = *Sinopodophyllum hexandrum* (Royle) T. S. Ying), *Picrorhiza kurroa* Royle ex Berth., *Berberis aristata* DC. and *Embelia ribes* Burm. f. were selected for the study under the all-India coordinated project on threatened species. The approach proved to be effective for bringing back the species from the verge of extinction.

Keywords: Bioactive metabolites, conservation strategy, medicinal plants, elite germplasm.

Introduction

CHEMICAL analysis of medicinal plant species is necessary to select elite germplasms for conservation action, mass propagation and release of elite materials to the farmers for commercial cultivation. At present, commercial production of secondary metabolites is achieved through cultivation, collection from the wild and tissue/cell culture of specific medicinal plants often supported by genetic engineering[1]. Information on quality and content of active constituents determines the market value of the harvested/collected raw drug. Development of sustainable harvest methods and use of substitutes are greatly supported by monitoring of active constituents. For the above purposes, qualifying and quantifying the reference and bioactive markers in medicinal plants are essential. The assessment of chemical constituents in threatened plants is crucial for identifying elite germplasm for large-scale commercial cultivation, which has been a successful strategy in conserving many threatened medicinal plants.

India is a reservoir of numerous high-valued medicinal plants and is recognized as one of the major medicinal plant-producing Asian countries. In India, 7637 plant-species are known for their medicinal value (http://envis.frlht.org/indian-medicinal-plants-database.php). Among these, temperate–alpine herbs such as *Aconitum heterophyllum* Wall. ex Royle, *Aconitum balfourii* Stapf, *Angelica glauca* Edgew., *Dactylorhiza hatagirea* (D. Don) Soo, *Podophyllum hexandrum* Royle (syn = *Sinopodophyllum hexandrum* (Royle) T. S. Ying), *Picrorhiza kurroa* Royle ex Berth. and *Berberis aristata* DC. are of considerable importance in terms of trade for their medicinal composition. Similarly, the tropical forests of India also host a rich diversity of endemic medicinal plant species which are widely traded and used in the traditional medicines and plant extracts industry. Species such as *Embelia ribes* Burm. f. and *Coscinium fenestratum* (Gaertn.) Colebr. are of conservation concern because they are traded in large volumes. A few are extracted for their active constituents and are subsequently used as precursors for the preparation/synthesis of several modern medicines. Certain plants that are endemic to the deserts of Gujarat and Rajasthan, such as guggul (*Commiphora wightii* (Arn.) Bhandari) have been overexploited[2]. Due to high value of the drugs derived from these plants and ever-increasing global market for the 'natural' bioactive compounds, these species are being subjected to reckless, often illegal harvesting, well beyond their natural regeneration capacity. This has pushed many species to the brink of extinction, and they have been listed in the *Red Data Book*.

Aconitine, berberine, embelin, podophyllotoxin and kutkin are bioactive molecules derived from *A. heterophyllum*, *B. aristata*, *E. ribes*, *P. hexandrum* and *P. kurroa* respectively. These plants are also well-known in Ayurvedic, Unani and other traditional systems of medicine. Several researchers have quantified the active ingredients in *A. heterophyllum*, *A. balfourii*, *P. kurroa*

*For correspondence. (e-mail: padma.venkat@tdu.edu.in)

and *P. hexandrum*[3–6]. The wide variation in quantity of bioactive compounds in different germplasm necessitates identification of elite germplasm[3–8]. The chemical and pharmacological similarities in substitutes for species such as *A. heterophyllum*, *Embelia ribes* and *B. aristata* have been recommended as strategies to augment conservation efforts[9,10].

Cultivation of medicinal plants with high-value bioactive compounds has been an important step in conservation action. In India, cultivation of certain species, including *Withania somnifera* (L.) Dunal (Ashwagandha) and *Asparagus racemosus* Willd. (Shatavari) has been taken up for meeting the needs of the drug industry, which in turn has contributed towards conservation of the species. However, a lot more efforts are needed for developing propagation and cultivation methods for several such threatened medicinal species.

Use of bioactive molecules is an important way to identify elite threatened species, monitor the quality and quantity of their yield and also identify other plant species or sources that have the same bioactive molecules and therefore, can be used as substitutes. The medicinal and aromatic properties of plants are usually attributed to the secondary metabolites produced by them. Plant secondary metabolites are broadly divided into the following five major groups: (i) Terpenoids: These are derived by repetitive fusion of branched five-carbon units based on isopentane skeleton[11]. About 30,000 terpenoids have been reported from plants[12]. Isoprene, eucalyptol, isoprenol, carotenoids, lycopene and rubber are some examples[13]. (ii) Essential oils: Natural aromatic and volatile oils are present in different parts of plants[14]. They are structurally very complex. Two major groups of essential oils are hydrocarbon terpenes and oxygenated and/or sulphured oils. (iii) Alkaloids: These are compounds containing basic nitrogen atoms with carbon and hydrogen[15]. They may also contain oxygen, sulphur and occasionally elements like chlorine, bromine and phosphorus[16]. They are mostly biosynthesized from amino acids like tyrosine[17]. About 12,000 alkaloids have been characterized till date[18]. Atropine, berberine, codeine, coniine, morphine, nicotine and solanine are some examples of plant alkaloids[15]. Several alkaloids have been reported to be toxic at certain concentrations. (iv) Phenolic compounds: Phenolics are characterized by the presence of hydroxylated aromatic rings[19]. More than 8000 phenolic compounds have been reported from the plant kingdom[20]. Phenolic compounds are classified into four groups[21], viz. phenolics with one or two aromatic rings, quinones and polymers. Phenolic compounds with more than one OH-group are called polyphenols. The predominant polyphenols in human diet are the flavonoids which contribute to the rich and vibrant shades of yellow and red in fruits and vegetables. Over 4000 flavonoids have been reported[22]. Catechin, apigenin, rutin, luteolin, quercetin, naringin, apigenidin and cyanidin are some of the plant flavonoids[23]. (v) Glycosides: These are phenol, alcohol or sulphur compounds characterized by the presence of a sugar moiety[24]. Saponins are a type of glycosides with distinct foaming characteristics. Their structure contains triterpene or steroid aglycone and one or more sugar chains.

This article aims to demonstrate that conservation of threatened species is possible through large-scale cultivation of elite germplasm identified through biochemical markers. In other words, the importance of chemical characterization of medicinal plants in guiding conservation research and actions has been demonstrated.

Materials and methods

A. heterophyllum and A. balfourii

Aconitum species (family Ranunculaceae) are distributed in the alpine and sub-alpine regions of the Indian Himalayan Region (IHR). About 300 species of *Aconitum* are found worldwide, of which 24 species are found IHR. The pharmaceutically significant compound present in the tuberous roots of *Aconitum* species is diterpenoid alkaloids[25]. These are classified into a strong toxic group (aconitine-type) and a weak toxic group (atisine-type). *A. heterophyllum* (Wall. ex Royle) and *A. balfourii* Stapf are important commercial species of this genus from the IHR region[26].

A. balfourii is a perennial herb with fleshy, spindle-shaped root containing pseudoaconitine, a highly toxic alkaloid as the principal component, and aconitine, benzylaconitine, picroaconitine and haemonepellene in traces. *A. heterophyllum* (*Ativisha*) is also a perennial herb distributed between 1800 and 4600 m amsl in the alpine and sub-alpine regions of the Himalaya; its root powder is used as a bitter tonic and febrifuge for controlling debility after fever and diarrhoea[27]. Among the *Aconitum* species, estimation of active ingredients in tubers in relation to altitude was carried out in *A. balfourii* and *A. heterophyllum*[4].

P. hexandrum

Podophyllum hexandrum Royle (family Podophyllaceae), a perennial herb found in the entire Himalayan region, is a prominent source podophyllotoxin which is used for the preparation of high value anti-tumour agents like etoposide, etopophos and teniposide[28–30]. The rhizomes of *P. hexandrum* contain three times more podophyllotoxin content in comparison to the other species, i.e. *P. peltatum*[31] and *P. sikkimensis*.

P. kurroa

Picrorhiza kurroa Royle ex Benth (family Scrophulariaceae), commonly known as 'Kutki', is a medicinal herb that grows in the elevation range of 2800–4800 m amsl.

B. aristata

Berberis aristata DC. (Berberidaceae) has been used in Ayurveda and traditional Chinese medicine for the past 3000 years[32,33]. In Ayurveda, it is called '*Daruharidra*' and is a key ingredient in formulations for eye care, wounds, skin diseases, jaundice, rheumatism and diabetes[32]. The genus *Berberis* is distributed throughout the Himalayan region, from Bhutan to Kunawar in India, and Sri Lanka[34]. *B. aristata* is a species of high trade sourced from temperate forests. The present annual production and supply of *Daruharidra* or *Daruhaldi* in India is predominantly from Himachal Pradesh[35]. *B. aristata* is an endangered species according to the IUCN list due to overexploitation and habitat degradation. Berberine, the bioactive compound present in *B. aristata* with diverse pharmacological effects, is also used as a nutraceutical[36–38].

E. ribes

Embelia ribes Burm F. (Myrsinaceae), popularly known as 'Vidanga' or 'Vaivding' in Ayurveda, is a Red-listed medicinal plant species[39]. *E. ribes* has great demand in Ayurveda and the pharmaceutical industry (>500 MT/year). Over exploitation has imposed tremendous pressure on natural populations in the Western Ghats and Eastern Himalaya of India. *E. ribes* is listed in the 'Priority Species List' for cultivation by the National Medicinal Plant Board. Embelin is the major bioactive phyto-constituent present in *E. ribes*, which has been shown to have anti-helmintic, anti-tumour, anti-fertility, anti-inflammatory and anti-microbial activities[40].

Analyses of secondary metabolites

The tubers/leaves, depending on the species, collected from geographically widespread populations were extracted, purified and analysed by HPLC and HPTLC fingerprinting for determining the active constituents in each species.

Results and discussion

A. heterophyllum and A. balfourii

The levels of aconitine estimated in tubers of *A. balfourii* ranged from 0.13% to 0.83% (% of dry wt basis), whereas in case of *A. heterophyllum*, they ranged from 0.13% to 0.75%. The data clearly indicate a wide variation amongst populations growing across altitudinal gradients in Garhwal and Kumaun Himalaya. The maximum (0.83%) and minimum (0.13%) levels of aconitine in *A. balfourii* were recorded at Phurkia Bugyal (3430 m) and Kedarnath (3600 m) respectively. However, in *A. heterophyllum*, maximum (0.75%) and minimum (0.13%) levels were recorded for Phurkia (3260 m) and Kafni (3400 m) respectively[4]. Based on these earlier works, elite populations were selected and mass propagation were undertaken using plants from Kafni in case of *A. heterophyllum* and Phurkia Bugyal in case of *A. balfourii* for the release of elite propagules to the farmers for cultivation. Multi-location trials are underway for assessing their consistent aconitine yield.

Podophyllum hexandrum

Presence of podophyllotoxin in the rhizomes of *P. hexandrum* from the IHR has been reported by several workers[3,6,41–43]; levels up to 9.53% have been reported[7]. Further, diversity analysis has been undertaken for characterizing better podophyllotoxin-rich populations[7,41]. A wide variation in podophyllotoxin content was observed in different altitudes/locations. The dried rhizome and roots of *P. hexandrum* are part of the Indian Pharmacopoeia and form the source of medicinal resin, i.e. podophyllotoxin[44].

The podophyllotoxin content in rhizome samples from populations in different locations in Garhwal and Kumaun Himalaya showed wide and significant variation ($P < 0.05$)[6] ranging from minimum (0.021%; Dayara, 3000 m amsl) to maximum (5.480%; Kedarnath, 3600 m amsl) levels. The podophyllotoxin content of root samples also showed significant variation ($P < 0.05$) and it ranged from a minimum of 0.090% (Phurkia; 3260 m amsl) to a maximum of 5.8% (Kedarnath; 3600 m amsl).

Under the present study in Kumaun region of Uttarakhand Himalaya (as a part of the DBT All-India Coordinated Project), estimation of podophyllotoxin in root and rhizome samples collected from different populations (2653–3640 m amsl) revealed that the content ranged from 0.238% to 0.489% in root samples and from 0.221% to 0.961% in rhizomes (Table 1). Maximum podophyllotoxin content was found in root (0.489%) and rhizome (0.961%) collected from Martoli Bugyal (3640 m amsl), and minimum in root (0.238%) and rhizome (0.221%) collected from Dunyadong (2653 m amsl).

Variation in the levels of podophyllotoxin among the populations has been attributed to edaphic factors such as soil physico-chemical characteristics, age of plant, genetic variability and different chemo-types in natural populations[3,44–46]. The observed chemo-diversity in different populations was useful for selection of elite populations for further mass propagation and conservation. Based on the results of the earlier studies as well as the present study (Table 1), six elite populations were identified, viz. Katheliya, ITBP Base Camp Milam, Martoli, Nanda Devi Base Milam, Martoli to Nanda Devi East Base, and Martoli Bugyal, which have been under cultivation by the farmers for multi-locational trials to

Table 1. Podophyllotoxin content in root and rhizome samples in different populations of *Podophyllum hexandrum* across Kumaun region, Uttarakhand

Populations	Altitude (m amsl)	Podophyllotoxin (% dry wt)	
		Rhizome	Root
Dunyadong	2653	0.221	0.238
Katiya	2850	0.365	0.345
Dwali	2935	0.254	0.284
Katheliya	3180	0.512	0.348
ITBP Base Camp Milam	3383	0.293	0.299
Martoli	3438	0.581	0.325
Nanda Devi Base Milam	3475	0.620	0.333
Martoli to Nanda Devi East Base	3640	0.805	0.449
Martoli Bugyal	3640	0.961	0.489

Table 2. List of plants in which berberine alkaloid is present

Family	Common name	Botanical name	Reference
Berberidacaeae	Tree Berberis; *Daruharidra* (name in Sanskrit); *Mara Manjal* (Tamil, Malayam)	*Berberis aristata*	47
		B. asiatica	48
		B. lyceum	49
		B. chitria	49
		B. concinna	50
		Mahonia leschenaultii	51
	Oregon grape	*Mahonia aquifolium*	52
	Berberis; European Barberry	*Berberis vulgaris*	53
Menispermaceae	Heart-leaved moonseed; *Guduci* (name in Sanskrit)	*Coscinium fenestratum*	54
		Tinospora cordifolia	55
		Coptis trifolia (syn. *Coptis groenlandica*)	56
Rutaceae	Amur cork tree	*Phellodendron* amurense	57
Ranunculaceae	Yellow root	*Xanorhiza simplicissima*	58
Papavaraceae		*Argemone mexicana*	59
		Eschscholzia californica	60

confirm the highest yield. Because of its high podophyllotoxin content and high market price, the species is popular among the farmers and is out of imminent threat of extinction.

P. kurroa

Very few reports exist on active ingredient levels (runner/rhizome) of *P. kurroa* collected from different locations in the IHR. Purohit *et al.*[47] investigated five high-altitude populations with morphological variations from the Garhwal Himalaya of Uttarakhand and reported that picrotin and picrotoxin content ranged from 1.00 to 6.05 mg/g. Subsequently, Sharma *et al*.[48] analysed seven populations of *P. kurroa* collected from the alpine region of Himachal Pradesh, and reported that major bioactive compounds, picroside I (3.5%) and picroside II (2.0%) were maximum in the population located at 3978 m amsl. In another study, picroside content was determined in rhizome of *P. kurroa* collected from Pithoragarh area of Uttarakhand, where picroside I and II levels were 1.26% and 0.48% respectively[8]. Based on these results, under the DBT Project elite populations were identified, micropropagated and released to the farmers for cultivation.

B. aristata

Studies from Foundation for Revitalisation of Local Health Traditions – TransDisciplinary University (FRLHT-TDU) laboratories (Figure 1) and other groups have shown that berberine is present in several plant species that are traded as *Daruharidra* (Table 2). For herbal formulations and nutraceuticals with berberine as the active principle, species other than the endangered *B. aristata* are also being used.

E. ribes

The study of nine different markets of India for *Vidanga* by FRLHT-TDU indicated that >90% of the traded samples were *Embelia tsjeriam-cottam* (Roem. & Schult.) A. DC, a species which is more commonly available than the

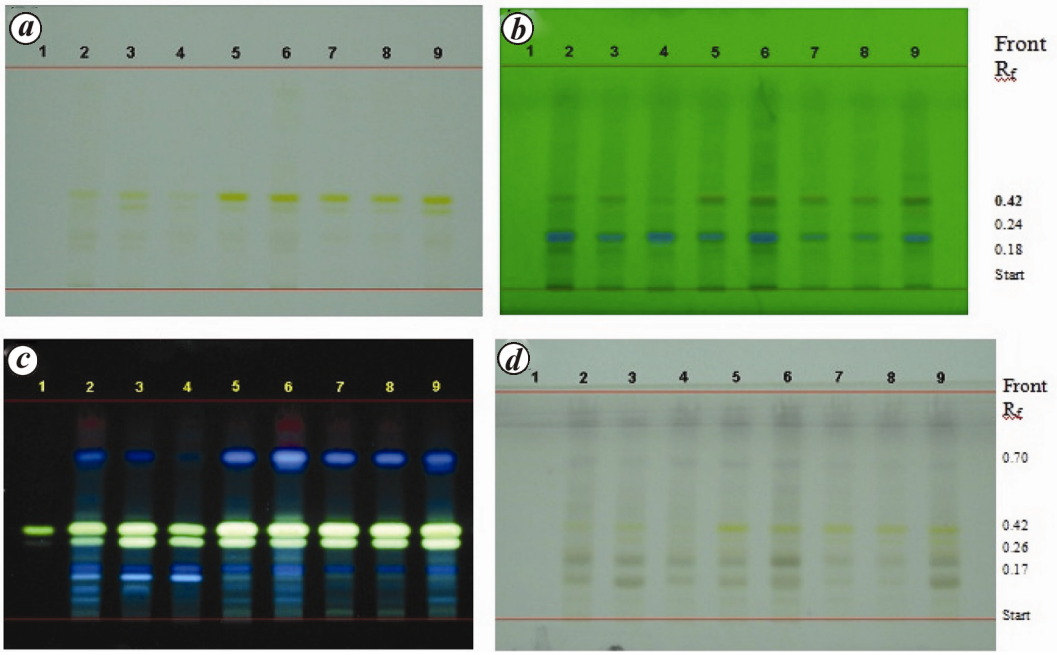

Figure 1. HPTLC fingerprint of *Berberis* spp. (methanol extract; mobile phase: *n*-butanol : glacial acetic acid : water at 7 : 1.5 : 2) ratio visualized under (***a***) white light, (***b***) UV 254 nm, (***c***) 366 nm, and (***d***) after derivatization with anisaldehyde-sulphuric acid reagent. Lane 1, Berberine standard (R_f 0.42); lanes 2–4, *Berberis aristata*; lanes 5 and 6, *Berberis asiatica*; lanes 7 and 9, *Berberis lycium* showing the presence of berberine.

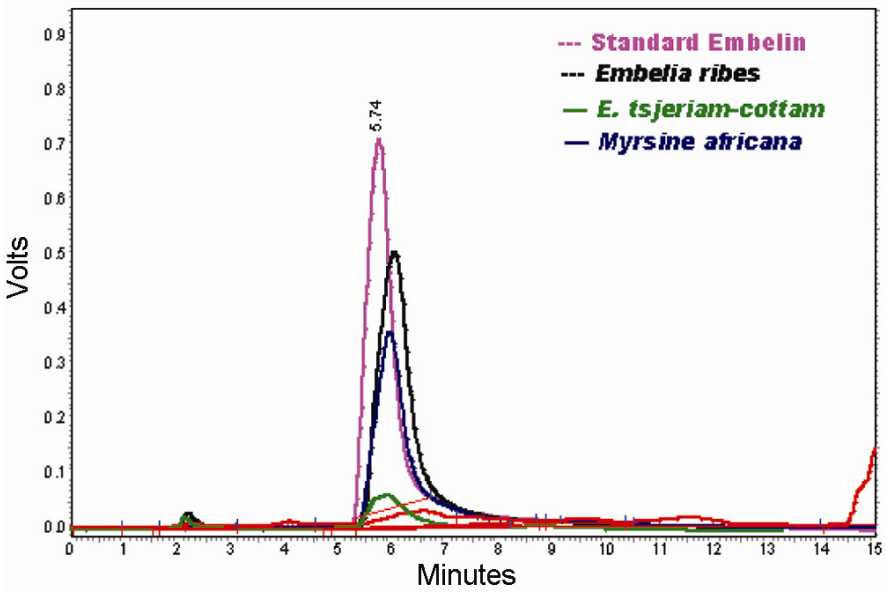

Figure 2. HPLC profile of *E. ribes*, *E. tsjeriam-cottam* and *Myrsine africana* with standard embelin. Overlay of HPLC profiles of the methanolic extracts of *E. ribes*, *E. tsjeriam-cottam* and *Maesa indica* with standard embelin. C18 column (Kromasil, 250 × 4.6 mm); methanol : water : acetic acid : tetrahydrofuran (85 : 15 : 3 : 0.1); 1.6 ml/min; 288 nm.

Red-listed *E. ribes* (http://envis.frlht.org/newsletters/mar2006.htm). *E. tsjeriam-cottam* is also mentioned as a substitute in the *Ayurvedic Formulary of India*[49,50]. *Myrsine africana* L. and *Maesa indica* (Roxb.) A.DC, both from the same family, i.e. Myrsinaceae are also used as *Vaividang* in Himachal Pradesh and North East India respectively[51]. Chromatographic studies indicated the presence of embelin in *E. ribes*, *E. tsjeriam-cottam* and *M. africana*, and not in *Maesa indica* (Figure 2 and Table 3). However, *M. indica* had a benzoquinone that was similar to embelin, which was isolated and named as kiritiquinone (Figure 3)[9].

Table 3. Embelin content in *Embelia ribes*, *Embelia tsjeriam-cottam* and *Myrsine africana* estimated by HPTLC

Botanical species	Embelin content (% w/w)
Embelia ribes	1.3–4.0
Embelia tsjeriam-cottam	1.0–4.8
Myrsine africana	2.1–4.4
Maesa indica	Not detected

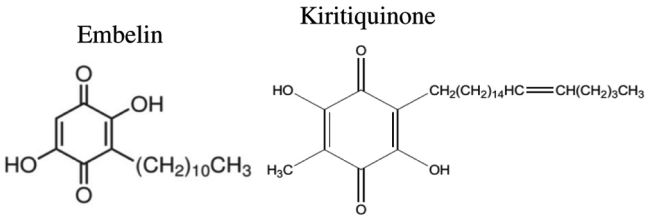

Figure 3. Chemical structures of embelin and kiritiquinone.

Conclusion

Conservation of threatened species through large-scale cultivation of elite germplasm identified using biochemical markers could be an effective method for bringing back the species from the verge of extinction. In the present study, this concept has been proved to be successful in the case of *A. balfourii*, *A. heterophyllum*, *P. hexandrum*, *P. kurroa*, *B. aristata* and *E. ribes*.

Plant cell culture may be used as an alternative for controlled production and supply of secondary metabolites, especially when the phytochemical of interest is known. This method is advantageous because once established it can produce consistent quality and yield of secondary metabolites of interest, under controlled conditions independent of external factors[52]. Several approaches are being tested for increasing the production of secondary metabolites from plant cell culture. Optimization of culture conditions (temperature, light, pH and oxygen), increasing the cell density, using high-yielding strains and addition of precursors, elicitors or bio-transformants are some such strategies[53].

Current trends are also to study the endophytic and rhizospheric flora from medicinal plants because of their ability to produce precursors and compounds of interest bearing functional similarity to the medicinal properties of the plant. Paclitaxel is an anticancer drug isolated from the Pacific yew, *Taxus brevifolia*. Caruso *et al.*[54] reported the production of paclitaxel from an endophytic actinomycete isolated from *Taxus wallichiana*. Golinska *et al.*[55] reviewed the potential use of endophytic actinomycetes isolated from various medicinal plant species for producing chemicals of interest.

Despite the advantages, there are also challenges in the standardization of methodologies for large-scale production of useful plant secondary metabolites, because the secondary metabolite production by a plant is dependent on edaphic, environmental and biological factors. Under controlled condition, the full expression of the entire bouquet of medicinal properties would not get expressed.

Destructive harvesting of vulnerable species from wild sources can pose a threat to the very existence of the species. In addition, sustainable harvesting method for optimum yield of the specific chemical compounds for a long-time period is crucial for conserving the species. The example of guggul (*Commiphora wightii*) in Rajasthan which became threatened due to faulty incision method of bark and overextraction of latex for guggulsterones, confirms the necessity for developing a sustainable harvesting method for bioactive chemical compounds to ensure the persistence of the species in nature.

Many species get pushed to the verge of extinction because the part of interest is crucial for the survival of the plant, like the root, bark, whole herbaceous plant, etc. In such cases, it helps to identify other parts of the same species and also other medicinal plant species which may have similar chemicals and bioactivities. Chemicals are useful to identify substitute parts and other species. Besides rhizomes, several studies have also reported the occurrence of podophyllotoxin from the leaves of *Podophyllum peltatum*[6,45,46,56–58].

Several secondary metabolites that enrich medicinal plants and are the reasons for their medicinal properties, are distributed across many related and unrelated species that are distributed across different geographical regions of the world. That is, many of the chemical compounds are not unique to one species or one region alone; they occur in many species. For example, berberine, an alkaloid has been reported from at least 54 plant species across several families. The curcuminoids are present in several *Curcuma* species (https://phytochem.nal.usda.gov/phytochem/chemicals/). Therefore, if a particular medicinal plant is facing conservation threat, the use of other species which have the same or similar chemical compounds can be explored as substitutes. This will wean the stress from the threatened species.

In view of the ever-growing demand for active constituents from these threatened medicinal plants leading to severe pressure on the natural populations, concerted efforts should now be directed towards selective propagation of elites. The categorization of elites based on their active constituents could serve as an attractive alternative for sustainable industrial utilization of medicinal plants. Therefore, substantial investment into research is required to understand the chemistry and biology of the medicinal plant species. Inter-population variations in these traits need to be understood in order to innovate new conservation strategies. In parallel, research into propagation, agrotechniques and novel biotechnology is vital to ensure continued supply of the medicinal plant

species and the phytocompounds. Needless to mention that the secondary metabolite profiling of the species has to be supported by pharmacological activities to claim functional eliteness.

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