Biological Approaches for Sustainable Utilization of Aconitum: A Genus Containing Valuable Medicinal Properties

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Abstract

Aconitum genus (Ranunculaceae) is well recognized for medicinal worth. Diterpenoid alkaloids (aconitines) are integral part of Aconitum which make them commercially valuable. The species of Aconitum genus have very small populations in different pockets of the globe; which need effective strategies for its conservation as an important natural resource that's why; the production of aconitines through biotechnological means is desirable. In the present review an attempt has been made to throw some focus on possible biogenesis pathway of aconitines and biological approaches to understand it. Upscale production strategies of aconitine which are going on and still need to be done correlating to other medicinal plants have been discussed. Our own efforts are also covered regarding gene mining of possible rate limiting steps with special reference to Aconitum balfourii Stapf. The aim of the present review is to give emphasis on recent biotechnological approaches to be applied which are lacking in Aconitum, however, these approaches are often being utilized in other medicinal plants for their valuable usages.

Keywords: Aconitum; Biochemical Pathway; Diterpenoid Alkaloids; Elicitors

Abbreviations: IPP: Isopentyl Di Phosphate; DMAPP: Dimethylallyl Diphosphate; MEP: 2-C-Methyl-D-Erythritol 4-Phosphate; GPP: Geranyl Pyrophosphate; FPP: farnesyl pyrophosphate; GGPP: gerenyl gerenyl pyrophosphate.

Introduction

Medicinal plants have been drawn considerable attention because people all over the world need medicines that should be safe and intrinsically good for health. Therefore consumption of herbal medicines is widespread and increasing [1]. India is gifted with huge biodiversity. The plant extracts have been used to make the Ayurvedic formulations for curing several ailments since ancient time. Himalayan region of India is considered as one of the hot spot of biodiversity. Of huge biodiversity in the Himalaya, Aconitum belonging to family Ranunculaceae pay a major contribution to it. The
pharmacologically significant compound present in the roots of Aconitum is a type of diterpenoid alkaloid [2]. Several different types of diterpenoids alkaloids (Figure 1) are present in the plant which is highly toxic in nature. A long history of their use in several systems of medicines in India, China and Tibet is evidenced from ancient literature. The most common medicinal application of aconite preparations is the management of pain associated with rheumatism, arthritis, gout, neuralgia, sciatica, migraine and cancer. In homeopathy, aconite is used to dispel fear, anxiety and stress [3].

Aconitines are the ingredient of several medicines but the low content found in wild plants is a serious limitation towards its effective utilization. Aconites natural population is very low while aconitines demand is very high as compare to their population and there is no report of commercial cultivation of Aconitum from any area [8]. Hence, all the demand of pharmaceutical companies is being entirely met by wild collection only. It poses heavy strain on wild habitat and due to continuous overexploitation Aconitum genus faces severe threat and leads into endangered category [9,10]. In the view of current scenario, it is essential to take the genus into consideration for various biological approaches to enhance the production of aconitines in vitro as well as in vivo. Several aspects viz evolutionary, pharmacological, toxicological, isolation and characterization of diterpenoid alkaloids, their phytochemical properties and conservation through micro propagation of various species of Aconitum have been undertaken so far by the scientists all over the world [11-19]. However, there is a lack of studies related to rate limiting gene(s) mining and their molecular characterization, gene over expression studies, and metabolic pathways study with the help of precursors, inhibitors and elicitors both at national and international level. We are working on Aconitun balfouri Stapf. One of the valuable Indian aconite. The rare cultures which need very specific habitat conditions were established successfully in in vitro facilities. Now, we are focusing our research on above mentioned lacking areas. In the present review we will discuss about possible biogenesis of diterpenoid alkaloids and biological approaches to understand the complex secondary metabolism of aconites and efforts of enhancing secondary metabolites of commercial values. These all areas will be discussed in the review correlating to other medicinal plants with the intention that these mentioned approaches might also be applied in Aconitum. It will surely prove beneficial for enhancing and understanding towards aconitines biogenesis which is an ultimate commercial product [20].

About 1358 species of this genus were reported all over the world (IPNI data). In the Himalayan regions it is distributed in Pakistan, India, Nepal, Bhutan and South Tibet, where aconites are used in local and traditional system of medicine [4]. A small group is also found in the Western North America and Eastern United States of America. In India the genus is represented by 24 species and is known as Indian Aconites. Out of two dozen aconitum plant species thirteen possess drug value [5,6] provided major contribution in the taxonomy of Indian aconites. He classified genus as existed in India and arranged the species in three sections on the basis of their root structures [7] (Table 1).
Diterpenoid alkaloids are derived in nature from tetracyclic or pentacyclic diterpenes in which carbon atoms 19 and 20 are linked with the nitrogen atom of the molecule of β-aminoethanol, ethylamine or methylamine to form a heterocyclic ring [23]. In the recent years it has been generally assumed that C20 and C19 skeleton of the alkaloids isolated from the plants of the Garraya, Aconitum and Delphinium species were terpenoids and as such produced, in vivo from acetate units by MVA pathway so well established for the biosynthesis of nonalkaloidal terpenes [24]. Thus these alkaloids are commonly referred as the diterpenoid alkaloids. The possible pathway by which nitrogen atom enters the skeleton remains obscure. It may be presumed that a di-aldehydic precursor accepts an ammonia molecule from a glutamine or lysine residue, as appears to be the case for the triterpenoid steroidal alkaloids, in a reductive amination process [25]. In current years studies proved that serine amino acid is an important in the biosynthesis and donate nitrogen atom to complete the structure of diterpenoid alkaloids [26].

### Table 1: Indian Aconites and their distribution pattern.

| Species             | Section         | Subsection       | Habitat                                      | Altitude(m) | Threat Status | Drug value |
|---------------------|-----------------|------------------|----------------------------------------------|-------------|---------------|------------|
| A. gymnandrum       | Gymnaconitum    | -                | Tibetan plateau                              | 3000-4500   | -             | -          |
| A. laeve Roye       | Lycoctonum      | -                | Jammu & Kashmir, Himanchal Pradesh, uttaranchal | 3200-4200   | -             | -          |
| Aluridum Hook.f. & Thomson | Lycoctonum | -                | Eastern Nepal to Chambai                      | 3600-4200   | Possess       |            |
| A. moschatum Stapf  | Lycoctonum      | -                | Jammu & Kashmir                              | -           | -             | -          |
| A. soongaricu Stapf | Napellus        | Napellus         | Kashmir, Central Asiatic province            | -           | -             | -          |
| A. chasmanthum Stapf | Napellus        | Napellus         | Western Himalaya from Chitral to Hazara, Himanchal Pradesh | 2100-3600   | Possess       |            |
| A. violaceum       | Napellus        | Napellus         | Kumaon Himalaya                              | 3000-4500   | VL Possess    |            |
| A. falconeri Stapf  | Napellus        | Napellus         | Alpine & Subalpine zones of Garhwal Himalaya | -           | CE Possess    |            |
| A. spicatum Stapf. | Napellus        | Napellus         | Sikkim Himalaya                              | -           | VL Possess    |            |
| A. laciniatum Stapf| Napellus        | Napellus         | Sikkim Himalaya                              | 3300-4200   | Possess       |            |
| A. ferox WALL. EX SERINGE | Napellus | Napellus         | Sikkim to Garhwal Himalaya, Assam            | -           | CE Possess    |            |
| A. heterophyllidos Staf | Napellus | Napellus         | -                                            | -           | -             | -          |
| A. leucanthum Stapf.| Napellus        | Napellus         | Himalaya of Nepal to Bhutan, SE tibet        | 3600-4500   | -             |            |

**Bioactive Compound of Genus Aconitum**

Before discussing the proposed biogenesis ofaconite type diterpenoid alkaloids, we are giving a brief introduction of alkaloids (bioactive compounds) [21] stated that bioactive compounds in plants can be defined as secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals. Bioactive compound of genus Aconitum are composed of several true and pseudoalkaloids collectively called as aconitines. Diterpenoid alkaloids fall into category of pseudoalkaloid and these were very well characterized by Rehman & Choudhary [22]. Recently again 10 year’s investigation (from 1998 to the end of 2008) on diterpenoid alkaloids was superbly compiled by Wang et al [15]. They covered more than 300 new diterpenoid alkaloids which have been isolated in ten years from different genera and focused on their structural relationships, and investigations into their chemical reactions, synthesis, and biological activities. They have classified diterpenoid as into three categories viz; C18, C19 and C20 type.

Diterpenoid alkaloids are derived in nature from tetracyclic or pentacyclic diterpenes in which carbon atoms 19 and 20 are linked with the nitrogen atom of a molecule of β-aminoethanol, ethylamine or methylamine to form a heterocyclic ring [23]. In the recent years it has been generally assumed that C20 and C19 skeleton of the alkaloids isolated from the plants of the Garraya, Aconitum and Delphinium species were terpenoids and as such...
pyrophosphate (GGPP) [27,28]. It is generally accepted that the cytosolic pool of IPP serves as a precursor of sesquiterpenes, triterpenes, sterols and polyterpenes whereas the plastid pool of IPP provides the precursors of mono-, di- and tetraterpenes [29]. Some exceptions have been described showing that interactions between the two biosynthetic pathways may exist. There is a compartmentalization of precursor isoprene unit during the synthesis of terpenoids and both the pathways are contributed precursors for the synthesis of terpenoids. There is a sign of crosstalk of two pathways during terpenoid synthesis in plants [30].

IPP pool is the building blocks on which several complex reactions take place and many structurally complex intermediates compounds are formed to complete the different pseudoalkaloids structure. In the formation of IPP two routes are followed; in MEP, the initial step of the plastidic 2C-methyl-D-erythritol 4-phosphate (MEP) pathway that produces isopentenyl diphosphate is catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase. Estevez, et al. [31] proved with the help of transgenic Arabidopsis that this enzyme catalyzes one of the rate limiting steps in MEP pathway. In MVA pathway the initial steps involve the fusion of three molecules of acetyl-CoA to produce 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). The HMG-CoA is then reduced to yield mevalonic acid in a NADPH-dependent double reduction. This step is catalyzed by mevalonate: NADP oxido reductase, CoA acylating; 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR; EC 1.1.1.34) [32]. However, the rate limiting nature of HMGR in plants and its regulation remain controversial [33,34]. Evidence for the contribution of HMGR as the rate limiting enzyme in isoprenoid biosynthesis has come from several investigators in which they have shown an induction in isoprenoid biosynthesis and a concomitant increase in HMGR activity [35-40]. DXS and HMGR are considered as rate limiting and the key players for synthesis of various isoprenoids in higher plants. Possible biogenesis of aconitines type of alkaloids is represented in Figure 2 [41-45].

![Figure 2: Possible biogenesis of aconitine type diterpenoid alkaloids.](image-url)
Recent Approaches Concerning Pathway Identification and Upscale Production Still Going on Tissue Culture

Plant cell culture technology is being used for commercial scale production of various secondary metabolites in which elicitation process has been effective at increasing the product yield. Hence challenging the cultures with elicitors facilitate to achieve a better understanding regarding pathway identification [46]. Elicitors could be used as enhancers of plant-secondary-metabolite synthesis and could play an important role in biosynthetic pathways to enhance production of commercially important compounds [47-50]. Methyl jasmonate and salicylic acids are two very common elicitors that have been used to study various plants species. Precursors and inhibitors studies are also proved beneficial to unravel the complex secondary metabolic pathways [51,52]. The studies related to this aspect on various medicinal plants are summarized in which is one of the missing areas of research on Aconitum. However a minor effort was made by us to see the effect of early step terpenoid pathway precursors and inhibitors on aconitine accumulation and effect on probable key enzyme activities 1-deoxy-D-xylulose phosphate synthase (DXS) and 3-Hydroxy- 3-methyl glutaryl Coenzyme A reductase (HMGR). It was concluded that there is possibility of crosstalk between mevalonate and non mevalonate pathways during isopentyl pyrophosphate (IPP) supply for aconitine biosynthesis. However, it seems that the mevalonate pathway plays a greater role IPP synthesis [53].

Several efforts have been carried out so far on conservation aspects, of Aconitum species through micro propagation to save it from extinction. It is very necessary to optimize the micro propagation protocol for mass multiplication and generation of material for research purpose for high altitude plants such as A. balfourii Stapf. Therefore, in vitro propagation protocols were optimized [54]. Study of population genetic diversity is another method that provides useful information for biological conservation. Four populations of A. balfourii Stapf were studied through polypeptide pattern and isoenzyme markers to locate the genetic diversity. They studied three enzyme systems such as: esterase, superoxide dismutase, and catalase. On the basis of isoenzyme variations and banding pattern, they have identified seven polymorphic loci in four populations.

Some efforts have been reported on Aconitum species in which hydroponics and suspension culture were tried in following mention studies. Hydroponics (from the Greek words hydro water and ponos labor) is a method of growing plants using mineral nutrient solutions, in water, without soil. For more than a decade research groups have been working on the development of a new biotechnological method for the industrial production of secondary metabolites using whole plants cultivated in soilless conditions. Such a technology may require not only agronomical knowledge but also know-how developed for in vitro and bioreactor processes [55,56]. In Aconitum balfourii Stapf an attempt has been made to increase the aconitine content through hydroponics. The plants regenerated from tissue culture were cultivated by Pathak et al hydroponically with a hydroponics apparatus placed in green house containing continuously running Hoagland’s solution .The plants cultivated hydroponically (after 27 days) got stronger and longer roots (4.8±0.5) in comparison to plants regenerated from tissue culture (root length 2.7±1.0). The hydroponically cultivated plants have greater dry weight of roots (0.05g/root) than tissue culture derived plants (0.032g/root). Aconitine content was checked through HPLC and it was found that the maximum percentage of aconitine 0.024% in hydroponics cultivated plants as compared to plants collected from wild (Tungnath) 0.015% and tissue culture derived plants 0.0123%. There is only single report on Aconitum suspension culture till date. In this study they have optimized the aconitine production in cell suspension culture of A. napellus. They investigated the effect of culture conditions on cell biomass and aconitine content. They have also observed the effect of salicylic acid and yeast extract on accumulation of aconitine. The results showed that proper culture conditions enhanced the content 2.5-3 folds as compared to control [57] (Table 2).

| Plant Source       | Secondary metabolites | Culture type | Elicitor/Precursor | References                  |
|--------------------|-----------------------|--------------|-------------------|-----------------------------|
| Gossypium barbadens| Gossypol              | Hairy roots  | SA, MJ            | Frankfater, et al., 2009    |
| Salvia miltiorrhiza| Tashinone             | Hairy root   | SL, YE            | Wu and Shi, 2008            |
| Salvia miltiorrhiza| Tashinone             | Hairy root   | ABA               | Ge and Wu, 2005             |
| Salvia officinalis | Carsonic acid         | Shoot cultures| MJ                | Gao, et al., 2011           |
| Euphorbia pekiensis| Isoeuphekinesis       | Suspension   | FE                | Grzegorczyk, 2009           |
Hairy root culture exemplifies a potential biotechnological tool to enhance secondary metabolite production in several medicinal plants. Root culture established by transformation with Agrobacterium rhizogenes is regarded as advantageous resource of useful compounds because of the rapid growth in culture media without phytohormones and relatively high productivity of secondary metabolites compared to undifferentiated calli or cell suspension, or in some case roots of mother plants. It is an emerging tool to enhance the pharmacologically active compound and have been tried in lots of medicinal plants. Giri, et al. [58] was reported hairy roots production in Aconitum heterophyllum Wall. They successfully transformed embryonic cell culture of Aconitum heterophyllum Wall. By using A. rhizogenes strains viz. LBA 9402, LBA 9360, AND A4 for the induction of hairy roots. It was found that total alkaloid (aconite) content transformed roots was 2.96%, which was 3.75 times higher compared to 0.79% in the non-transformed roots. Agrobacterium rhizogene mediated transformation method has been used to enhance the secondary metabolites in several other plant species [59-62].

**Gene Mining and Functional Characterization**

We have previously illustrated the role of mevalonate and non mevalonate pathways in proposed biogenesis of aconitine type alkaloids. Therefore, the gene mining and their functional characterization of rate limiting steps of concerning pathways are very necessary [63,64]. It is supposed to be providing several clues for targeting genetic manipulation for upscale production of medicinally significant components in vitro as well as in vivo. Several studies have been carried out so far on isolation and characterization of rate limiting genes in several other medicinal plants. The discoveries are summarized in Table 3. These types of studies are also needed in case of Aconitum genus. Therefore, an attempt has been successfully made in our laboratory to partially isolate and functionally characterized both rate limiting genes i.e. HMGR and DXS from A. balfourii Stapf. (Accession nos are KC425323.1 and KC51434.1 respectively). Bioinformatics analysis was carried out and the phylogenetic relationship (N-) method of genes from other plants genes retrieved from database is shown in Figures 3 & 4. To investigate the expression of HMGR and DXS in different tissues, total RNA was isolated from leaves, shoots and roots and subjected to RT-PCR and Real time PCR analysis [65-69]. The result of RT-PCR and real time PCR were almost parallel to each other [70-73]. Expression profiling of AbDXS with reverse transcriptase PCR indicated constitutive expression in all tissues i.e. leaf, shoot and roots, however, differential expression patterns were obtained [74-77] (Figure S).
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Table 3: Cloning and characterization of rate limiting genes of isoprenoid pathway.

| Plant source     | Gene | Substrate binding motif | NADPH/TPP binding motif | References                      |
|------------------|------|--------------------------|--------------------------|---------------------------------|
| Nicotiana sylvestris | HMGR | EMPGYVQIP                | DAMGMNM                  | Genschik, et al., 1992          |
| Camptotheca acuminata | HMGR | EMPGYVQIP                | DAMGMNM                  | Maldonado, et al., 1997         |
| Ustilago maydis | HMGR | EMPGYVQIP                | DAMGMNM                  | Croxen, et al., 1994            |
| Gossypium hirsutum L. | HMGR | EMPGYVQIP                | DAMGMNM                  | Loguercio, et al., 1999         |
| Morus alba | HMGR | EMPGYVQIP                | DAMGMNM                  | Jain, et al., 2000              |
| Taxus medica | HMGR | EMPGYVQIP                | DAMGMNM                  | Liao, et al., 2004              |
| Eucomia ulmoides | HMGR | EMPGLFLQIP               | GTVGGGT                  | Shen, et al., 2006              |
| Corylus avellana | HMGR | EMPGLFLQIP               | DAMGMNM                  | Wang, et al., 2007              |
| Solanum nigrum | HMGR | EMPGYVQIP                | DAMGMNM                  | Jose, et al., 2008              |
| Jatropha curcas | HMGR | EMPGYVQIP                | DAMGMNM                  | Liao, et al., 2009              |
| Euphorbia pekinensis | HMGR | EMPGYVQIP                | DAMGMNM                  | Lin, et al., 2009               |
| Michelia chapensis | HMGR | EMPGYVQIP                | DAMGMNM                  | Cao, et al., 2010               |
| Panax geneseng | HMGR | EMPGYVQIP                | DAMGMNM                  | Wu, et al., 2012                |
| Catharanthus roseus | HMGR | EMPGYVQIP                | DAMGMNM                  | Abdin, et al., 2012             |
| T. brevicorniculatum | HMGR | EMPGYVQIP                | DAMGMNM                  | Deenen, et al., 2012            |
| Arabidopsis | DXR | LPADSEHSAI               | GSTSGIT                  | Paulet, et al., 2002            |
| Ginkgo biloba | DXS  | PSD, DRAG                | LNDN, GDG                | Gong, et al., 2006              |
| Glycine max(Soybean) | DXS | PSD, DRAG                | LNDN, GDG                | Zhang, et al., 2009             |
| Salvia Miltiorrhiza | DXR | LPADSEHSAI               | GSTSGIT                  | Wu, et al., 2009                |
| Amomum villosum | DXS  | PSD, DRAG                | LNDN, GDG                | Yang, et al., 2012              |
| Amomum villosum | DXR  | LPADSEHSAI               | GSTSGIT                  | Yang, et al., 2012              |

Figure 3: Phylogenetic tree of HMGR protein from different plant species. Sequence analysis was performed using Clustal X, and the nearest neighbor-joining method was applied to create trees. Aconitum balfourii HMGR and related proteins Coffee arabica (ADR51242.1), Nicotiana tabacum (AAAB87727.1), Morus alba (AAD03789.1), Populus trichocarpa (XP 002317026.1), Ricinus communis (XP 002510733.1), Panax quinquefolius (ACV65036.1), Camptotheca acuminata (AAB69726.1), Glycine max (XP 003519474.1), Medicago truncatula (XP 003629056.1), Sorghum bicolor (XP 002445887.1), Brachypodium (XP 003572378.1), Physcomitrella patens (XP 001771547.1), Tanacetum parthenium (AER00469.1), Eucommia ulmoides (AAV54051.1), Andrographis paniculata (AAP14352.2), Aquilaria sinensis (AFU75319.1).
Gene Over Expression

Gene over expression analysis is also being successfully used to find out the correlation studies between related key gene(s) transcript level and secondary metabolite accumulation in several plant species [77,78]. Studies were carried out over expression studies in which HMG-Co A reductase gene (hmgr) from Catharanthus roseus (L) G. Don and amorpha-4, 11-diene synthase (ads) gene from A. annua L. were over-expressed in A. annua L. plants to study their effects on artemisinin yields [79-83]. The expressions of hmgr and ads at the transcriptional level were also confirmed in each transgenic line employing RT-PCR assays [84]. The HPLC analyses showed that the artemisinin contents were significantly increased in these transgenics. One of the transgenic line, TR4, was found to contain 7.65-fold higher (1.73 mg/gDW) artemisinin than the non-transgenic plant (W) [85]. The increased artemisinin levels were found to be correlated with HMG-CoA reductase and amorpha-4, 11-diene synthase enzymatic activities in the biochemical analyses [86,87]. Singh, et al. [88] studied the expression analysis of isoprenoid pathway in the presence of two inhibitors mevinolin and fosmidomycin in suspension cultures of Arnebia euchroma. They have suggested a positive correlation between shikonins content and expression of 3-hydroxy-3-methylglutaryl-CoA reductase (AeHMGR) and AePGT suggesting critical role played by these genes in shikonins biosynthesis [89].

Conclusion and Future Perspectives

In last few decades efforts have been made by scientist all over the world to understand plant secondary metabolism and its regulation. The marginal success in this field is due to structural complexity and tedious regulatory mechanism of secondary metabolic pathways. It is still a long way to go how these compounds are synthesized in plants and how their biosynthesis is regulated. Diterpenoid alkaloids (aconitines) are integral part of genus Aconitum which make it commercially valuable. However, the current knowledge regarding the biogenesis of aconitines is very limited; exemplify the major hurdle for successful metabolic engineering and commercialization. So, it is an essential requirement to consider the genus Aconitum for optimization of recent genetic engineering techniques which are being employ successfully in other medicinal plants. The different approaches involved in understanding biogenesis might be helpful for getting higher aconitines yield and saving the genus from extinction as well.
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