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DNA FINGERPRINTING OF *Valeriana jatamansi* BY USING RAPD AND SSR MARKERS

Enrollment numbers : 091565 & 091704

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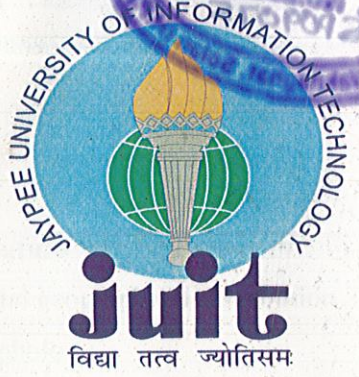

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Submitted in partial fulfillment of the Degree of

Bachelor of Technology

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT

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CERTIFICATE

This is to certify that the work titled "**DNA Fingerprinting of *Valeriana jatamansi* by using RAPD and SSR markers**" submitted by "**Vikas Singh and Shashank Ranjan Srivastav**" in partial fulfillment for the award of degree of B.Tech Biotechnology of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Signature of Supervisor

Name of Supervisor Dr. R. S. Chauhan

Designation Head of Department

Date 25/05/2013

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We take this opportunity to thank our esteemed mentor and supervisor Dr. R. S. Chauhan, Head of Department of Biotechnology and Bioinformatics, JUIT, for lending me stimulating suggestions, innovative quality guidance and creative thinking. His practicality, constructive criticism, constant encouragement and advice helped us in all stages of the project. His scientific views and scientific approach will always be the source of motivation for us. We are grateful to him for the support, he provided us in doing things at my pace and for being patient with our mistakes.

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SUMMARY

Valeriana jatamansi, a high altitude medicinal plant, is known for its drug content called valerenic acid and valepotriates. In the present study, DNA-based molecular marker techniques, viz. simple sequence repeats (SSR) and RAPD (random amplified polymorphic DNA) markers were used to study polymorphism in *Valeriana jatamansi.* Twelve strains of *Valeriana jatamansi* were obtained from HFRI Shimla which shows valepotriates content in all twelve strains is varying from 1.6 % - 3% where as valerenic acid content is absent in all strains except three strains. These valepotriates and valerenic acid are secondary metabolites which are used in medicine. 79 SSR and 10 RAPD primers were tested on genomic DNA of Valeriana strains, out of which 71 SSR primers and 5 RAPD markers showed amplification. 8 SSR primers and 1 RAPD primers showed polymorphism. results showing less polymorphism occurring in strains of *Valeriana jatamansi.*

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Signature of Student: - Shashank

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List of Abbreviations

DNA: deoxyribose nucleic acid.

dH20: Distilled Water.

EDTA: Ethylene Diamine Tetra-acetic Acid.

EtBr: Ethidium Bromide.

LB: Luria broth.

PCR: Polymerase Chain Reaction.

TE: Tris EDTA Buffer.

TAE: Tris Acetic acid EDTA.

RAPD: Rapid Amplification of Polymorphic DNA.

SSR: Simple Sequence Repeats.

CTAB: Cetyltrimethyl Ammonium Bromide.

HFRI: Himalayan Forest Research Institute.

FRSs: Forest Research Stations.

RFLP: Restriction Fragment Length Polymorphism.

AFLP: Amplified Fragment Length Polymorphism.

dNTP: Deoxyribonucleoside triphosphate.

V. jatamansi: Valeriana jatamansi.

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CHAPTER - 1

INTRODUCTION

Valeriana jatamansi (family Valerianaceae), commonly known as 'Tagar' or 'Indian valerian', grows wild in the temperate Himalayan region between an altitude of 1000–3000 m asl (above sea level). The species is characterized as rhizomatous, occasionally stoloniferous, perennial herb with erect, unbranched flowering stem revealing considerable morphological variability. It is dioecious, polygamous or occasionally polygamomonoecious species (Prakash, 1999), well known for its medicinal and other ethnobotanical values (Mathela et al., 2005; Singh et al., 2010). The active ingredient – valerian, derived from its rhizomes is used for the treatment of various diseases (Prakash, 1999). The roots and rhizomes of this species are also used for the preparation of phytomedicines with mild sedative action (Houghton1999).

The species is phytochemically well investigated (Mathela et al., 2005; Singh et al., 2006, 2010). More recent reports have established its antioxidant potential (Kalim et al., 2010), which can largely be attributed to the presence of polyphenols and their free radicals scavenging properties (Parr and Bolwel, 2000; Scalbert and Williamson, 2000) and known protective effects against certain chronic and degenerative diseases (Meyer et al., 1998; Record et al., 2001).

The genetic diversity studies of chloroplast SSR markers in case of Valeriana wall-rothii (Grassi et al., 2004). However, no reports are available on the association of molecular markers with biochemical traits of *V. jatamansi*. The use of SSR markers has advantage over others (i.e. RAPD, AFLP) as it involved PCR amplification of DNA by a single 11–18 bp long primer composed of a repeated sequence. This method has comparative advantage of being highly reproducible and cost effective (Powell et al., 1996), as compared to reported low reproducibility and low polymorphism of RAPD markers (Karp et al., 1997; Virk et al., 2000).

It is an important medicinal herb of Northern Western Himalayas being used in the treatment of epilepsy, leprosy, hysteria and asthma. The active principle of this plant besides having antibacterial and antiprozal activity can be taken as a remedy for snake bite as well as

scorpion sting (Chopra *et al.*,1956) It can also be used as potential anti-tumor agents (Bounthanh*et al.*, 1981).

Keeping in view the immense medicinal importance and critically endangered status of *Valeriana jatamansi.* it becomes necessary to study various reproductive constraints if any which in turn will prove to be helpful in planning the conservation strategies. Nonetheless, the mounting demand of this plant species from various sources necessitates its domestication and propagation in a big way outside its natural habitat. A thorough understanding of their reproductive and growth biology as well as identification of biological and ecological constraints leading to their reducing fitness, restricted distribution, or even extinction is, therefore essential so that one is able to predict their behavior under ex situ cultivation and develop strategies for their successful conservation. This devised programme of the study on Valeriana jatamansi can help in developing certain protocols to combat the problems that impede regeneration. Further, the study of reproductive biology can provide important paradigm for conservation, reclamation and restoration of *Valeriana jatamansi.*

AIM- DNA Fingerprinting of *Valeriana jatamansi* by using the RAPD and SSR markers.

Objectives

1. Molecular characterization of *Valeriana jatamansi* strains by using RAPD and SSR markers.

CHAPTER- 2

REVIEW OF LITERATURE

Valeriana jatamansi or Indian Valerian of the family Valerianaceae is an important medicinal plant used in several indigenous systems of medicine practiced in India (Anonymous, 1976). This species has been listed as critically endangered species in Western Himalayas (Kaul& Handa, 2000) and is therefore of considerable interest for various studies which centre around the following aspects.

2.1. Taxonomy and geographical distribution:

Taxonomy and conservation go hand in hand. We cannot conserve organisms that we cannot identify and our understanding the consequences of environmental change and degradation are compromised fatally, if we cannot recognize and describe the interacting components of natural ecosystems (Mace, 2004). The familial status of Valerianaceae is debatable in light of recent studies (Bell and Donoghue, 2005). Judd *et al.* (1994) on the basis of morphological and anatomical similarities merged it in Caprifoliaceae.

Valerianaceae comprises of 350 species distributed throughout the World (except Australia and New Zealand), mostly at high elevations and with many species in alpine zones (Backlund and Moritz, 1998). Bell (2004) labeled Valerianaceae as a natural group of 350 species of cosmopolitan distribution comprising of 13 genera with 200 species chiefly confined to temperate regions. *Valeriana jatamansi* Jones (Syn. *Valeriana wallichi)* popularly known as Indian Valerian (English), Mushkibala (Hindi), Suganthdhawal or Tagara (Sanskrit), is distributed in all the temperate regions except Australia, (Jain, 12 1968; Bennet, 1987).

Several species of *Valeriana* have also been reported from Andean Chile, Brazil, South Africa and Sub-tropical Asia. About 12 species of genus *Valeriana* have been reported from India (Anonymous, 1976). Rao *et al.* (1977) have reported 10 species of *Valeriana* from India. Out of these *Valeriana jatamansi* has been reported to be widely distributed in temperate Himalayas at an altitude ranging from 1500 m in Khasi Hills to 3000m in Jammu and Kashmir, Himachal Pradesh and Bhutan (Kritikar and Basu, 1975).

Mukerjee, (1953) while assessing distribution of *Valeriana officinal is* reported it to be growing only in restricted sites of North Kashmir at an altitude of 2400 to 2700m. However,

Valeriana jatamansi according to Chauhan and Khosla (1988) is sporadically found in whole Northern Western Himalayas.

Polunin and Stainton, (1987) reported the distribution of *Valeriana jatamansi* from Afghanistan to South west China and Burma. The herb was seen to grow at an altitude of 1500 to 2600m where in the surroundings were mainly comprising of Bedula (*Fiscus* species), Laliguron (*Rhododendron arboreum*). The herbarium records of KASH, Department of Botany, University of Kashmir represents 5 species of the genus *Valeriana* reported from different localities of Kashmir valley which include Shajnar, Dara, Harwan, Gulmarg, Yusmarg, Dacksun, Ferozpur, Sonamarg (Naqashi and Dar, 1982-1986- KASH Herbarium Collection).

2.2. Species morphology:

Valeriana jatamansi is a perennial herb with pubescent stem, radical leaves, several long petiolated cordate-ovate, cauline few or much smaller entire or pinnate, fruits hairy or nearly glabrous. Root stocks thick, horizontal with thick descending fibers; stem 6-18cm, radical leaves often 1-3cm in diameter, deeply cordate, usually acute toothed. Cymes corymboselypanicled; bracts small oblong or linear persistent, calyx limb in flower obscure, unrolling in fruit into 5-15 plumose bristles united at base equal or sub-gibbous, flowers white, stamens 3, ovary 3celled, 1 ovuled, stigma shortly 2-3 fid or sub-entire, fruits oblong lanceolate, compressed crowned by persistent pappus calyx (Hooker, 1881). Kokwar, (1968) described Valerianaceae member as annuals (occasionally biennial) or perennial herbs, rarely subshrubs; often with strongly scented rhizomes. Leaves opposite sometimes forming basal rosettes, exstipulate, often pinnately much divided but sometimes entire, cauline leaves sometimes few, small or none; basal leaves pinnatifid, base often sheathing. Inflorescence a many flowered compound dichasial cyme, thyrse or monochasium, sometimes condensed and capitate, bracteate and usually bracteolate.Flowers hermaphrodite or unisexual by abortion (plants then dioecious as in some Valeriana spp.), irregular or almost regular usually 5-merous. Calyx often small or absolute at the time of flowering, sometimes enlarging as the fruit matures, and then variously lobed; lobes often forming a pappus. Corolla funnel shaped or tubular, often attunated at the base. Stamens 1-4, epipetalous, alternating with the corolla lobes, anthers versatile, 2 or 4-lobed, 2 or 4-thecous, pollen grains tricolpate, echinate ovary inferior, tricarpellate, 3 locular but only one locule fertile; ovule solitary and pendulous, anatropous, style single and slender; stigma 2-3 lobed, fruit a 1- seeded achene. Polunin and

Stainton, (1987) reported corolla of *Valeriana* as funnel shaped, limb 5 lobed, spreading, stamens 3, style slender, undivided, fruit is indehiscent bearing a single hairy achene.

2.3. Breeding system:

Plants cannot choose their mates; nevertheless they have diverse methods by which genetic structure of their populations and the patterns of their evolution is influenced by their mating patterns. Plant breeding systems are under genetic control and can themselves be selected for. They are fluid and respond to selection pressures in an infinite variety of subtle and interrelated ways because breeding systems are genetically controlled and affect genotype structure(Richards, 1986). Thus breeding system is the purposeful manipulation of plant species in order to create desired genotypes and phenotypes for specific purposes. Mating strategies in flowering plants are governed by several classes of floral adaptations. Floral design and display primarily influence the quantity and quality of pollen dispersed during pollination, whereas physiological mechanisms operative in pistil screen pollen receipt by rejecting certain male gametophytes, especially self pollen, (Barret, 1998), thus selective mechanisms that influence the evolution of plant mating strategies include inbreeding depression, pollen discounting and optimal allocation of resources to female and male function.

Layton and Ganders (1984) investigated the genetic consequences of contrasting breeding systems in *Plectris* (Valerianaceae). The study revealed the consequences of contrasting breeding systems in two closely related taxa; *Plectriscongesta* (Lindl) D.C. and *Plectrisbrachystemon* F & M (Valerinaceae). The studies revealed that due to large flowers and a nectiferous spur in the former, insect (pollinator) visitation was active. Thus, even though self-compatible, it was strongly protandrous also. The numerous flowers on the inflorescence may thus open simultaneously which will facilitate geitenogamous pollination. In contrast, *Plectrisbrachystemon* bears small flowers with spurred corolla though in some population the spur is reduced to a mere swelling, the flower were not protandrous and hence pollinators were not dominantly visiting the flowers. The differences in floral character strongly suggest contrasting breeding strategies which was further investigated by Gander *et al.* (1977 a and b).

Wyatt, (1982), Richards (1986) and Harder and Barret, (1996) while assessing reproductive biology of different plant species reported that plant mating systems and pollinator behavior are influenced by many morpho-reproductive characters, like phenology, self incompatibility and inflorescence architecture.

2.4. Seed Biology

In most angiosperms, flowering date varies among plants within a population, and such variation may result in differences in reproductive output among plants like fruit: flower ratio (Dieringer, 1991; Kelly and Levin, 2000). seed: ovule ratio (Widden, 1991), number of seeds produced by a plant (Schmitt, 1983; Kelly and Levin, 2000) and number of seeds per fruit produced by a plant (Widden, 1991). The reproductive strategies among plants may evolve from fitness tradeoff between seed dispersal and seedling establishment. Large seeds of small crops generally are not dispersed as compared to small seeds of large crops, but large seeds contain more reserves for seedling establishment (Preciak, 2002). However, variation in seed size vs. number can reflect a compromise between seed dispersal (i.e., movement of seeds away from parent plants) and seedling establishment including seed germination and seedling emergence, growth and survival (Sollabanks, 1992). Seeds play an important role in the development of civilization by supplying food, feed and natural products and traditional medicines, thus acquiring knowledge of seed biology has been a priority for most cultures (Jaimie *et al.,* 2005). Weins, (1984) on the basis of studies on seed productivity reported that seed ovule ratio of out crossing perennials tends to be lower than selfing species. However, Argen and Willson, (1991) have reported that in gynodioecious species, seed production in hermaphrodite plants was lower than in female plants. It was reported that in gynodioecious *Gerniummaculatum* female plants produce 1.6 times more seeds than hermaphrodites while in *Geranium sylvaticum*, females usually produce fewer and smaller seeds than hermaphrodites 22 Lubbers and Christensen, (1986) analyzed the intra seasonal variation in seed production among flowers and plants of *Thalictrum thalictroids*. The studies revealed that the mean seed number and percent seed set were lower in the flowers that open late in the season than in those that open earlier. Further the low seed set was primarily reported in flowers that are positioned laterally and open later than the centrally located flowers. The studies also revealed that the plants flowering earlier in the season produce more flowers, ovules and seeds than those flower latter. However, the percent seed set per plant did not change indicating that the temporal differences in total seed output can be traced largely to variation in total ovule number. The authors concluded that seed output may also be influenced by limitations which in turn will have greatest effect on total seed production. According to Aswanthaiah *et al.* (1993) germination test is useful in evaluating the planting value of a seed lot. Gorbunov, (1979) while studying the biomorphic characteristics of 5 *Valeriana* species reported better percentage of seed germination at 3-4°C, 7-9°C alternatively with 18-20°C than the germination at constant temperature of 18-20°C. The

polyploidy species viz. *Valeriana cardamines* and *Valeriana eriophylla*, according to the author are more suitable for Cultivation than the diploid *Valeriana alpestris* and *Valeriana allarifolia*. Vashist and Kant, (1998) conducted studies on seed viability, seed germination and seed storage of *Nardostachys jatamansi*. They report that GA3 Treatment at 100 ppm resulted in 85% germination. They further reported that freshly harvested seeds showed 65% germination after 20 days. After one month storage % age decreased to 60% for seeds in cold storage and 45% for those stored at room temperature and corresponding values of germination after six months Storage are 25% and 10% respectively.

Mattana *et al.* (2010) on the basis of studies on *Centranthus* (Valerianaceae) reported that the effects of a range of constant temperature 5-25oC and two 23 alternating regimes 25/10oC and 30/15oC on seed germination. They argued that seed and seedling mass of *Centranthus ruber* were higher than for *Centranthus amazonum* and the lack of a persistent soil seed bank detected for *Centranthus amazonum* increases vulnerability to extinction for this species.

2.5. *In vivo/In vitro* studies

Propagation is the practice of rapidly multiplying stock plant material to produce large number of progeny plants. It is used to multiply novel plants such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets which do not produce seeds or do not respond well to vegetative reproduction (Horn, 1992).

Kaur*et al.* (1999) through *in vitro* propagations established productive method for rapid and mass multiplication of *Valeriana jatamansi* through induction of shoot proliferation from shoot buds. The optimum results were obtained by using solid media supplemented with benzyl adenine alone or in combination with the indole acetic acid or naphthalene acetic acid. Further, culturing on media supplemented with BA and IAA or NAA facilitated the shoot formation which produced roots on the same .medium within 3-4 weeks. The survival of progenials was hundred percent.

Luissa *et al.* (2002) developed a practical method for the multiplication of *Valeriana glechomifolia*. They also assessed the valtrate synthesis in the plantlet. Doing their study the auxiliary buds and shoot tips were cultured in 0.3µ Murrashige and Skoog basal medium supplemented either with 0.4mg BAP or without plant growth regulators. The cultured segment grew on both media producing roots after 3-4 weeks.

Singh *et al.* (2005) assessed the impact of transplantation time on the growth and yield of *Valeriana jatamansi*. The maximum plant yield in terms of height, aerial biomass, underground biomass, rhizome yield, and root yield was obtained after 9 months of transplantation. The assessment of impact of habitat conditions depicted highest values for plant height, biomass, underground biomass, rhizome yield and root when plantation was carried out on ridges.

2.6 Medicinal properties

Chopra *et al.* (1956) reported that rhizome and roots of *Valeriana jatamansi* contain an essential oil-valepotriate, which has antibacterial and antiprozoal activity. The isolation of valepotriates and determination of its medicinal properties resulted in wide spread use of this compound as a sedative in west Germany under the trade name "valmane" (Thies, 1966). The valmane comprises of standarized mixture of valepotriates containing valtrate (15%), didrovaltrate (80%) and acevaltrate (5%). Although some clinical testing of these alkaloids were carried out earlier also, but the first report on their medicinal properties was published by Von Eickstedt and Rehman, (1969).

Uniyal *et al.* (1967) on the basis of collection of ethenic information reported that the tribes of Tehri-Garwal regard *Valeriana* as a sacred plant. It is used in the preparation of ubtan (a cosmetic) in certain ceremonies and also used as an insect repellent.

Kritikar and Basu, (1975) have reported that the roots and rhizomes of *Valeriana* are useful in the treatment of epilepsy, hysteria and asthma. They also reported that roots of *Valeriana jatamansi,* in combination with other drugs as a remedy for snake bite and scorpion sting.

Bounthanh *et al.* (1981) conducted tests on the cytotoxity of some valepotriates for their use as potential anti-tumor agents. *In vitro* experiments, using cultural rat hepatoma cells, showed that the valtrate and didrovaltrate suspension were highly cytotoxic but Baldrinal did not have such activity. Didrovaltrate was found to reduce tumor size after 24 hours of its application.

Pande*et al.* (1994) reported roots of *Valeriana jatamansi* are acrid and bitter with a flavor. These are used as carminative, laxative, antiperiodic, hypnotic and aphrodisiac. The roots are also used for curing diseases of blood, burning sensation, leprosy, cholera, skin disease throat troubles and ulcers. Further the roots increase the lusture of eyes, promote growth and blackness of hair and also useful in the treatment of cough, chest pain and kidney troubles. Prakash and Mahrotra (1994) reported that *Valeriana jatamansi* rhizome and roots have been

used in the treatment of hysteria, epilepsy, asthma. However, Gupta et al., (1996) assessed that the drug Valerian extracted from roots of *Valeriana* is being used immemorial for curing different diseases. Valerian is also used as a traditional sedation, antispasmodic and tranquilizer (Diapher and Hindwarch, 2004).

2.7. Conservation status

The ever increasing exploitation of medicinal plants and habitat destruction are the main causes of plant extinction. The growing public awareness of resources exploitation and its impact on surrounding biodiversity has necessitated creation of suitable protective strategies so that natural wealth is maintained.

Wyatt (1981) while assessing the status of *Valeriana* reported that *Valeriana jatamansi* and *Valeriana officinalis* are over exploited due to their use in wide range of diseases nationally and internationally. The population assessment of *Valeriana* has revealed that on an average there is a decrease of about 30-40 plants per 100 square meter. Which is increasing with every passing year. A study by Wyatt (1981) reveals that they are totally wiped out from some previously recorded localities, comparatively at lower altitude while their distribution in the inaccessible terrain has dissected and shrunken. The authors suggested both *in-situ* and *ex-situ* conservation strategies to protect the genus from extinction. Verma *et al.* (2011) reported that *Valeriana wallichi* is exploited for its rhizome, which is the source of active principle-valepotriates, for which plant is sought after and has been depleting from its natural habitats at a fast pace and hence is of immediate concern to observe conservation strategy.

2.8. Uses of *Valeriana jatamansi*

Valerian is frequently listed among the ten most widely used herbal supplements (Morris and Avorn 2003). Historically, valerian has been used as a sedative, anti-spasmodic, carminative and mild analgesic (Davidson and Connor 2000). The use of valerian to alleviate insomnia and nervous disorders has been reported (Kiesewetter and Muller 1958; Last 1969; Houghton 1999; LaFrance, Lauterbach et al. 2000; Krystal and Ressler 2001). It is often combined with hops (humulus lupus), lemon balm (Melissa officinalis), chamomile (Chamaemelumnobile), Kava kava (Piper methysticum) or St. John's wort (Hypericumperforatum)(Davidson and Connor 2000). Table 1 summarizes the various pathological conditions treated with valerian.

Table 1 Summary of pathophysiological conditions treated with *Valeriana jatamansi.*

Sedative effects	*Antispasmodic*	*Gastrointestinal*
Emotional Stress	Muscular pain	Digestive disturbances
Insomnia	Menstrual cramps	Stomach cramps
Nervousness	Bronchial spasms	Diarrhea
Anxiety	Coughs	Bloating
Nervous heart conditions		Colic
Children's anorexia		
Restlessness		
Trembling		
Tension headaches		
Graves disease		
Hypochondria		
Excitability		

2.9. Pharmacognosy and chemistry:

Valerian belongs to the family Valerianaceae and genus *Valeriana. Valeriana officinalis* is the species commonly used in the commercial preparations. Other species such as *V. wallichi* (Indian valerian), *V. edulis* (Mexican valerian) and *Nardostachyschinensis* (Chinese) are also used medicinally (Schultz and Eckstein 1962; Martinez 1969; Holzl and Jurcic 1975; Bagchi, Oshima et al. 1988; Herrera-Arellano, Luna-Villegas et al. 2001). Extracts from the roots and rhizomes are used in the preparation of phytomedicines. The presence of valerenic acid and its derivatives (acetoxyvalerenic acid and hydroxyvalerenic acid) are characteristic to *V. officinalis* and its subspecies (Bos, Woerdenbag et al. 1997; Letchamo, Ward et al. 2004). Other constituents in valerian include epoxy iridoid esters (valepotriates), their decomposition products such as baldrinal and homobaldrinal, amino acids (arginine, GABA, glutamine, tyrosine), essential oils (borneol, pinene, camphene) and alkaloids (Hansel and Schulz 1981). Recent chemical investigations have identified the presence of lignans and a bi-cyclic sesquiterpene acid (epoxy valerenic acid) (Dharmaratne, Nanayakkara et al. 2002; Schumacher, Scholle et al. 2002).

It was established that the therapeutic action of valerian is due to the presence of valepotriates and ethereal oil sesquiterpenoids, including valerenic acid, valerenolic acid, acetyl valerenic acid, valeranol, etc. (O.A. Konovalova,1991). This knowledge opened the way to development of more reliable methods for standardization of the valerian preparations.

At present, the quality of raw material and final preparations is assessed by a number of methods based on determining both the total content of extractable substances, certain groups of compounds, and separate components of the total

Extract (Moscow 1987). However, the absence of standard samples of the active components still hinders characterization of the raw materials and ready-to-use preparations on a sufficiently precise scale. A pronounced biological activity is inherent in valepotriates. Although several methods were suggested for the quantitative determination of this component in valerian, the highly labile character of valepotriates (accountingfor their missing from galenics) makes these methods inexpedient in the characterization of raw materials and preparations. Sesquiterpene derivatives contained in the ethereal oilare more stable than valepotriates. The most stable of these derivatives is valerenic acid (H. Hendriks

et al. 1981), which was originally isolated in 1957 (H. Hendriks et al. 1981). Subsequent investigation allowed the chemical structure and biological activity of this compound to be determined (*J. Am. et al.1960*). It was found that valerenic acid produces a pronounced spasmolytic action and nonspecific inhibition of the central nervous system, thuscontributing to the total effect of valerian preparations [H.wagner et al. 1979]. It was reported that the spasmolytic activity of valerenic acid is three times that of papaverine (O.A.Konovalova et al. 1991). The high content of valerenic acid is an important sign of the most therapeutically valuable kinds of raw material and is typical of the European populations of *Valeriana officinalis.* Presently, it is recommended to use the valerenic acid content for evaluating the quality of both raw materials and preparations of common valerian (B. Hazelnoff et al. 1979).

2.10. Random Amplification of Polymorphic DNA (RAPD):

RAPD marker is stripped-down version of PCR but uses a single sequence in the design of the primer (i.e. two primers are still needed for PCR: the same primer is used at either end).

The primer may be designed specifically, but could be chosen randomly and is used to amplify a series of samples which will include both the material of interest as well as other control samples with which the experimental material needs to be compared. Choice of primer length will be critical to the determination of band complexity in the resulting amplification pattern. Eventually a particular probe will be found that is able to distinguish between the sample of interest (e.g. a pit-bull terrier) and those that are different (e.g. a Staffordshire bull terrier).

While the RAPD method is empirical, its simplicity of use and the eventual identification of some stretch of DNA, albeit unknown, to facilitate discrimination, makes it a popular means of identifying breeds.

2.11. Simple sequence repeats (SSR):

Simple sequence repeat (SSR) markers are repeats of short nucleotide sequences, usually equal to or less than six bases in length, that vary in number (Rafalski et al., 1996). SSR markers have become quite useful in various aspects of molecular genetic studies in the past decade, including assessment ofgenetic diversity (Amsellem et al., 2001; Ashley et al.,

2003),fingerprinting (Rongwen et al., 1995), ecological-genetic studies (Li et al., 2000), marker-assisted selection (Fazio et al., 2003), gene flow characterization (Aldrich and Hamrick, 1998; Chase et al., 1996), and genetic linkage mapping (Akkaya et al., 1995; Broun and Tanksley, 1996). They are desirable because they are often co-dominant, highly reproducible, frequent in most eukaryotes, and reveal high allelic diversity (Mohan et al., 1997). SSR markers are amplified using the PCR, thus allowing for the rapid generation of data from a relatively small amount of plant tissue. However, SSR markers are also expensive to generate and can be very laborious in certain species. Because of these expense and labor issues, many researchers have attempted to use SSR primers developed from one species for studies on related species and genera. Several studies have indicated successful inter-species and inter-genera results within Rosaceae (Ashley et al., 2003; Cipriani et al., 1999; Decroocq et al., 2003; Dirlewanger et al., 2002; Graham et al., 2002; Lewers et al., 2005) and other plant families (Bowers et al., 1996; Cordeiro et al., 2001; Decroocq et al., 2003; Echt et al., 1999; Guilford et al., 1997; Huang et al., 1998; Kijas et al., 1997; Selvi et al., 2003; Sharon et al., 1997).

Table 2 Characterizations of DNA markers

Marker	RFLP	RAPD	AFLP	SSR
Based on	Hybridization	PCR	PCR	PCR
Degree of polymorphism	High	High	high	Very high
Cost	High	Low	high	High
Reproducibility	High	Low	high	high
Obtaining data	Easy	Easy	complex	Very easy

CHAPTER - 3

3.0Materials and methods

3.1Materials:

3.1.1 Plant Sample: The freshly harvested healthy plant sample of Valeriana jatamansi was obtained from HFRI, Distt. Shimla (H.P.).

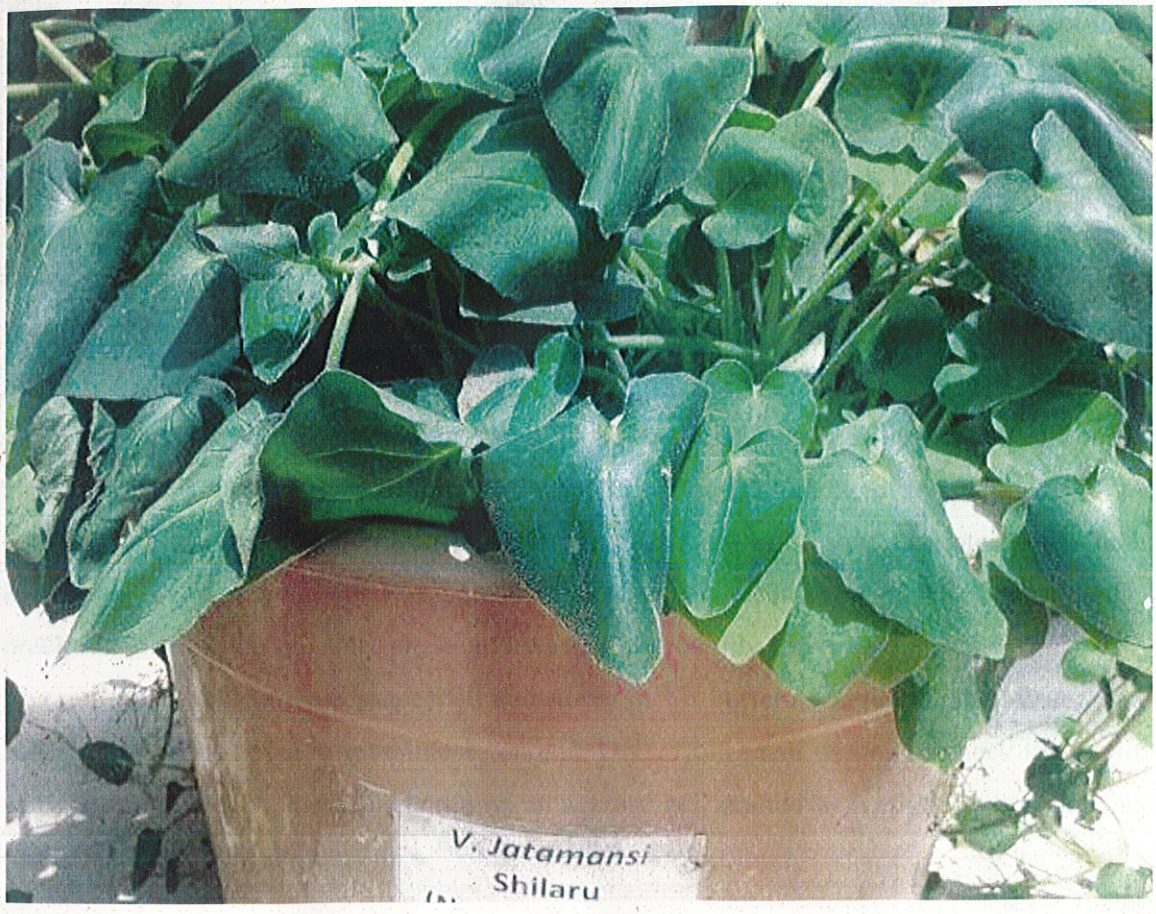

Fig.1 Plant sample of *Valeriana jatamansi.*

3.1.2 Chemicals:

The chemicals used in the present study were obtained from Merck Limited and Qualigens fine chemicals Limited. The various media used in the study namely Agerose gel, Potato Dextrose Agar, Nutrient Broth, Potato Dextrose Broth and Luria Broth were manufactured by HiMedia Laboratories Pvt. Ltd. (Appendix-A).

3.2 Methods:

3.2.1 Media preparation:

(Appendix-A) were used as solid or liquid media for growth of endophytes which were prepared by mixing a desired concentration of media in distilled water and then followed by autoclaving at 121oC and 15 lb/in2 pressure for 15-20 minutes.

3.2.2 Plant (*Valeriana jatamansi*) Genomic DNA Extraction using CTAB:

The search for a more efficient means of extracting DNA of both higher quality and yield has lead to the development of a variety of protocols, however the fundamentals of DNA extraction remains the same. DNA must be purified from cellular material in a manner that prevents degradation. Because of this, even crude extraction procedures can still be adopted to prepare a sufficient amount of DNA to allow for multiple end uses. DNA extraction from plant tissue can vary depending on the material used. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated. Once the tissue has been sufficiently ground, it can then be resuspended in a suitable buffer, such as CTAB. In order to purify DNA, insoluble particulates are removed through centrifugation while soluble proteins and other material are separated through mixing with chloroform and centrifugation. DNA must then be precipitated from the aqueous phase and washed thoroughly to remove contaminating salts. The purified DNA is then resuspended and stored in TE buffer or sterile distilled water. This method has been shown to give intact genomic DNA from plant tissue. To check the quality of the extracted DNA, a sample is run on an agarose gel, stained with ethidium bromide, and visualized under UV light.

3.2.3 Plant genomic DNA Isolation Method:

- Grind 100 mg of plant tissue to a fine paste in approximately 700µl of CTAB buffer.
- Transfer CTAB/plant extract mixture to a microfuge tube.
- Incubate the CTAB/plant extract mixture for about 1 hour at 60° C in a recirculating water bath.
- After incubation, spin the CTAB/plant extract mixture at 10000 rpm for 10 min to spin down cell debris. Transfer the supernatant to clean microfuge tubes.

- To each tube add 700µl of Chloroform: Iso-Amyl Alcohol (24:1) and mix the solution by inversion. After mixing, spin the tubes at 10,000 rpm for 10 min.
- Transfer the upper aqueous phase only (contains the DNA) to a clean microfuge tube.
- To each tube add 500µl of chilled isopropanol.
- Invert the tubes slowly several times to precipitate the DNA. Generally the DNA can be seen to precipitate out of solution. Alternatively the tubes can be placed for 1 hr at -20°C after the addition of ethanol to precipitate the DNA.
- Following precipitation, the DNA can be pipetted off by slowly rotating/spinning a tip in the cold solution. The precipitated DNA sticks to the pipette and is visible as a clear thick precipitate. Centrifuse it 10,000 rpm for 10 min.nTo wash the DNA, transfer the precipitate into a microfuge tube containing 200µl of ice cold 70 % ethanol and slowly invert the tube. Repeat. ((Alternatively the precipitate can be isolated by spinning the tube at 10000 rpm for a minute to form a pellet. Remove the supernatant and wash the DNA pellet by adding two changes of ice cold 70 % ethanol)).
- After the wash, spin the DNA into a pellet by again centrifuging at 10,000 rpm for 1 min.
- Remove all the supernatant and allow the DNA pellet to dry (approximately 15 min).
- Do not allow the DNA to over dry or it will be hard to re-dissolve.
- Resuspend the DNA in sterile DNase free water (approximately 50-100µl H2O; the amount of water needed to dissolve the DNA can vary, depending on how much is isolated). RNase A (10µg/ml) can be added to the water prior to dissolving the DNA to remove any RNA in the preparation (10µl RNase A in 10ml H2O).
- After resuspension, the DNA is incubated at 65°C for 20 min to destroy any DNase that may be present and store at 4°C.
- Agarose gel electrophoresis of the DNA will show the integrity of the DNA, while spectrophotometry will give an indication of the concentration and cleanliness.

3.2.4 Treatment of all the samples by RNase:

All the DNA sample were treated by RNase to removing the RNA contamination. After that the all the sample were run in Gel Electrophoresis and quantified by Nano Drop (Thermo Scientific).

Table 3.1 RAPD primers names and sequences:

Primer	Nucleotide Sequence	Amplification
OPD-14	5'-CTTCCCCAAG-3'	NO
OPD-20	5'-ACCCGGTCAC-3'	YES
OPJ-01	5'-CCCGGCATAA-3'	YES
OPJ-11	5'-ACTCCTGCGA-3'	NO
OPC-06	5'-GAACGGACTC-3'	NO
OPC-15	5'-GACGGATCAG-3'	YES
OPA-04	5'-AATCGGGCTG-3'	YES
OPA-05	5'-AGGGGTCTTG-3'	NO
OPD-12	5'-CACCGTATCC-3'	NO
OPA-14	5'-TCTGTGCTGG-3'	YES

Table 3.2 SSR Primers names and sequences:

Primer	Repeat Unit		Nucleotide Sequence	Amplification
PKSTS				
M1	(A)14		5'-CTTTCAAGATTTATCGACCG-3' 5'-ACGACAAAAAGAACTCCTGA3'	NO
M2	(A)12		5'-CTACCTGCTTCTACGGAAGA-3' 5'-GCTATGCTATTGCCCTACTG-3'	Yes
M3	(A)14		5'-CTTGGAGATCCGACTACAAG-3' 5'-TTTAGGTGGTTCTCGAAAAA-3'	Yes
M4	(A)12		5'-AGAGAATAGGCCTGTCACCT-3' 5'-TAGAACCACGATGATTCACA-3'	Yes
M5	(A)14		5'-ACAAAGACAACAGACGCTCT-3' 5'-GATTCGCAAGAATTGAGAAG-3'	Yes
M7	(A)12		5'-TTAACTTTCAATACCCCCAA-3' 5'-AGAAGCTGATACAGCGAAAG-3'	Yes
M8	(T)12		5'-TTGATGCACACAAGAGTGAT-3' 5'-CTATTCGGTTGAGCATAAGC-3'	Yes
D2			5'-AATGTAGCTTGCTTTGGTGT-3' 5'-AAGAGAAGTTTTGCGTCAAC-3'	Yes
D5	(CT)12		5'-ACAGGTTCTCTCACGAACAC-3' 5'-CAGCAATGAATCTCAAATGA-3'	Yes
T2	(TTC)11		5'-GGTAATTTCCCCTTCTGTTT-3' 5'-TATCCAAATCGAAAGGAAGA-3'	Yes
T4	(CTT)5		5'-CCATCTTGAGAAGTGATGGT-3' 5'-GCATTCAGGTGAAGAAGAAG-3'	Yes
T5	(TGC)5		5'-GATTCTGACGTAATTGGAGC-3' 5'-CTCGAAGAATCCAAACTCAC-3'	Yes
T7	(TCG)5		5'-TTCAAATCAAATTCAGGGAC-3' 5'-AAATTCTCTCTCCCAACCTC-3'	Yes
T9	(AAG)5		5'-CAGTAGAGTCGTCAAGTGCA-3 5'-CCCTTTTCCAATAATCTCCT-3'	Yes
T11	(GCA)6		5'-CTTTGGATAAGTTCCTGCTG-3' 5'-TCCGAATCAATATTGGAGAC-3'	Yes
P10	(GTGTT)3		5'-AAAAAGGTAAGAAATGCGG-3' 5'-GATGGGACAAGAGATCTCAA-3'	Yes
P15	(CAAGA)3		5'-AAGAGTGCTAGGACACCTGA-3' 5'-GACGAAGTTTTCACTGCTTC-3'	Yes
H1	(TTTTTC)3		5'-AATGACCATTAAATTGTGCG-3' 5'-TCTTTCTATTGTTTTTGCCC-3'	Yes
H2	(GAAA		5'-TCTTTCTATTGTTTTTGCCC-3'	Yes

	AA)3		5'-AATGACCATTAAATTGTGCG-3'	
H3	(GGTG AA)3		5'-CAAAGTCAACAAGAAGGGAG-3' 5'-CTTGCTGATCTTCTTAGCGT-3'	Yes
H4	(GAAA GA)3		5'-CGGGACACCTTAATCATTTA-3' 5'-CCTAGACACTCTAGGAGGGG-3'	Yes
H5	(CGGG AA)8		5'-AAGGTCTTCTTTACGCTCCT-3' 5'-CTATCTCTTTCCCGTACCCT-3'	Yes
H6	(GTAG CA)3		5'-GAAGTTGAAGCTCTGGCTTA-3' 5'-TCCTTGACCTTGCTTCTAAG-3'	Yes
H7	(AGAT CG)3		5'-GCTGTCAAATGTTGACAGTG-3' 5'-TAGCAATTATGTTGCTGACG-3'	Yes
H8	(CTCC AG)4		5'-GTTTTATTCGTGTTTACCGG-3' 5'-TAACATACCGACCAGGATTC-3'	Yes
H9	(AGAT GA)3		5'-CCGTCTGTAGACCATGCTAT-3' 5'-CGTATCATCCTCTTCACCAT-3'	Yes
H10	(TCTG GC)3		5'-CTGACCAGCTAGCTTCTTGT-3' 5'-GGAAGAGGAGAAGGTCAAGT-3'	Yes
H11	(TCGA GC)3		5'-TCGTCATACCTAGAGCCTTC-3' 5'-TCTCACACAACACACACACA-3'	Yes
H12	(AAAT CC)4		5'-TTCATTTTGGCACATGAGTA-3' 5'-TGCAAACACTCAAACTTTAACT-3'	Yes
H13	(GAAC CT)5		5'-TTAGAGGAGGAAGCTAGGGT-3' 5'-TAAGTCAGCTTCTCAGGCTC-3'	Yes
H14	(AGAT GA)3		5'-TTTGTAGCTTGCAATGAATG-3' 5'-GCAGAAATCGTATCGTCTTC-3'	Yes
H15	(GGTG GA)3		5'-GAATTCGCTTGACGAATTAC-3' 5'-TTTGATTCCTTAAACAGGGA-3'	Yes
		PKR25		
M1	(T)14	photosystem ii 47 kda protein	5'-TACTGGTCTTAGAACCCGAA-3' 5'-ATGATTTCCAACGAAATCAG-3'	Yes
M2	(A)12	ribosomal protein s18	5'-CATAGATTCGATCGTGGTTT-3' 5'-TCTTCTTCTTCGGACACATT-3'	Yes
M3	(A)14	ribosomal protein s18	5'-TAATCTAAAGGGAGTTCCCC-3' 5'-AAACAATTTGAAAGAACCGA-3'	Yes
M4	(T)12	basic helix-loop-helix domain-containing protein	5'-CTGGCCTAACTAAACATTGG-3' 5'-AAATCACACATTAACTCGGC-3'	Yes
M5	(T)12	hydroxymethylglutaryl- lyase	5'-GTTGCGATATGGTGGTAGTT-3' 5'-AAGTTTAGCAAAAGCATTGC-3'	Yes
M6	(A)18	glycoprotein precursor	5'-CCACAATCAAGTCATAACGA-3' 5'-TTAGCTGCGTTATTTGCATA-3'	Yes
M7	(T)20		5'-GGGATTGCAAAACTGTTTA-3' 5'-ATATGACGAACAATCCATTAT-3'	Yes
M8	(T)12	unnamed protein product [Tetraodonnigroviridis]	5'-AAAGAAGACAATCCCACTCA-3' 5'-AAAAAGCCTAAACCTTTGCT-3'	Yes
M9	(A)18	c6 zinc finger domain protein	5'-AAGACAACCGCAAATAAAGA-3' 5'-TTTGTTCGTTCCTTCTGTTT-3'	NO
M10	(A)12		5'-GTTGCTTACATCCCAATCAT-3' 5'-CCACATGTTTATCCGGTAGT-3'	Yes
M11	(A)14	photosystem ii 47 kda protein	5'-AGGTGGAGTCGAAATGACTA-3' 5'-GGATTCTCGGATTTGTAGAA-3'	Yes
M12	(C)12		5'-AAAACACAGTAAAACTCGGC-3' 5'-ACCAGTGTTGGATAGGTTTG-3'	Yes
M13	(T)14	-domain protein	5'-CCCCAAGCAAAGTACAAGTA-3' 5'-TATGAATCTGAACCACTGGG-3'	Yes
M14	(T)12	nadh dehydrogenase subunit 2	5'-AAAAGGCAAAAATCCTTTCT-3' 5'-AAAGAAAGGCAAAAATCCTT-3'	Yes
M15	(A)12	hypothetical chloroplast rf1	5'-ATGACCTGATCAATTCGAAC-3' 5'-CCTCCCCTACAGTATCATCA-3'	Yes
D1	(TA)8		5'-TAGGCGATTTATGATTTCGT-3' 5'-CGTGAACATATTACGTGACG-3'	NO
D2	(AT)8		5'-GAATGCTTGCACTTAGGATC-3'	Yes

			5'-CATGACGTGAACATATTTCG-3'	
D3	(TA)12		5'-TTCGCAATCACAATAAACTG-3' 5'-CCTATAAAAAGGGGAGGAAA-3'	Yes
D4	(AT)7		5'-TTTTTCAATGAAATATCGTGAA-3' 5'-GGATTTGAACCGATGACTTA-3'	Yes
D5	(TA)7	exopolysaccharide synthesis protein	5'-CAATAGCACTGGCACTCATA-3' 5'-GCATTTTCTTTTGCAATCTC-3'	Yes
D6	(AT)10		5'-TGGTATTCATAACACTGGCA-3' 5'-CTTCACAAATCGTGTCAATG-3'	Yes
D7	(AT)7		5'-GGCGTTACTCTACCACTGAG-3' 5'-TTTTTCAATGAAATATCGTGAA-3'	Yes
D8	(TA)9		5'-TCAATATTGCACGTACACGT-3' 5'-CGTGAATCAGATTTGTGTTG-3'	Yes
D9	(AT)10		5'-TGTACAAATCCAATGAACCA-3' 5'-CTACTTCCCAAATATTCCCC-3'	Yes
D10	(AC)7		5'-CAAATTTTGACTACCCAAGC-3' 5'-TTTTCTTTTCTTGTTTTCGC-3'	Yes
D11	(AT)10	phospholipase d delta isoform	5'-GGTAGGGTACAGGTATGCAA-3' 5'-AAAAATGCGACAGAGAGATG-3'	Yes
D12	(AT)8	transposon en spm sub-class	5'-TAAATCGTCCCCAAAGTAGA-3' 5'-TTCGATTCTATGGAGCATCT-3'	Yes
D13	(AT)8	reverse transcriptase	5'-TTTAGAGGATTTGGTGCAAT-3' 5'-TTGGGTAAGAGTTGCAAGTT-3'	Yes
D14	(TA)10		5'-AGAAGGAGTGCAAGCTTATG-3' 5'-ATAAGAGAAGGAGGTGGAGG-3'	Yes
D15	(GA)8	n-acetylmuramoyl-l-alanine amidase	5'-TATCTCAGTGCCCAACTTTT-3' 5'-GTGACATTTTGGCTTTTCTC-3'	Yes
H4			5'-ATACGTAAAACTCGGCCATA-3' 5'-AGACTTTCCAAACACACCAC-3'	Yes
H7			5'-GTGTCGACGTCTCCTACAAT-3' 5'-TTACTTGTCGACCGTGTACA-3'	Yes
H8			5'-AGAGGCCGTAAGAGAGTTCT-3' 5'-CTCCAATTCAACCATTGATT-3'	Yes
H9			5'-CTCCAATTCAACCATTGATT-3' 5'-AGAGGCGGTAAGAGAGTTCT-3'	Yes
T11			5'-CCCCAATAAATAAACAGTCG-3' 5'-AATCTCTAGAAGCTCCCTTGA-3'	Yes
TT2			5'-TTTCTGGTGGGTATCCTTAA-3' 5'-TCGACTATATCCGCATTTTT-3'	Yes
TT4			5'-GAATGAAGCCAAGTCTGAAG-3' 5'-TGGCTTCTTTAATTTCTTCG-3'	Yes
TT5			5'-GAAAGCGTGTCATCT , TCTC-3' 5'-AAGTGGAAAAATGCCATTTA-3'	Yes
TT11			5'-AATCTTCGGACCCTTTAGTC-3' 5'-CCAAGAAGAAACAGGAAATG-3'	Yes
T9			5'-AAGAGGAAGGTGAAAAGACC-3' 5'-GGCACATTCTTGAGATCTGT-3'	Yes
T13			5'-ATGCTGTTGGATTTTTACGT-3' 5'-ATCTTGAAAGCCTTCAATCA-3'	Yes
Total= 74				

Table 3.3 PCR Cycle

SERIAL NO.	STAGE	STEP	TEMPERATURE	TIME
1.	STAGE 1 *1 CYCLE		94° C	4mins
2.	STAGE 2 *30 CYCLES	**STEP 1**	94° C	30 sec
		STEP 2	52° C	1 min
		STEP 3	72° C	1min
3.	STAGE 3 *1 CYCLE		72° C	7mins
4.	STAGE 4		4° C	∞

Table 3.4 -PCR Master Mix

MATERIALS	1X
Template DNA (50 ng)	2 µl
Primers (10µm)	0.5µl(each)
10X PCR Buffer(with Mgcl2)	1.25 µl
10mM DNTPs	0.25 µl
Autoclaved Water	8.875µl
Taq DNA Polymerase	0.125 µl
TOTAL	12.5µl

To perform several parallel reactions, master mix was prepared containing autoclaved distilled water, buffer, dNTPs, primers and Taq DNA polymerase in a single tube, and aliquoted into individual tubes. Template DNA solutions was added. The solutions were gently vortexed and centrifuged after thawing before addition. Master-Mix was added, in a thin-walled PCR tube, on ice. Samples were vortexed again in order to collect all drops from walls of tube. Samples were the placed in a thermocycler PCR was started. Products were resolved using 1X TAE, 1.8% agarose gel.

3.2.5 Agarose gel electrophoresis of PCR Products

The amplified PCR product 8µl mixed with 2µl of gel loading dye was loaded on 1.2 % agarose gel and electrophoresed in 1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel vas visualized and photographed in GelDoc (BioRad).

3.2.6 Polymorphism detected by PCR

We were used PCR to amplify a fragment of the DNA sampleand the resulting product to detect polymorphisms. This is a gold-standard for determining genotype particularly in genes where multiple polymorphisms occur, it is less time consuming and inexpensive method. We will use a series of other PCR-based methods to detect specific polymorphisms without sequencing. These illustrate different methods used commercially and in research. PCR provides the ultimate in sensitivity — single DNA molecules can be detected and analyzed for sequence content (Li et al., 1988; Arnheim et al., 1990).

CHAPTER - 4

Results and Discussion:

4.1 Isolation of DNA sample by using CTAB method

DNA of 12 different strains of *Valeriana jatamansi* was extracted by using the CTAB method.

1-Yungpa

2-Nauradhar

3-Rumsu

4-Nichar

5-KUFRI

6-khirki

7-Haripurdhar

8-Shilaru

9-Torani Nala

10-Baktot

11-Chur Dhar

12-Jalori Jot

Table 4.1 Existing Strains of *Valeriana jatamansi* available with HFRI, Shimla at different FRSs (H.P.):-

S. No.	SOURCE CODE*	VALEPOTRIATES %	VELERENIC ACID %
1	VJ-1	1.8	ABSENT
2	VJ-2	2.1	ABSENT
3	VJ-3	2.4	ABSENT
4	VJ-1-6	1.6	ABSENT
5	VJ-1-8	2.0	ABSENT
6	VJ-2-7	2.6	ABSENT
7	VJ-3-6	2.2	ABSENT
8	VJ-3-7	2.0	ABSENT
9	VJ-1-15	2.0	IN TRACES
10	VJ-2-15	2.4	IN TRACES
11	VJ-1-17	3.0	IN TRACES

4.2 Molecular Characterization of *Valeriana jatamansi* strains.

4.2.1 DNA Isolation of *12* strains of *Valeriana jatamansi*

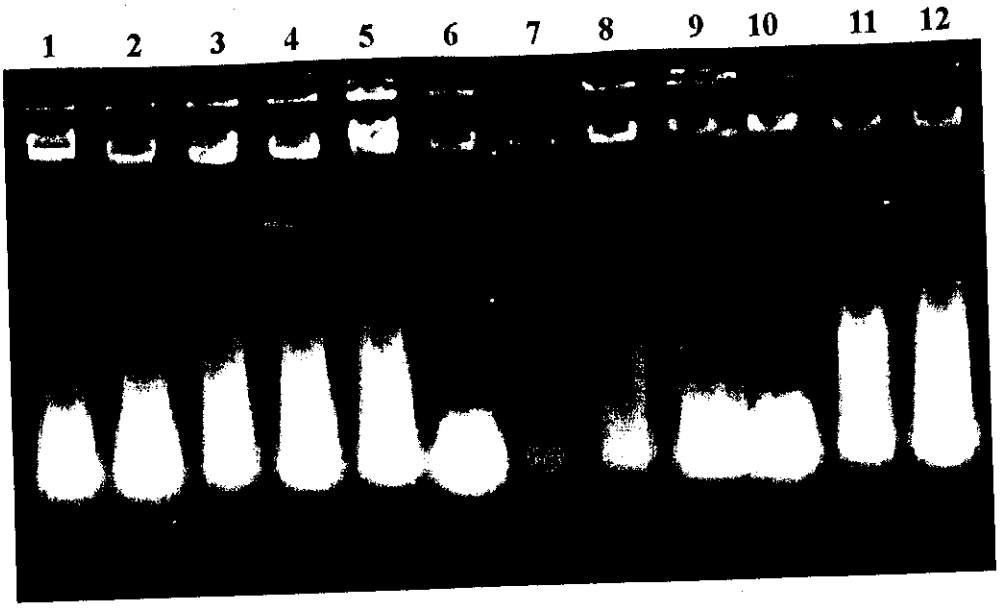

Fig. 2 DNA isolation from 12 strains of *Valeriana jatamansi.*

The isolated DNA 3 µl mixed with 1µl of gel loading dye was loaded on 0.8% agarose gel and electrophoresed in 1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (Bio-Rad).

4.2.2 PCR amplifications of *Valeriana jatamansi* strains by using RAPD primers.

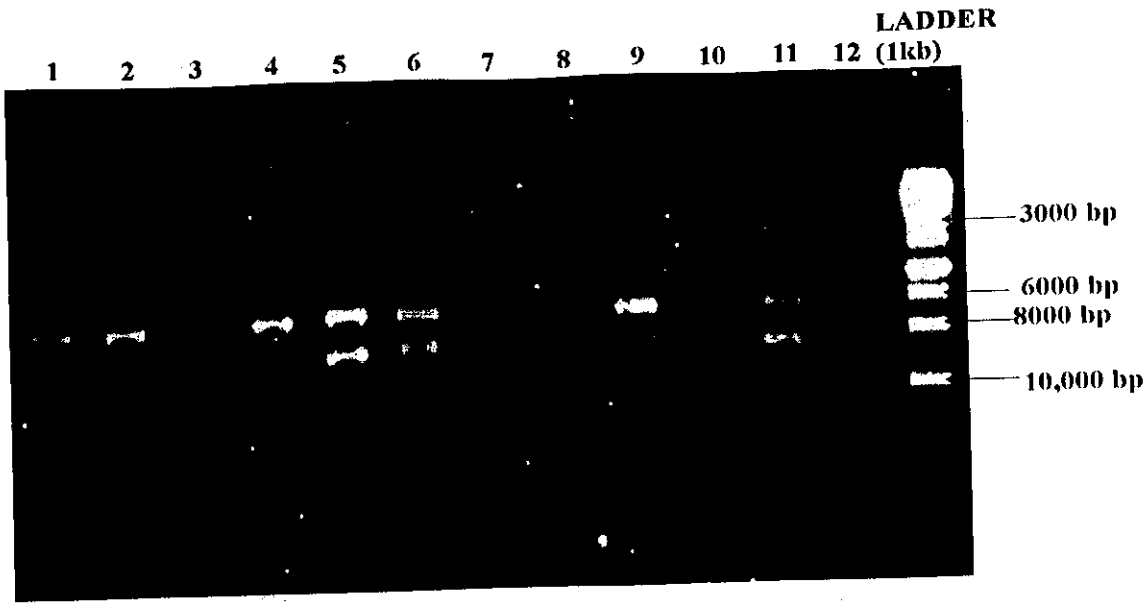

Fig. 3 PCR amplification of 12 strains of *Valeriana jatamansi* by using OPD-20 primer

The PCR product 12.5 µl mixed with 2µl of gel loading dye was loaded on1.2% agarose gel and electrophoresed in 1X TAE buffer at 75 V for 1-2h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml).After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

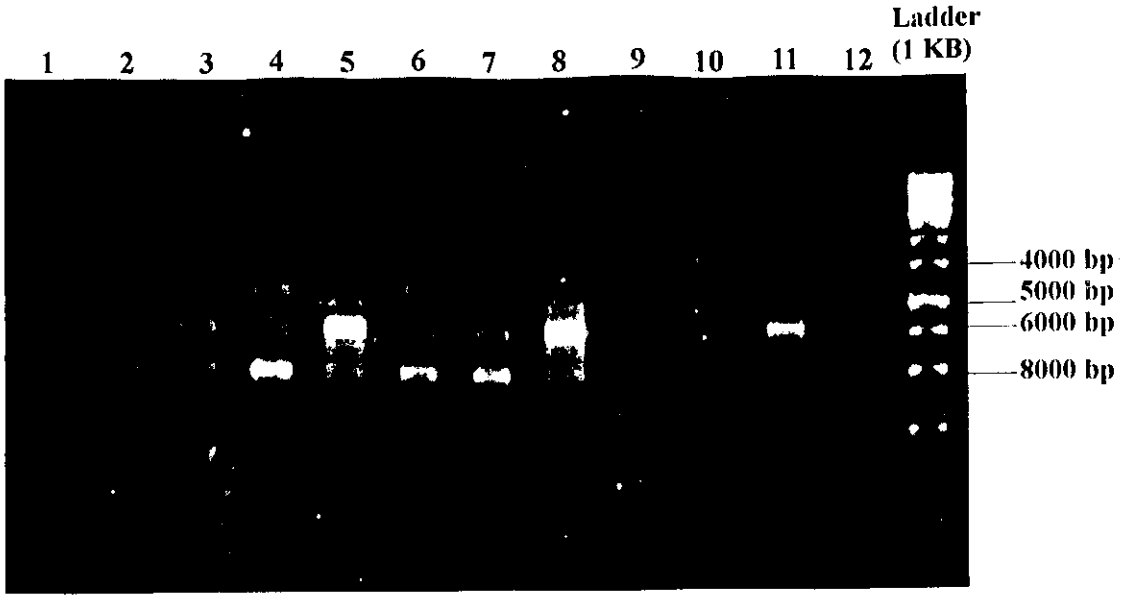

Fig. 4 PCR amplification of 12 strains of *Valeriana jatamansi* by Using OPA-02 primer.

The PCR product 12.5 µl mixed with 2µl of gel loading dye was loaded on 1.2% agarose gel and electrophoresed in 1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

4.2.3 PCR amplifications of *V. Jatamansi* strains by using SSR primers.

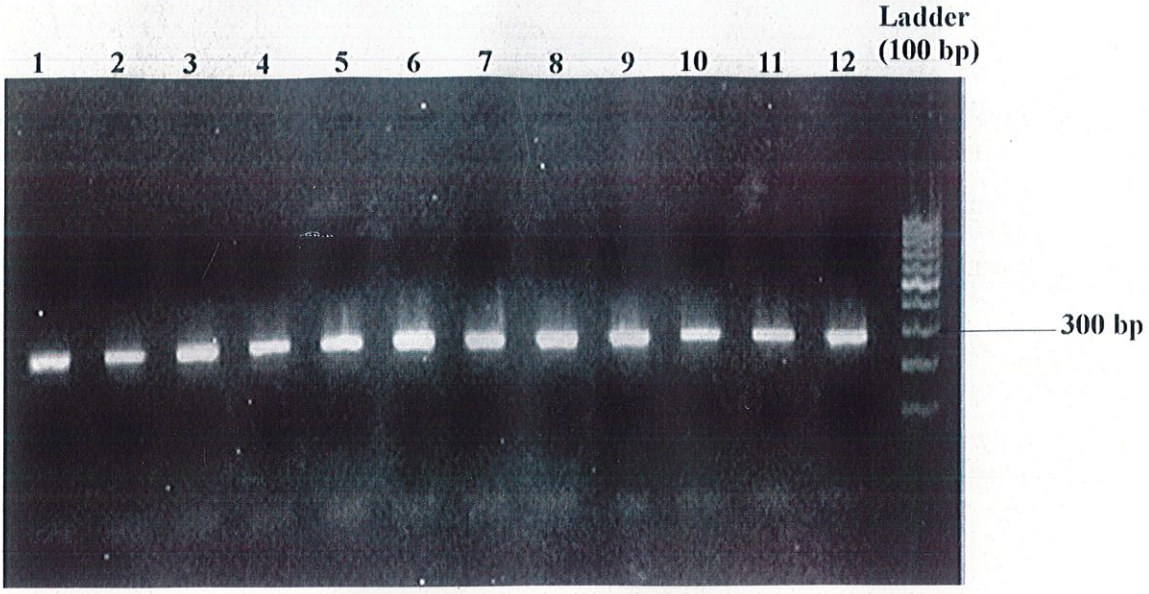

Fig. 5 PCR amplification of 12 strain of *Valeriana jatamansi* by using PKSTS M3 primer.

The PCR products 12.5µl mixed with 2µl of gel loading dye was loaded on 2.5% agarose gel and electrophoresed in1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

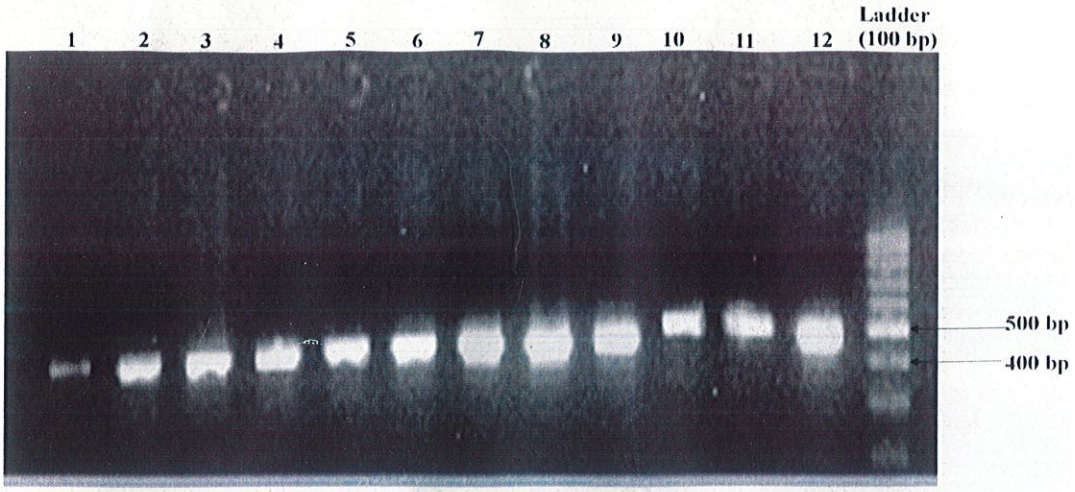

Fig. 6 PCR amplification of 12 strains of *Valeriana jatamansi* by using PKSTS M4 primer.

The PCR products 12.5µl mixed with 2µl of gel loading dye was loaded on 2.5% agarose gel and electrophoresed in1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

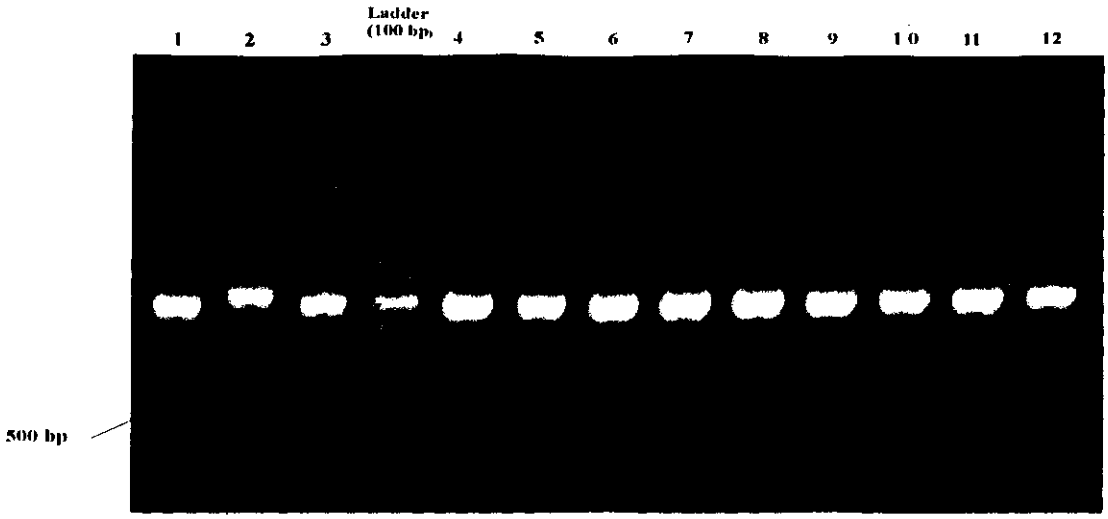

Fig. 7 PCR amplification of 12 strains of *Valeriana jatamansi* by using PKSTS M5 primer.

The PCR products 12.5µl mixed with 2µl of gel loading dye was loaded on 2.5% agarose gel and electrophoresed in1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

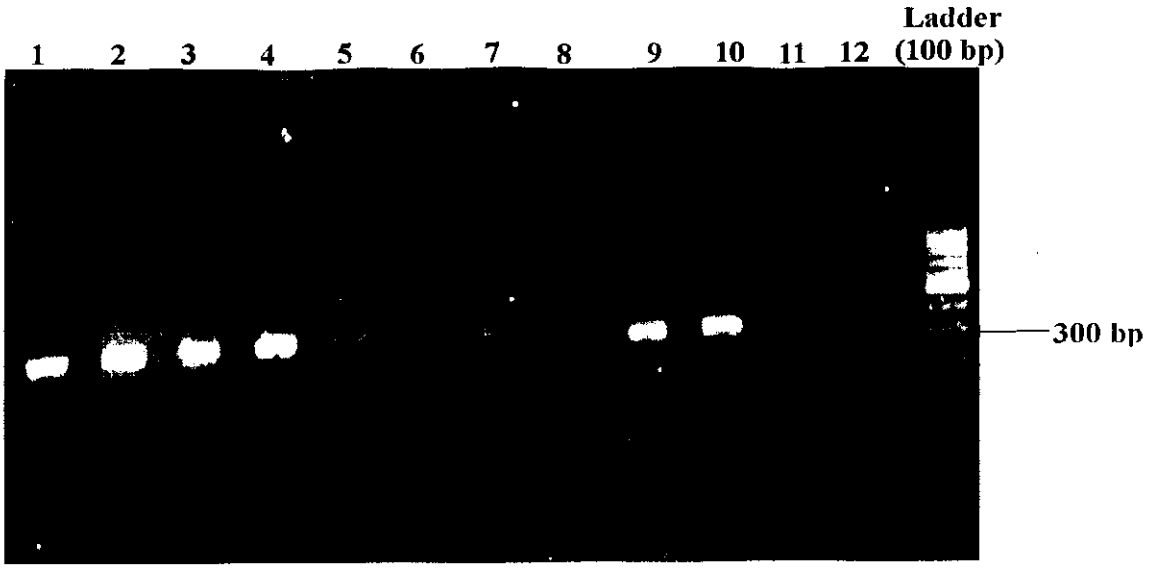

Fig.8 PCR amplification of 12 strain of *Valeriana jatamansi* by using PKR25 M2 primer.

The PCR products 12.5µl mixed with 2µl of gel loading dye was loaded on 2.5% agarose gel and electrophoresed in1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

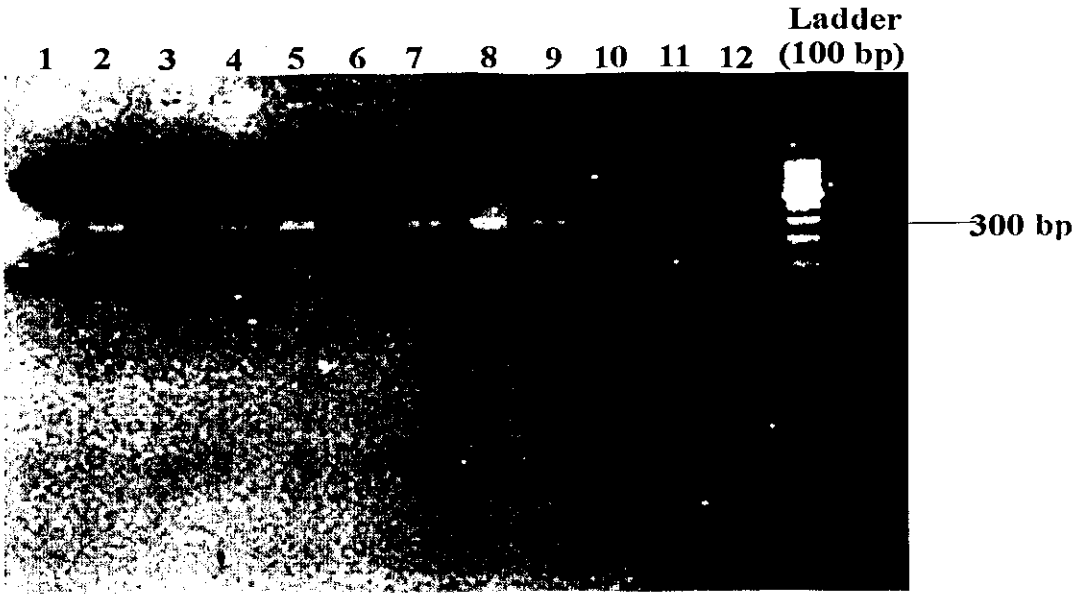

Fig. 9 PCR amplification of 12 strains of *Valeriana jatamansi* by using PKR25 M9 primer.

The PCR products 12.5µl mixed with 2µl of gel loading dye was loaded on 2.5% agarose gel and electrophoresed in1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

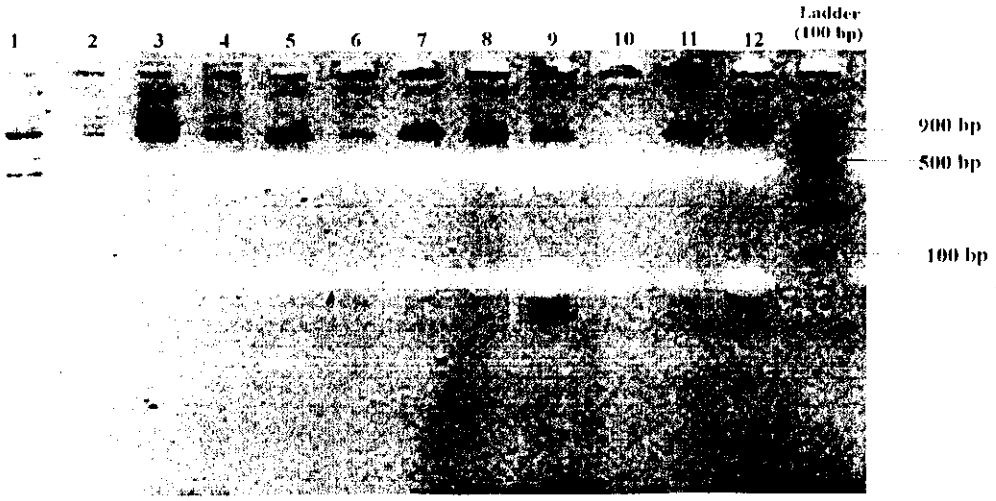

Fig. 10 PCR amplification of 12 strains of *Valeriana jatamansi* by using PKR25 M14 primer.

The PCR products 12.5µl mixed with 2µl of gel loading dye was loaded on 2.5% agarose gel and electrophoresed in1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

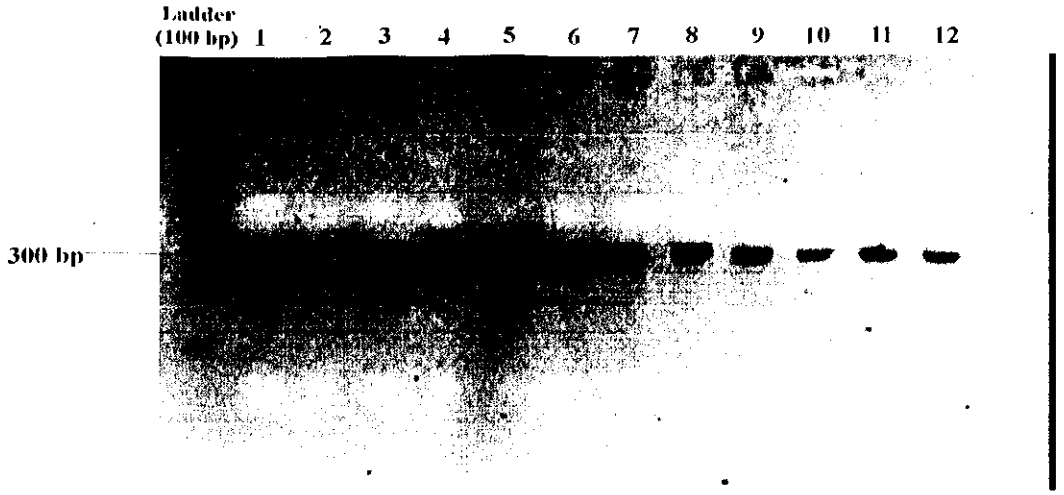

Fig. 11 PCR amplification of 12 strains of *Valeriana jatamansi* by using PKR25 M15 primer.

The PCR products 12.5µl mixed with 2µl of gel loading dye was loaded on 2.5% agarose gel and electrophoresed in1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

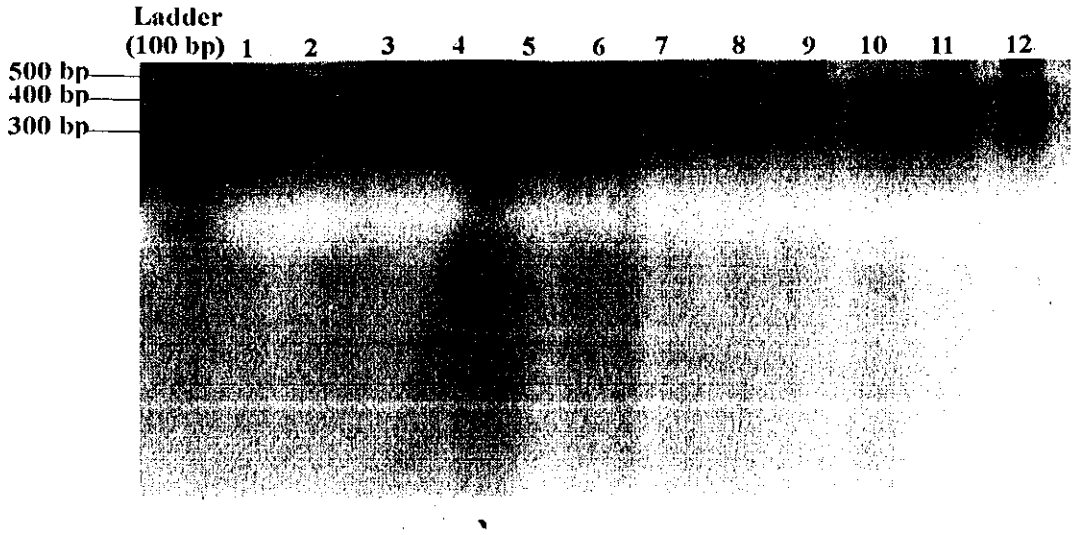

Fig. 12 PCR amplification of 12 strains of Valeriana jatamansi by using PKS25 T9 primer.

The PCR products 12.5µl mixed with 2µl of gel loading dye was loaded on 2.5% agarose gel and electrophoresed in1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

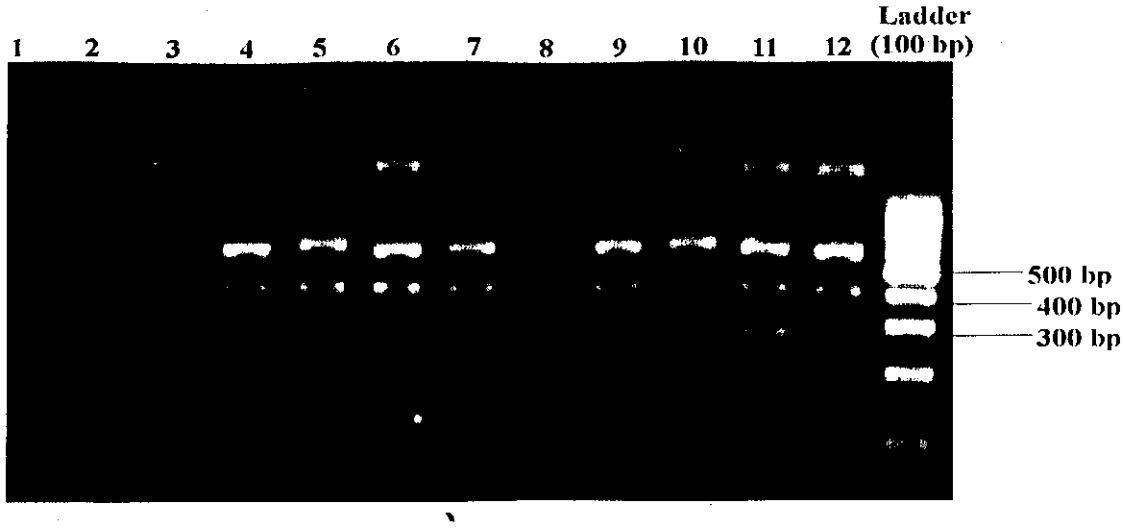

Fig. 13 PCR amplification of 12 strains of Valeriana jatamansi by using PKSTS P10 primer.

The PCR products 12.5µl mixed with 2µl of gel loading dye was loaded on 2.5% agarose gel and electrophoresed in1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

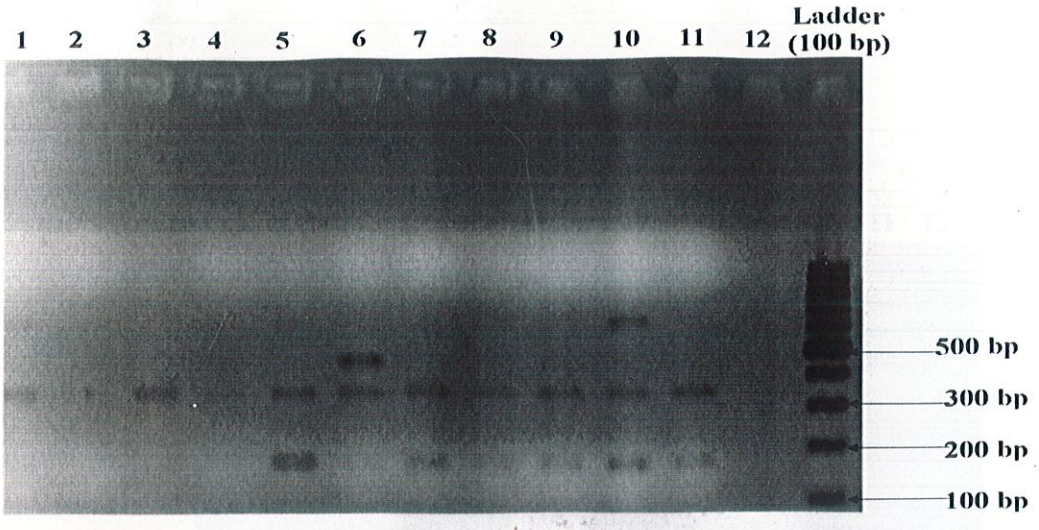

Fig. 14 PCR amplification of 12 strains of Valeriana jatamansi by using PKSTS P15 primer.

The PCR products 12.5µl mixed with 2µl of gel loading dye was loaded on 2.5% agarose gel and electrophoresed in1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

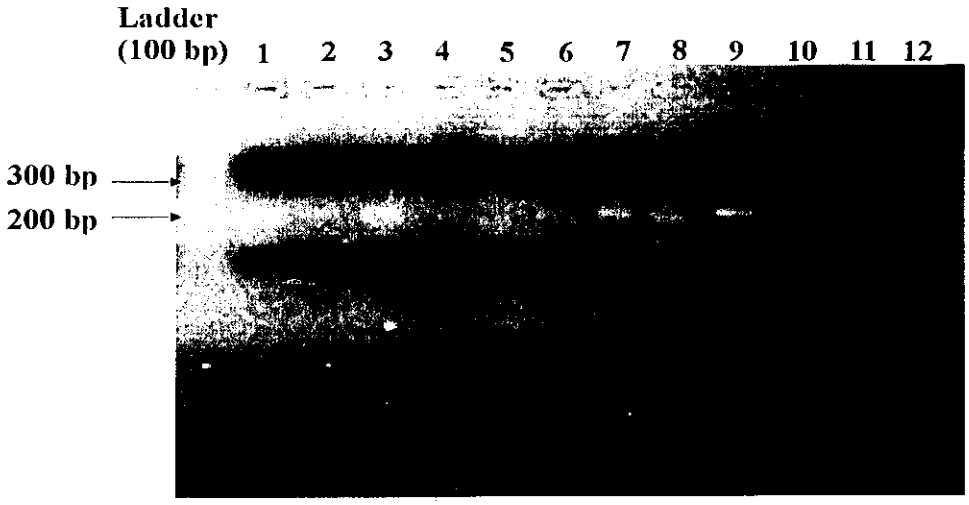

Fig. 15 PCR amplification of 12 strains of Valeriana jatamansi by using PKSTS H5 primer.

The PCR products 12.5µl mixed with 2µl of gel loading dye was loaded on 2.5% agarose gel and electrophoresed in1X TAE buffer at 75 V for 1-2 h. During gel preparation, gel was stained by adding ethidium bromide (0.5µg/ml). After electrophoresis, the gel was visualized and photographed in GelDoc (BioRad).

4.3 DISCUSSION:

4.3.1 Results obtained by RAPD primers:

- **OPD 20:** This primer amplified the band at 8000 bp & 6000 bp in all the genotype except genotype 3, 8 & 10. Genotype 5 & 11 also amplified at 3000 bp.
- **OPA 02:** This primer amplified the band at 8000 bp & 6000 bp in all the genotype except intensity of genotype 1, 2 & 10 are very low. Genotype 3, 4, 6 & 7 are also amplified at 4800 bp while genotype 5 & 8 are amplified at 5000 bp. Genotype 10 is also amplified at 3800 bp.

4.3.2 Results obtained by SSR primers:

- **PKSTS M3:** This primer amplified the band at 300bp in all the genotype.
- **PKSTS M4:** This primer amplified the band at 500bp in all the genotype except genotype 10 and 11. Genotype 10 and 11 are amplified at 520bp.
- **PKSTS M5:** This primer amplified the band at 500bp in all the genotype except genotype 2. Genotype 2 is amplified at 520bp.
- **PKR25 M2:** This primer amplified the band at 300bp in all the genotype except genotype 12 while genotype 5 & 11 are also amplified at 280bp.
- **PKR25 M9:** this primer amplified the band at 300bp in all the genotype except genotype 6 & 12.
- **PKR25 M14:** This primer amplified the band at 900bp in all the genotype except genotype 10. Genotype 1, 2, 3 & 4 are also amplified at some other places.
- **PKR25 M15:** This primer amplified the band at 300bp in all the genotype.
- **PKSTS P10:** This primer amplified the band at 420bp & 600bp in all the genotype except genotype 3 & 8. Genotype 4, 6, 9, 10, 11 & 12 are also amplified at some other places.
- **PKSTS P15:** This primer amplified the band at 350bp in all the genotype except genotype 2 & 12. Genotype 5, 7, 8, 9, 10 & 11 are also amplified at 180bp and genotype 6 also amplified at 500bp.
- **PKSTS H5:** This primer amplified the band at 200bp in all the genotype except genotype 4, 5, 6 & 8. Genotype 4 & 8 are amplified at 300bp. Genotype 8 & 10 are also amplified at 180bp.

CHAPTER - 5

CONCLUSION

Valeriana jatamansi is a well known herb which has abundant medicinal properties. It is found in Himalayan regions at an elevation of 1,000-3,000 meters asl (above sea level) and has traditionally been used to treat used as a sedative, anti-spasmodic, carminative and mild analgesic (Davidson and Connor 2000). The use of valerian to alleviate insomnia and nervous disorders etc. The active constituents are obtained from the dried roots and rhizomes. The roots and rhizomes of this species are also used for the preparation of phytomedicines with mild sedative action.

In the present study we have isolated DNA from the healthy Plant leaves of *Valeriana jatamansi* by using the CTAB method. We have also used DNA-based molecular marker techniques, viz. simple sequence repeats (SSR) and RAPD (random amplified polymorphic DNA) markers were used to study polymorphism in *Valeriana jatamansi*. Twelve strains of *Valeriana jatamansi* were obtained from HFRI Shimla which shows valeopotriates content in all twelve strains is varying from 1.6 % - 3% where as velerenic acid content is absent in all strains except three strains. These valeopotriates and veleranic acid are secondary metabolites which are used in medicine. 79 SSR and 10 RAPD primers were tested on genomic DNA of Valeriana strains, out of which 71 SSR primers and 5 RAPD markers showed amplification. 8 SSR primers and 1 RAPD primers showed polymorphism. Our results showing less polymorphism occurring in strains of *Valeriana jatamansi.*

APPENDIX-A

List of chemicals:

1M Tris:

30.28 g of Tris in 250 ml of dH2O and adjust the final pH to 8.

0.5M EDTA:

46.53 g of EDTA in 250 ml of dH_2O and adjust the final pH to 8.

TE Buffer(1M)

Composition of TE Buffer (100ml)

0.2 M Tris base	50ml
0.1M EDTA	50ml

Phenol – Chloroform – Isoamyl alcohol (25:24:1) – 10ml

Phenol	5ml
Chloroform	4.8ml
Isoamyl alcohol	0.2ml

Extraction Buffer (100ml)

1% CTAB	1gm
50 mMTrisHcl	5ml of 1M
50mM EDTA	10ml of 0.5M
700Mm Nacl	70ml of 1 M
Water	Raise volume to 99ml and autoclave
1% β-Mercaptoethanol (added after autoclaving)	1ml

TAE Buffer

50X Stock solution of TAE was prepared by adding the following:

Composition of TAE Buffer

Tris base	24.2gm
Glacial acetic acid	5.71ml
EDTA(0.5M, pH 8.0)	10ml
Distilled water	Make up the vol. to 100ml

Gel loading dye(6X)

Composition of Gel loading dye

Bromophenol Blue	0.25%(w/v)
Xylene Cyanol	0.25%(w/v)
Glycerol in DW	30%(v/v)

Ethidium bromide (0.5 mg/ml)

0.5 mg of ethidium bromide dissolved in 1 ml of autoclaved ddH_2O.

APPENDIX - B

List of instruments:

Micro litre Centrifuge Z233 MK-2 (Hermle Labor Technik)

Microwave MS-283mc (LG)

Vortex (BangloreGenel)

Serological Water bath NSW-125

Laminar Flow Bench (Complab)

Autoclave (NSW, India)

Thermal cycler (Applied Biosystem)

Micropipette (Eppendorf and Axygen)

Microtips (Axygen)

Glassware (Borosil)

Gel Doc (Biorad)

Centrifuge (Elteck)

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Brief Bio-data of Students

Title of Thesis	: DNA Fingerprinting of *Valeriana jatamansi* by Using RAPD and SSR markers.
Name of Students	: Vikas Singh
Enrollment Number	: 091565
Name of Supervisor	: Dr. R. S. Chauhan
Major Field	: Biotechnology
Minor Fields	: i) Genetic Engineering
	: ii) Plant Biotechnology
Degree Awarded	: B.Tech (Biotechnology)
Year of Award of Degree	: 2014
No. of Pages in Thesis	: 54

Title of Thesis	: DNA Fingerprinting of *Valeriana jatamansi* by Using RAPD and SSR markers.
Name of Student	: Shashank Ranjan Srivastav
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Minor Fields	: i) Genetic Engineering
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Year of Award of Degree	: 2014
No. of Pages in Thesis	: 54