# STUDY OF GENETIC POLYMORPHISM IN *ARTEMISIA ANNUA* USING CAPS AS A GENETIC MARKER


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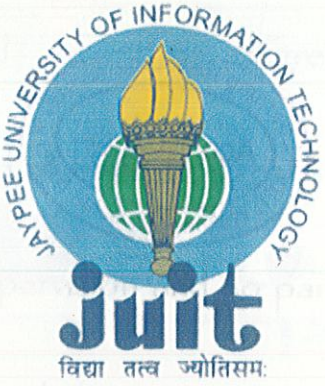
**A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF**

## BACHELOR OF TECHNOLOGY





**JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY**

**WAKNAGHAT, SOLAN 173215, HIMACHAL PRADESH, INDIA**

**MAY 2010**

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# CERTIFICATE

This is to certify that the thesis entitled "**Association study of genetic polymorphism with Artemisinin yield in *Artemisia annua* population**" submitted by **Ms.Nikita Maheshwari and Ms. Meera Batra** to the Jaypee University of Information Technology, Waknaghat in fulfillment of the requirement for the award of the degree of **Bachelor of technology(Science)** is a record of bona fide research work carried out by them under my guidance and supervision and no part of this work has been submitted for any other degree or diploma.

(Dr. P.K. Naik)

# DECLARATION

We hereby declare that the work presented in this thesis has been carried out by us under the supervision of Dr. Pradeep Kumar Naik, Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan-173215, Himachal Pradesh, and has not been submitted for any degree or diploma to any other university. All assistance and help received during the course of the investigation has been duly acknowledged.

Ms.Meera Batra

Ms.Nikita Maheshwari

# Acknowledgement

We would like to express our gratitude to our supervisor, Dr. Pradeep Naik, whose expertise, understanding, and patience, added considerably to our undergraduate experience. We appreciate his vast knowledge and skill in many areas (e.g., vision, aging, ethics, interaction with participants), and his assistance in writing reports, making presentations, etc. always making us do work efficiently. We would like to thank Dr. Harvinder Singh for the assistance they provided at all levels of the research project. Finally, we would like to thank Jitender sir from DRDO for taking time out from his busy schedule to serve as my external reader.

*I am indebted to Dr. Y. Medury (Vice Chancellor, JUIT), Brig. (Retd) Balbir Singh (Registrar, JUIT) and Prof. R. S. Chauhan (Head of the department) for having provided all kinds of facilities to carry out research.* It was through their persistence, understanding and kindness that we are encouraged to apply for graduate training. We doubt that we will ever be able to convey our appreciation fully, but we owe them our eternal gratitude.

*We would also like to thank members of the lab and colleagues from other labs for their constant support. It was a pleasure to work with them. We also thank Somlata mam, Payal mam, Arti mam, Ismail sir, Ravikant sir, Baleshwar sir and Mamta mam for their assistantance.*

We would also like to thank our family for the support they provided us through our entire life and in particular, we must acknowledge our mother, without whose love and encouragement, we would not have finished this thesis.

In conclusion, we recognize that this *work has been supported by funds from Department of Science and Technology, Government of India, in the form of young scientist scheme to Dr. Naik and is duly acknowledged.*

*[Meera Batra and Nikita Maheshwari]*

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# ABSTRACT

Although CAPS markers have been used to study the genetic diversity and association of traits of many plant species like wheat etc with their genetic make up, but no such studies have been carried out on *Artemisia annua*. CAPS in candidate genes involved in the biosynthesis of artemisinin of *Artemisia annua* will be of immense practical importance in genetic improvement, both by association mapping and molecular-marker-assisted selection of high artemisinin content trait in Artemisia. High artemisinin content genotypes can be recommended for direct large scale plantation or even be included in Artemisia genetic improvement programmes. CAPS can also be used in the development of genetic map of Artemisia.

CAPS markers basically involve genomic DNA extraction, quantification of DNA extracted, PCR amplification and finally restriction digestion of amplified DNA by restriction endonucleases, which was carried out to get the results. Though no polymorphism was observed in this study the explaination may be attributed to highly conserved transcribed region and low number of restriction enzymes used for CAPS analysis.

Finally extraction and estimation of Artemisinin content from leaves and inflorescence of the plants was done for this analysis of presence of Artemisinin by Spectrophotometric and Gas-chromatographic techniques {GC-FID}carried out.

# CHAPTER 1

# INTRODUCTION

## 1.1 ECOLOGY, TAXONOMY AND BIOLOGY

*Artemisia annua*

***Artemisia annua***, also known as **Sweet Wormwood**, **Sweet Annie**, **Sweet Sagewort** or **Annual Wormwood** is a common type of wormwood that is native to temperate Asia, but naturalized throughout the world.

**Characteristics**

It has fern-like leaves, bright yellow flowers, and a camphor-like scent. Its height averages about 2 m tall, and the plant has a single stem, alternating branches, and alternating leaves which range 2.5–5 cm in length. It is cross-pollinated by wind or insects. It is a diploid plant with chromosome number, 2n=18.

## 1.2 MEDICINAL USES

Sweet Wormwood was used by Chinese herbalists in ancient times to treat fever, but had fallen out of common use, but was rediscovered in 1970 when the Chinese Handbook of Prescriptions for Emergency Treatments (340 AD) was found. This pharmacopeia contained recipes for a tea from dried leaves, prescribed for fevers (not specifically malaria). In 1971, scientists demonstrated that the plant extracts had antimalarial activity in primate models, and in 1972 the active ingredient, artemisinin (formerly referred to as arteannuin), was isolated and its chemical structure described. Artemisinin may be extracted using a low boiling point solvent such as diethylether and is found in the glandular trichomes of the leaves, stems, and inflorescences, and it is concentrated in the upper portions of plant within new growth. It is commonly used in tropical nations which can afford it, preferentially as part of a combination-cocktail with other antimalarials in order to prevent the development of parasite resistance. Artemisinin itself is a sesquiterpene lactone with an endoperoxide bridge and has been produced semi-synthetically as an antimalarial drug. The efficacy of tea made from A. annua in the

treatment of malaria is contentious. According to some authors, artemesinin is not soluble in water and the concentrations in these infusions are considered insufficient to treatment malaria. Other researchers have claimed that *Artemisia annua* contains a cocktail of anti-malarial substances, and insist that clinical trials be conducted to demonstrate scientifically that Artemisia tea is effective in treating malaria. This simpler use may be a cheaper alternative to commercial pharmaceuticals, and may enable health dispensaries in the tropics to be more self-reliant in their malaria treatment. The plant has also been shown to have anti-cancer properties. It is said to have the ability to be selectively toxic to some breast cancer cells and some form of prostate cancer, there have been exciting preclinical results against leukemia, and other cancer cells.

**Scientific classification:**

**Kingdom:** Plantae

Angiosperms

Eudicots

Asterids

**Order:** Asterales

**Family:** Asteraceae

**Genus:** Artemisia

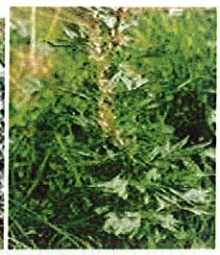
**Species:** A. annua

## 1.3 Genetic Diversity of *Artemisia annua* species

***Artemisia absinthium***

***Artemisia** vulgaris*

*Silver **Artemisia***



*Mound **Artemisia** vulgaris*

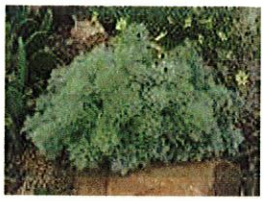
***Artemisia***

***Artemisia** tridentata*

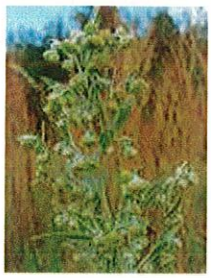
***Artemisia** vulgaris*

***Artemisia***

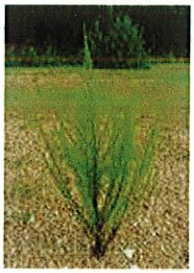
***Artemisia** absintium*



***Artemisia** schmidtiana*



***Artemisia** absinthium*



***Artemisia** caudata Michx.*

## 1.4 Importance of *Artemisia annua*

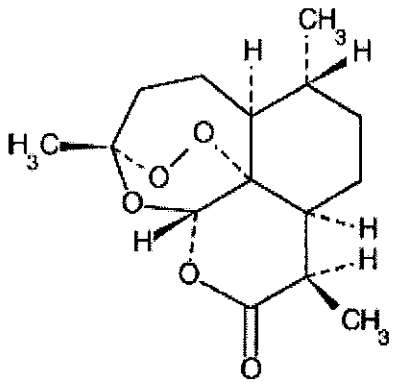
*Artemisia annua* L. (annual wormwood, sweet wormwood, sweet Annie), a highly aromatic annual herb of Asiatic and eastern European origin, is widely dispersed throughout the temperate region (Bailey and Bailey 1976, Simon et al. 1984). The species has naturalized in the United States and is sold on a limited scale as a dried herb for the floral and craft trade where it is used as an aromatic wreath. The plant has traditionally

been grown in China as a medicinal and, more recently in Europe for its aromatic leaves which are used in flavoring beverages.

Recent research in the Peoples Republic of China with traditional herbal medicine has brought attention to *A. annua,* the source of *qinghaosu* (artemisinin), a compound that shows promise as an anti-malarial agent Artemisinin has also been reported to be a potent plant inhibitor with potential as a natural herbicide. Artemisinin and its derivatives, artemether and artesunate, have been studied for their efficacy as antimalarial agents. In in vitro trials conducted in China (WHO 1981), all three compounds have been effective against the erythrocytic stages of two chloroquine-resistant Hainan strains of *Plasmodium falciparum,* the malarial parasite, at lower minimum effective concentrations than chloroquine, the most commonly used drug. Artemisinin and its derivatives have effectively treated malaria and cerebral malaria in human subjects with no apparent adverse reactions or side effects (Klayman, 1985). With *P. falciparum* developing resistance to chloroquine and pyrimethamine/sulfonamide (WHO 1981), alternative treatments based on new compounds such as artemisinin and its derivatives are actively being sought. While artemisinin and its derivatives may be synthesized, the synthetic compounds are unlikely to be economically competitive with the naturally derived plant products.

The relatively low content of artemisinin in cultivated European and New World types of *A. annua* has been a limiting factor for the isolation and evaluation of artemisinin on a technical scale. Artemisinin yields of 0.06% have been extracted from samples of *A. annua* collected in the United States (Klayman et al. 1984) which are low for commercial exploitation. Yields of extracted artemisinin from the above-ground portions of the plant have ranged from 0.01% to 0.5% (w/w) in the People's Republic of China (WHO 1981). Although artemisinin yield varies with environmental and management conditions (WHO 1981), specific effects are unknown. The extent of genetic variation on artemisinin content was also poorly understood.

## 1.5 CHEMISTRY OF ARTEMISININ

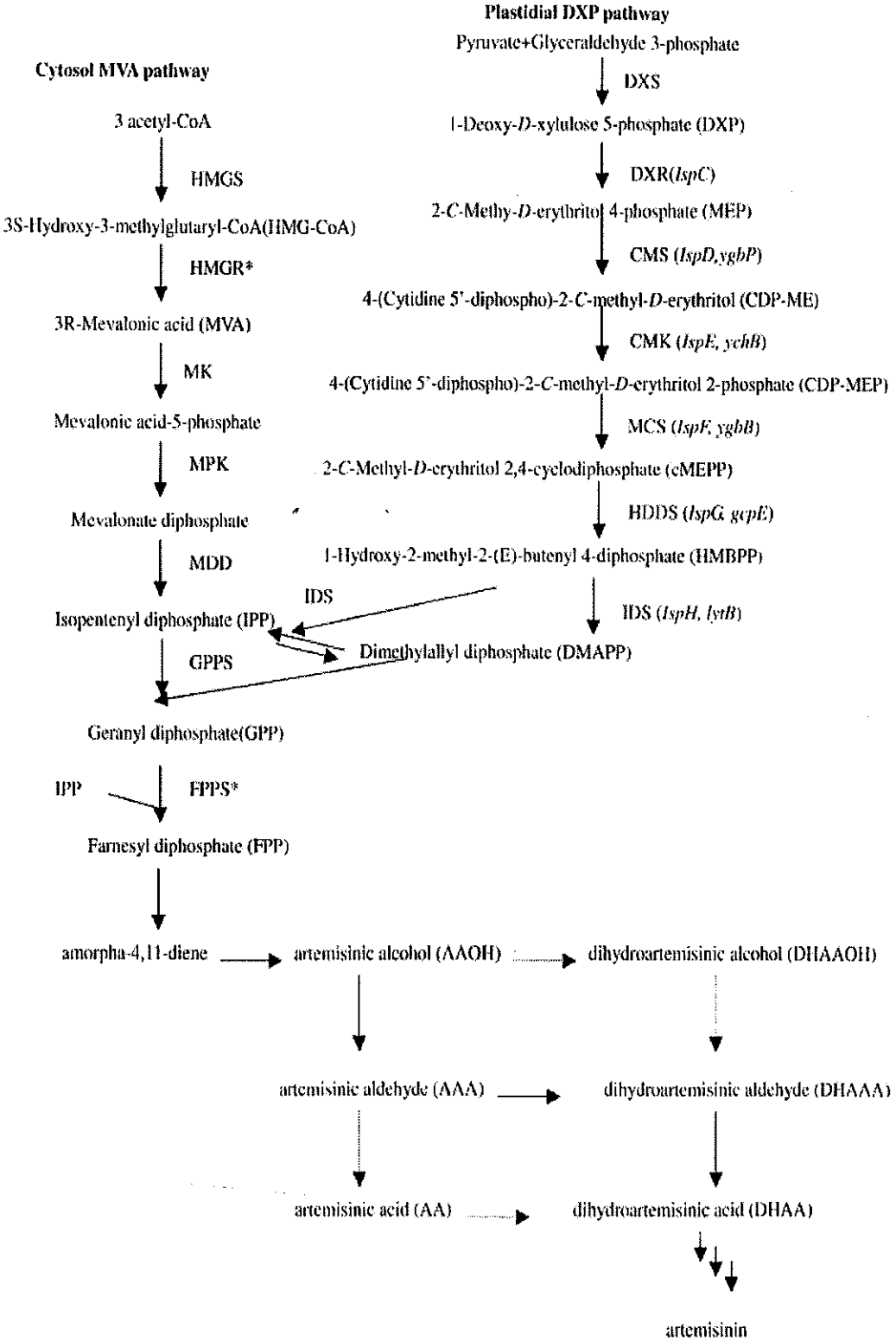


Formula: $C_{15}H_{22}O_5$
Mol. Mass: 282.332 g/mol

Superclasses:
secondary metabolite -> terpenoid ->sesquiterpenoid

### 1.5.1 Synthetic Pathway of Artemisinin

Artemisinin is a secondary or natural plant metabolite identified as a sesquiterpene lactone endoperoxide (Klayman et al. 1984). Analysis of artemisinin is difficult because the compound is unstable, concentrations in the plant low, the intact molecule stains poorly, and other compounds in the crude plant extracts interfere in its detection. Artemisinin is an endoperoxide sesquiterpene lactone which belongs to isoprenoid group of compounds. The isoprenoid pathway is one of the most important biosynthetic pathways in plants . Terpenoids are derived from two common precursors, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP). It has been established that higher plants have two independent biosynthetic pathways leading to the formation of IPP: the cytosolic mevalonate pathway and the plastid-localized mevalonate-independent pathway. Recently, two gene clones encoding for deoxy-Dxylulose- 5-phosphate synthase (DXPS) and deoxy-D-xylulose- 5-phosphate reductoisomerase (DXPR) isolated from transformed hairy roots of A. annua demonstrated the plastid-localized terpenoid biosynthetic pathway. But, the mevalonate pathway is not thought to be the sole route to the synthesis of artemisinin in A. annua.


Plastidial DXP pathway
Pyruvate+Glyceraldehyde 3-phosphate
DXS
Cytosol MVA pathway
3 acetyl-CoA
1-Deoxy-D-xylulose 5-phosphate (DXP)
HMGS
DXR(IspC)
3S-Hydroxy-3-methylglutaryl-CoA(HMG-CoA)
2-C-Methy-D-erythritol 4-phosphate (MEP)
HMGR*
CMS (IspD,ygbP)
3R-Mevalonic acid (MVA)
4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol (CDP-ME)
MK
CMK (IspE, ychB)
Mevalonic acid-5-phosphate
4-(Cytidine 5'-diphospho)-2-C-methyl-D-erythritol 2-phosphate (CDP-MEP)
MPK
MCS (IspF, ygbB)
2-C-Methyl-D-erythritol 2,4-cyclodiphosphate (cMEPP)
Mevalonate diphosphate
HDDS (IspG, gcpE)
MDD
1-Hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBPP)
IDS
IDS (IspH, lytB)
Isopentenyl diphosphate (IPP)
Dimethylallyl diphosphate (DMAPP)
GPPS
Geranyl diphosphate(GPP)
IPP
FPPS*
Farnesyl diphosphate (FPP)
amorpha-4,11-diene
artemisinic alcohol (AAOH)
dihydroartemisinic alcohol (DHAAOH)
artemisinic aldehyde (AAA)
dihydroartemisinic aldehyde (DHAAA)
artemisinic acid (AA)
dihydroartemisinic acid (DHAA)
artemisinin

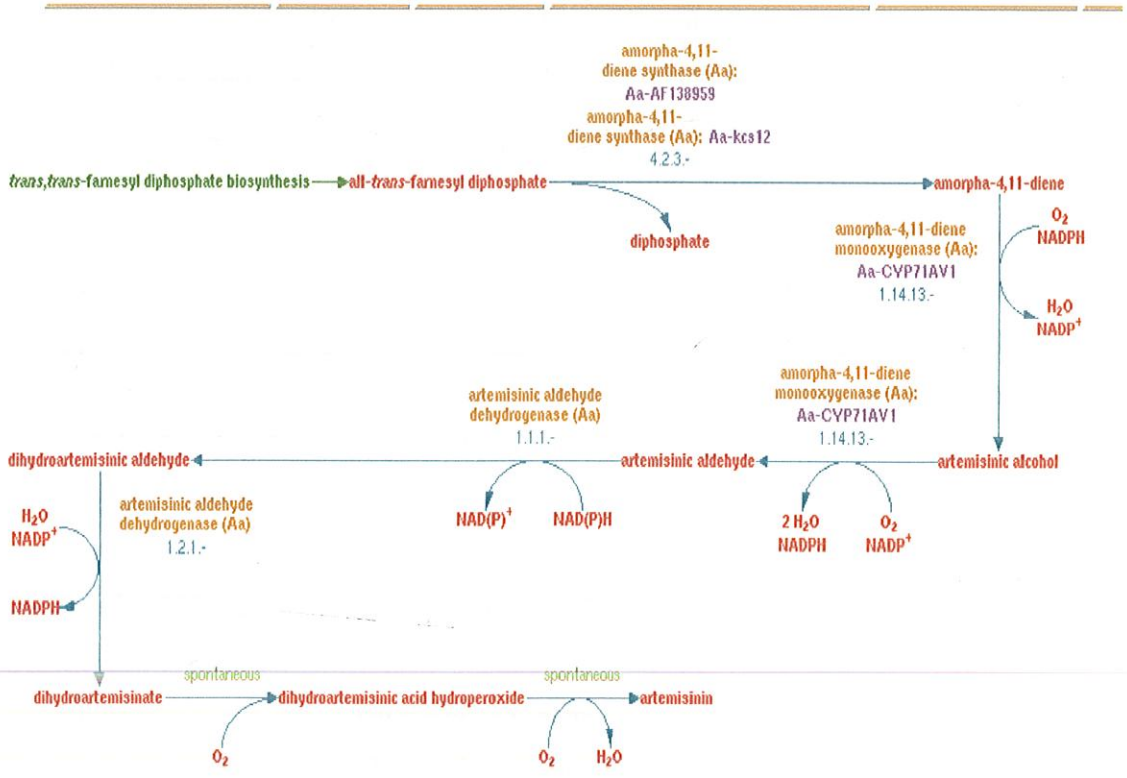
### 1.5.2 Location of Natural Products

Essential oils and artemisinin were assumed to be associated with secretory cells based on the association of mono- and sesquiterpenes with well-defined secretory structures. The relative distribution of artemisinin is shown in. Leaves had 89% of the total artemisinin in the plant with the uppermost foliar portion of the plant (top 1/3 of growth at maturity) containing almost double that of the lower leaves. Kelsey and Shafizadeh (1980) had reported that 35% of the mature leaf surface is covered with capitate glands which contain most of the monoterpenes and virtually all of the sesquiterpene lactones. Essential oils from *A. annua* are similarly distributed, with 36% of the total from the upper third of the foliage, 47% from the middle third, and 17% from the lower third, with only trace amounts in the main stem side shoots, and roots.

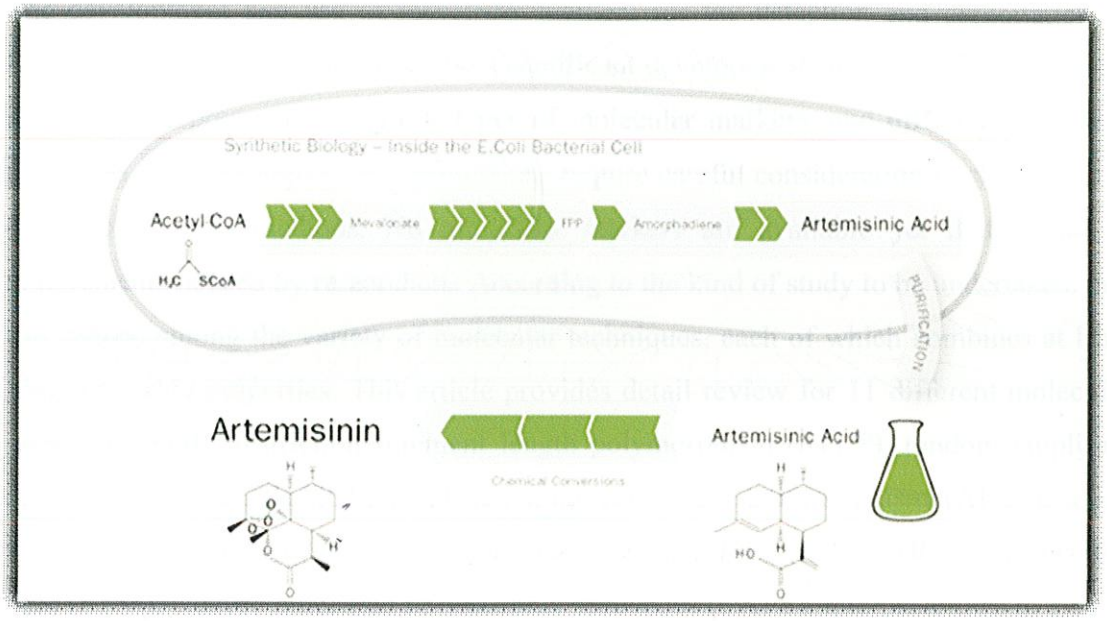
**Field view of Artemisia annua in India, Ladhak region.**

### 1.5.3 Artemisinin Synthesis Pathways

Artemisinin is an endoperoxide sesquiterpene lactone which belongs to isoprenoid group of compounds. The isoprenoid pathway is one of the most important biosynthetic pathways in plants . Terpenoids are derived from two common precursors, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP). It has been established that higher plants have two independent biosynthetic pathways leading to the formation of IPP: the cytosolic mevalonate pathway and the plastid-localized mevalonate-independent pathway. Recently, two gene clones encoding for deoxy-Dxylulose- 5-phosphate synthase (DXPS) and deoxy-D-xylulose- 5-phosphate reductoisomerase (DXPR) isolated from transformed hairy roots of A. annua demonstrated the plastid-localized terpenoid biosynthetic pathway. But, the mevalonate pathway is not thought to be the sole route to the synthesis of artemisinin in A. annua.



**Alternative For: Synthesis of Artemisinin (by synthetic biology)**




The alternative synthesis of artemisinin pathway is focused on increasing and stabilizing the supply of artemisinin to reduce the cost of life-saving artemisinin-based combination therapies (ACTs) by lowering the production cost of artemisinin derivatives. Here, synthetic biology is being used, to produce artemisinin, a natural compound traditionally extracted from *Artemisia annua*, or the sweet wormwood plant. The diagram shows a semisynthetic artemisinin production system, into which the genes from *A. annua* are being transferred. Using synthetic biology, the metabolism of *Escherichia coli* (*E. coli*) is engineered to produce artemisinic acid, a precursor to the important antimalarial artemisinin. Starting from acetyl-CoA, an abundant product of *E. coli* central metabolism, the bacteria produce, in turn, mevalonate, farnesyl pyrophosphate (FPP), amorphadiene and finally, artemisinic acid. The artemisinic acid is released from the bacteria and purified from the culture media. The artemisinic acid is then chemically converted to artemisinin. Once the artemisinin is produced, it must be further chemically converted into a derivative such as artesunate or artemether, which is then integrated into ACTs. The production process to make semisynthetic artemisinin is expected to take weeks rather than the months required to grow and extract botanically-derived artemisinin.

### 1.5.6 Molecular Markers Analysis:

The development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant developments in the field of molecular genetics. The presence of various types of molecular markers, and differences in their principles, methodologies, and applications require careful consideration in choosing one or more of such methods. No molecular markers are available yet that fulfill all requirements needed by researchers. According to the kind of study to be undertaken, one can choose among the variety of molecular techniques, each of which combines at least some desirable properties. This article provides detail review for 11 different molecular marker methods: restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter-simple sequence repeats (ISSRs), sequence characterized regions (SCARs), sequence tag sites (STSs), cleaved amplified polymorphic sequences (CAPS), microsatellites or simple sequence repeats (SSRs), expressed sequence tags (ESTs), single nucleotide polymorphisms (SNPs), and diversity arrays technology (DArT).

The differences that distinguish one plant from another are encoded in the plant's genetic material, the deoxyribonucleic acid (DNA). DNA is packaged in chromosome pairs, one coming from each parent. The genes, which control a plant's characteristics, are located on specific segments of each chromosome. All of the genes carried by a single gamete i.e., by a single representative of each of all chromosome pairs is known as genome. Although the whole genome sequence is now available for a few plant species such as *Arabidopsis thaliana* and rice, to help identify specific genes located on a particular chromosome, most scientists use an indirect method called genetic markers.

A genetic marker can be defined in one of the following ways:

(a) A chromosomal landmark or allele that allows for the tracing of a specific region of DNA;

(b) A specific piece of DNA with a known position on the genome

(c) A gene whose phenotypic expression is usually easily discerned, used to identify an individual or a cell that carries it, or as a probe to mark a nucleus, chromosomes, or locus.

### 1.5.7 Types of Molecular Markers

Genetic markers fall into one of the three broad classes:

1. Those based on visually assessable traits (morphological and agronomic traits),
2. Those based on gene product (biochemical markers),
3. Those relying on a DNA assay (molecular markers).

#### 1.5.7.1 Hybridization Based Markers:

RFLP is the most widely used hybridization-based molecular marker. The technique is based on restriction enzymes that reveal a pattern difference between DNA fragment sizes in individual organisms.

Although two individuals of the same species have almost identical genomes, they will always differ at a few nucleotides due to one or more of the following causes: point mutation, insertion/deletion, translocation, inversion and duplication. Some of the differences in DNA sequences at the restriction sites can result in the gain, loss, or relocation of a restriction site. Hence, digestion of DNA with restriction enzymes results in fragments whose number and size can vary among individuals, populations, and species.

#### 1.5.7.2 PCR-Based Markers

Arbitrary or semi-arbitrary primed PCR techniques that developed without prior sequence information RAPD, AFLP, ISSR

- Site-targeted PCR techniques that developed from known DNA sequences EST, CAPS, SSR,STS

Advantages of PCR techniques over hybridization-based Methods:

- ⊡ A small amount of DNA is required.
- ⊡ Elimination of radioisotopes in most techniques.
- ⊡ Ability to amplify DNA sequences from preserved tissues.
- ⊡ Accessibility of methodology for small labs
- ⊡ No prior sequence knowledge is required for many applications, such as RAPD, AFLP and ISSR.

- High polymorphism that enables to generate many genetic markers within a short time
- Ability to screen many genes simultaneously

(African Journal of Biotechnology Vol. 5 (25) et al.)

### 1.5.2 CAPS Markers To Study Genetic Diversity

Primers of length 300-400 bp from the conserved regions were designed and the restriction enzymes involved in the metabolic pathways were predicted using in silico methods. Then PCR analysis was done and DNA fragments were amplified from conserved regions. The amplicons were separately digested with one or more restriction enzymes predicted. And finally the screening of the digested amplicons for polymorphism on gels stained with ethidium bromide was done.

40 Samples each were taken from Leh and Nubra valley at an altitude of 11500ft and 10500ft respectively.

**Table 1: Comparison of CAP and RLPF Techniques:**

| **Cleaved Amplified Polymorphism (CAP)** | **Restriction Fragment Length Polymorphism (RFLP)** |
|---|---|
| *ADVANTAGES:* | *ADVANTAGES:* |
| 1.Numerous | 1. Numerous |
| 2.Codominant | 2.Codominant |
| 3.Easy to assay | 3.Reproducible |
| 4.Reproducible | 4.Relatively polymorphic |
| 5.Polymorphic | |
| | |
| *DISADVANTAGES:* | *DISADVANTAGES:* |
| 1. laborious and expensive to develop | 1.laborious |
| 2. requires upfront discovery of restriction site polymorphisms or sequencing | 2.complex patterns |
| | 3. automation is difficult |

**Table 2: Comparison of Application in Conservation Genetics for different Molecular Markers:**

| | CAPS | RFLPS | SSRS | AFLPS | SNPS | RAPDS |
|---|---|---|---|---|---|---|
| **Measuring genetic variation** | Good | Limited | Good | Good | Good | Good |
| **Measuring population genetic structure and differentiation** | Good | Limited | Good | Good | Good | Good |
| **Estimating introgressive hybridisation, migration or gene flow** | Good | Limited | Limited to within species analysis | Poor | Poor | Good |
| **Studying phylogeny or taxonomy** | Good | Limited to closely related taxa | Poor | Limited | Limited | Limited |
| **Characterizing mating systems and paternity analysis** | Good | Limited | V.good | Poor | Limited | Poor |
| **Individual identification or DNA fingerprinting** | Good | Good | V.good | Poor | Good | Poor |
| **Genetic maps** | Good | Limited | Good | V.good | Good | Poor |

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 INTRODUCTION

Primer designing, DNA extraction, quantification, PCR amplification followed by restriction is the crux of this project. This part of the project took the major amount of time and precision. The protocol was revised again and again so as to finally standardise to get the best possible results.

## 2.2 SEQUENCES OF GENES INVOLVED IN ARTEMISISIN SYNTHESIS PATHWAY:

**TABLE 3:**

GENES RELATED TO ARTEMISININ BIOSYNTHESIS IN *A. ANNUA* L.

| Enzyme | Gene | Function | Location | GenBank accession number | Reference |
|---|---|---|---|---|---|
| Deoxyxylulose synthase | *dxs* | 1-Deoxy-D-xylulose-5-phosphate synthase activity | Plastid | AF182286 | Souret et al., 2002, 2003 |
| Deoxyxylulose reductase | *dxr* | Isomerase and oxidoreductase activity | Plastid | AF182287 | Souret at al., 2002, 2003 |
| 3-Hydroxy-3-methylglutaryl coenzyme A reductase | *hmg* | Catalyzes the two-step reduction of (S)-3-hydroxy-3-methylglutaryl coenzyme A (S-HMG-CoA) into *R*-mevalonate | Cytosol | AF142473 | Souret et al., 2002, 2003 |
| Farnesyl diphosphate synthase | *fps* | Synthesis of FDP | Cytosol | AF112881 | Chen et al., 2000; Souret et al., 2002, 2003 |
| Sesquiterpene cyclases: | | Catalyzes cyclization of FDP to: | All likely in cytosol | | |
| Epicedrol synthase | *eps* | 8-Epicedrol | | AJ001539 | Mercke et al., 1999; Hua and Matsuda, 1999 |
| Amorphadiene synthase | *ads* | Amorpha-4,11 diene | | AJ251751 | Mercke et al., 2000; Chang et al., 2000 |
| β-caryophyllene synthase | *cs (qhs1)* | β-Caryophyllene | | AF472361 | Cai et al., 2002 |
| β-farnesene synthase | *fs* | β-Farnesene | | AY835398 | Picaud et al., 2005a |
| Putative sesquiterpene cyclases | *cas125* | Isoprenoid biosynthesis and lyase activity | Isolated from: flowers, | AJ271792 | Van Geldre, 2000 |
| | *cas34* | Isoprenoid biosynthesis and lyase activity | leaves and flowers | AJ271793 | Van Geldre, 2000 |
| | *ses* | Reaction product not determined | young leaves | AAD39832 | Liu et al., 2002 |
| Squalene synthase | *ads* | Farnesyl-diphosphate farnesyltransferase activity | Endoplasmic reticulum | AY445506 | Liu et al., 2003 |
| Squalene synthase fragment | *sqs1* | Transferase activity | Cytosol | AF182286 | Souret et al., 2003 |
| CYP71AV1 | *na* | Catalyzes 3 steps post ADS | Trichomes | DQ315671 | Teoh et al., 2006 |
| PsbA [Fragment] | *psba* | Act as barcode for flowering plants | Chloroplast | DQ006143 | Kress et al., 2005 |
| Ribulose-1,5-biphosphate carboxylase/oxygenase | *rbcl* | Carbon dioxide fixation; barcoding for flowering plants | Chloroplast | DQ006057 | Kress et al., 2005 |
| Peroxidase 1 | *prx1* | Favored the bioconversion of artemisinic acid to artemisinin | Roots, stems and leaves | AY208699 | Zhang et al., 2004 |
| Beta-pinene synthase | *qh6* | Circadian pattern of expression | Juvenile leaves | AF276072 | Lu et al., 2002 |
| (3R)-linalool synthase | *qh1* | lyase activity | Leaves and flowers | AF154125 | Jia et al., 1999 |
| Isopentenyl transferase | *ipt* | Biosynthesis of the cytokinin phytohormones | Transferred into *A. annua* L. via *A. tumefaciens* | M91610 | Su et al., 2001 |

## 2.3 DESIGNING OF PRIMERS: PROTOCOL

The first step was to search for the pathway for the biosynthesis of Artemisinin. The pathway for the biosynthesis of Artemisinin was not found on NCBI or KEGG. So the search for the pathway was carried on metacys, where entries for the pathway as well as the compound were found. Then the enzyme sequences involved in the synthesis pathway were downloaded from NCBI database. Sequences from animals were rejected. Sequences from only Artemisia Genus were taken, rest all the sequences were rejected. Then the similarity between enzyme sequences and EST sequences was found using standalone nucleotide blast. MSA was done on those sequences which had BIT score> 149 by using DNAstar megalign. Conserved regions were taken from consensus sequence after MSA. Then the left and right primers were designed with the help of the consensus sequence by using online available primer designing tool "PRIMER3".

## 2.4 DIFFERENT PRIMERS DESIGNED

### 1. PRIMERS FOR "ads" GENE

Different sequences taken:

1. AF138959 *Artemisia annua* amorpha-4, 11-diene synthase mRNA, complete cds
2. AF327526 *Artemisia annua* amorpha-4, 11-diene synthase (AMS1) mRNA, complete cds
3. AJ251751.1 *Artemisia annua* mRNA for amorpha-4, 11-diene Synthase
4. AY006482.1 *Artemisia annua* amorpha-4, 11-diene synthase mRNA, complete cds
5. DQ241826.1 *Artemisia annua* amorpha-4, 11-diene synthase mRNA, complete cds
6. EF197888.1 *Artemisia annua* amorpha-4, 11-diene synthase mRNA, complete cds
7. GO502359 Mdrtc1033G07.g1 Apple_EST_Mdrtc Malus hybrid rootstock cDNA 5' similar to gb|AAF98444.1| amorpha-4, 11-diene synthase [*Artemisia annua*], mRNA sequence

| Oligo | Start | Length | Tm | GC% | Sequence | Prod. size |
|---|---|---|---|---|---|---|
| Left primer | 8 | 20 | 59.85 | 50.00 | GTTCCGTCTTATGCGAAAGC | 437 |
| Right primer | 444 | 20 | 60.12 | 45.00 | AAGCTTTCCACCATTTGCAC | |

1 CCTTATG***GTTCCGTCTTATGCGAAAGCAAGGATATTATGTTACATGTGATGTTTTCAATA***

***61 ACTATAAAGACAAAAATGGAGCGTTCAAGCAATCGTTAGCTAATGATGTTGAAGGTTTGC***

***121 TTGAGTTGTACGAAGCAACTTCTATGAGGGTACCTGGGGAGATTATATTAGAAGATGCTC***

***181 TTGGTTTTACACGATCTCGTCTTAGCATTATGACAAAAGATGCTTTTTCTACAAACCCCG***

***241 CTCTTTTTACCGAAATACAACGGGCACTAAAGCAACCCCTTTGGAAAAGGTTGCCAAGAA***

***301 TAGAGGCGGCGCAGTACATTCCTTTCTATCAACAACAAGATTCTCATAACAAGACTTTAC***

***361 TTAAACTTGCTAAGTTAGAGTTCAATTTGCTTCAGTCATTGCACAAGGAAGAGCTCAGCC***

***421 ATGTGTGCAAATGGTGGAAAGCT***TTCGATATCAAGAAGAACGCACCTTGTTTAAGAGATA

481 GAATTGTTGAATGCTACTTTTGGGGACTAGGTTCAGGCTATGAGCCACAGTATTCCCGGG

541 CTAGAGTTTTCTTCACAAAAGCTGTTGCTGTTATAACTCTTATAGATGACACTTATGATG

601 CGTATGGTACTTATGAAGAACTTAAGATCTTTACTGAAGCTGTTGAAAGGTGGTCAATTA


## 2. PRIMERS FOR "caryophyllene synthase" GENE

Different sequences taken:

AF472361 *Artemisia annua* beta-caryophyllene synthase QHS1 (QHS1) mRNA, complete cds

NO OTHER SEQUENES FOUND FOR THIS GENE

| Oligo | Start | Length | Tm | GC% | Sequence | Prod. size |
|---|---|---|---|---|---|---|
| Left primer | 1063 | 20 | 60.05 | 50.00 | GGGAAAAGAGGGAAA AGCAC | 436 |
| Right primer | 1498 | 20 | 59.93 | 50.00 | CACAAGGGATTCTCGG GTTA | |

1 AGACATGTCTGTTAAAGAAGAGAAAGTAATTCGGCCCATTGTCCACTTTCCTCCTAGCGT

61 TTGGGCAGACCAGTTTCTCATCTTCGATGACAAGCAAGCAGAGCAAGCCAATGTAGACA

121 AGTAGTCAACGAGTTAAGAGAAGATGTGAGAAAAGATTTAGTGTCATCTTTAGATGTTCA

181 AACGGAGCACACAAATTTGCTAAAACTTATTGATGCTATCCAACGTCTTGGTATAGCGTA

241 TCATTTTGAAGAGGAGATTGAGCAAGCCTTACAACATATTTATGATACATATGGTGATGA

301 CTGGAAAGGCAGAAGCCCTTCCCTTTGGTTTCGAATCCTTCGGCAACAAGGTTTTTATGT

361 TTCTTGTGATATTTTTAAAAACTATAAAAAAGAGGATGGTTCATTTAAGGAATCCCTCAC

421 CAACGATGTAGAAGGCTTGCTTGAGCTGTATGAGGCGACATATTTGAGAGTGCAAGGCGA

481 AGGGGTTCTAGATGATGCTCTTGTTTTCACAAGGACTTGTCTTGAGAAAATAGCAAAGGA

541 TCTTGTTCACACCAACCCAACACTATCTACCTACATACAAGAAGCACTAAAACAGCCGTT

601 ACATAAAAGGTTGACAAGACTAGAGGCATTGCGTTACATTCCTATGTACGAACAACAAGC

661 ATCTCATAATGAGTCTTTACTCAAACTTGCGAAGCTAGGTTTCAACTTACTTCAATCATT

721 GCATAGAAAGGAGCTTAGCGAAGTTTCCAGGTGGTGGAAAGGTCTTGATGTCCCAAACAA

781 TCTACCTTATGCAAGAGATAGAATGGTTGAATGTTATTTTTGGGCACTAGGTGTCTATTT

841 TGAGCCAAAATATTCTCAAGCTAGGATCTTTTTAGCAAAAGTAATTTCGCTAGCAACTGT

901 TCTTGACGACACTTATGATGCTTATGGAACCTATGAAGAACTTAAGATCTTTACTGAAGC

961 AATTCAAAGGTGGTCGATTACATGCATAGATATGCTTCCAGAATACCTGAAACTATTATA

1021 CCAAGGAGTCTTGGATATATACATAGAAATGGAAGAAATAAT***GGGAAAAGAGGGAAAAGC***

***1081 ACATCATCTTAGCTATGCTAAAGAATCTATGAAAGAGTTTATTAGAAGCTATATGATGGA***

***1141 AGCAAAATGGGCAAATGAGGGGTATGTACCAACAGCAGAGGAGCATATGTCAGTTGCGTT***

***1201 TGTAAGCAGCGGGTATAGCATGCTTGCAACAACATGTTTTGTTGGCATGGGTGATATCGT***

***1261 TACAGATGAAGCATTCAAATGGGCTCTGACAAAACCTCCTATCATCAAAGCTTCATGTGC***

***1321 AATTGCTAGACTCATGGATGATATTCACTCTCAAAAGGAGGAGAAAGAAAGAATACATGT***

***1381 TGCATCTAGTGTTGAAAGTTACATGAAGCAATATGATGTGACAGAGGAGCATGTCCTTAA***

***1441 AGTATTTAACAAGAAAATCGAGGATGCGTGGAAAGATATAACCCGAGAATCCCTTGTGCG***

1501 TAAAGATATTCCGATGCCTCTGATGATGCGAGTGATTAACTTGGCACAGGTGATGGATGT

1561 TTTATATAAACATAAAGACGGTTTCACTAATGTGGGAGAAGAACTCAAGGATCATATTAA

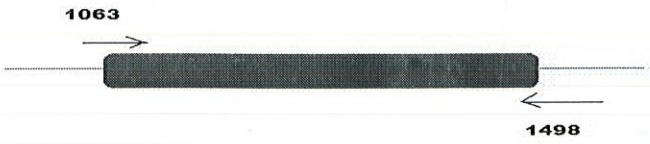
1621 ATCTTTGCTCGTTCATCCTATACCTATATAAGTTCCGAAACTTCATCTCGTTTTTAGCCT

1681 CCGATACACTCAACAACTCAACTTTCTCTCATCTTCAAATGGTTGGTTGTGGCAAATCTT

1741 AAGTCCTTGGGTGATTGAAATCTTCAAATGCGTGTTTGTTACTACATGCATGTGATTTTT

1801 TTCCCCCTCTTCTGTGTGGCATTTGTATTTGGATGATTTAATAATAAATAAAGGTGTGTT

1861 GTTAAAAAAAAAAAAAAAAA



3. **PRIMER FOR "dxs" gene**:

Different sequences taken:

AF182286.2| *Artemisia annua* 1-deoxy-D-xylulose-5-phosphate synthase (DXS1) mRNA, partial cds

DR993486 Mdas9003G19.g1 Apple_EST_Mdas Malus x domestica cDNA similar to gb|AAD56390.2|AF182286_1 1-deoxy-D-xylulose-5-phosphate synthase [*Artemisia annua*], mRNA sequence

GO518868 Mdfrs3137J04.g1 Apple_EST_Mdfrs Malus x domestica cDNA 5' similar to gb|AAD56390.2|AF182286_1 1-deoxy-D-xylulose-5-phosphate synthase [*Artemisia annua*], mRNA sequence

GO521454 Mdfrb3145P14.g1 Apple_EST_Mdfrb Malus x domestica cDNA 5' similar to gb|AAD56390.2|AF182286_1 1-deoxy-D-xylulose-5-phosphate synthase [*Artemisia annua*], mRNA sequence

GO566164 Mddb5016H18_e2123.g1 Mddb Malus x domestica cDNA 5' similar to gb|AAD56390.2|AF182286_1 1-deoxy-D-xylulose-5-phosphate synthase [Artemisia annua], mRNA sequence

| Oligo | Start | Length | Tm | GC% | Sequence | Prod. Size |
|---|---|---|---|---|---|---|
| Left primer | 23 | 20 | 59.86 | 50.00 | ACCACCAACTCCACTTTTGG | 477 |
| Right primer | 499 | 20 | 60.10 | 50.00 | ATGGCTTCGTATGCTTGTCC | |

1GAGGTGAATATCATTCTCAAAG**ACCACCAACTCCACTTTTGGACACTATTAATTACCCTA**
*61 TCATATGAAGAATTTGTCTGTTAAGGAACTGAAGCAACTAGCTGACGAGCTTCGATCTG*
*121 TGTGATCTTTAACGTTTCTAAAACCGGTGGCCATTTGGGTTCAAGTCTTGGTGTTGTTG*
*181 GCTCACCGTTGCGCTTCATTATGTATTTAATACGCCGCAAGATAAGATACTTTGGGACG*
*241 GGTCATCAGTCTTATCCACATAAAATATTGACTGGAAGAAGAGACCAAATGCATACCA*
*301 AGACAGACAAATGGGTTGGCGGGATTCACCAAACGCTCTGAAAGTGAACATGATTGTT*
*361 GGCACGGGTCATAGCTCTACTACCATTTCTGCAGGATTAGGGATGGCTGTGGGACGGG*
*421 TTAAAGGGAGGAACAAACGACGTAGTTGCTATAATAGGTGACGGTGCTATGACAGCT*
*481 GACAAGCATACGAAGCCATGAACAACGCCGGTTACTTAGATTCAGACATGATAGTCATCC*
541 TAACGACAACAAACAAGTATCTTTACCTACTGCCAATCTTGACGGTCCTATACCACCCG
601 TTG

## 4. PRIMER FOR "epicedrol synthase" GENE

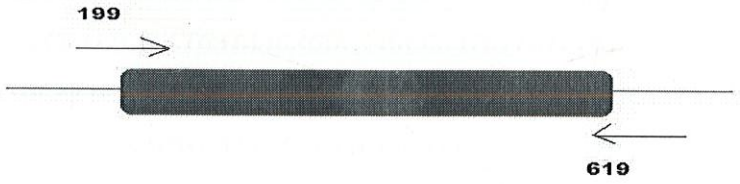
Different sequences taken:

AF157059 Artemisia annua 8-epicedrol synthase (Ecs1) mRNA, complete cds

AJ001539.1| Artemisia annua mRNA for epi-cedrol synthase

| Oligo | Start | Length | Tm | GC% | Sequence | Prod.size |
|---|---|---|---|---|---|---|
| Left primer | 199 | 20 | 59.96 | 45.00 | AATCGAACGCCTTGGTATTG | 421 |
| Right primer | 619 | 20 | 60.18 | 60.00 | GTAGCGCAACGCCTCTAGTC | |

1 TATAGTAGAGGATGTCATACGCCCCAATGCAAATTTTCCTTCTGAAATTTGGGGAGATCA
61 GTTTCTTGCCTATGACCAGGATGAGCAAGAGGGAGTTGAACAAGTAATCAAAGATTTGAA
121 AGAAGAAGTGAAGAGCGAACTATTGACAGCTCTAAATTCTCCGACTCAGCATACGGAGTT
181 GTTGAAATTTATTGACGC***AATCGAACGCCTTGGTATTGCGTATTATTTTGAAGAGGAGAT***
***241 CAACCAAGTATTCCAACACATGTATACTGCATATGGTGATAAGTGGACAGGTGGTAATAC***
***301 TTCCCTTTGGTTTCGACTCATGCGACAACATGGATTTTTCGTTTCAAGCGATATTTTTAG***
***361TACCTACAAAGACAAAGAGGGACGTTTTAAGGAGTCTTTAGAAAAGGACGTTCACGGGTT***
***421ACTTGAGTTGTATGAGGCGGCATATATGTTTGTGCCTGGGGAAGGGATACTAGATGATGC***
***481 TCTTGTTTTTACAAGAACTTGTCTTGATGAAATAGCGAAAAATCCTAGTCTAAGTAACTC***
***541 TGCTGTTTCTTCCCAAATACGTGAAGCTCTAACGCAACCTTTACATAAAAGATTACCGAG***
***601 ACTAGAGGCGTTGCGCTAC***ATACCTTTCTACCAACAACAAGCTTCTCACAGTGAGACATT
661 GTTAAAACTGGCCAAATTAGGGTTCAACCAACTTCAATCACT




## 5. PRIMERS FOR "fransyl diphosphate synthase (fps)" GENE

Different sequences taken:

AF112881 Artemisia annua farnesyl diphosphate synthase mRNA, complete cds

AF136602 Artemisia annua farnesyl diphosphate synthase (fps2) mRNA, complete cds

AF149257 Artemisia annua farnesyl diphosphate synthase gene, partial cds

AY308476.1| Artemisia tridentata subsp. spiciformis farnesyl diphosphate synthase (FDS-2) mRNA, complete cds

AY308477.1| Artemisia tridentata subsp. spiciformis farnesyl diphosphate synthase (FDS-1) mRNA, complete cds

AY308478.1| Artemisia tridentata subsp. spiciformis chrysanthemyl diphosphate synthase (FDS-5) mRNA, complete cds

U36376.1|AAU36376 Artemisia annua farnesyl diphosphate synthase (fps1) mRNA, complete cds

AY835398.1| Artemisia annua (E)-beta-farnesene synthase mRNA, complete cds

| Oligo | Start | Length | Tm | GC% | Sequence | Prod.size |
|---|---|---|---|---|---|---|
| Left primer | 4 | 20 | 61.21 | 50.00 | CCCGAGGTGATTGGAAAGAT | 401 |
| Right primer | 404 | 20 | 59.87 | 50.00 | TACAGACAACATCGGCTTGC | |

1 GCT*CCCGAGGTGATTGGAAAGATTGGAACTGATATTGAAGACTTTAAGTGCTCCTGGTTA*

*61 GTTGTCAAGGCATTGGAACTCGCCAATGAGGAACAAAAGAAAGTCCTACATGAGAACTAT*

*121 GGGAAAAAGGACCCCGCGTCTGTTGCTAAAGTGAAGGAAGTATACCACACTCTCAATCTT*

*181 CAGGCTGTATTCGAAGATTACGAGGCCACAAGTTACAAGAAGCTTATCACATCGATTGAA*

*241 AATCGCCCAAGCAAAGCAGTCCAAGCGGTGCTGAAATCTTTCTTGGGTAAAATCTACAAG*

*301 AGGCAAAAGTAGATGTCTTGTACCAACTGATTTCTGATTTTGAGTCTAGCGAGTGGAAAA*

*361 TCATTCGCATTCTCCTGATAAGAGGCAAGCCGATGTTGTCTGT*ATCTTTTGTTATTTGTA

421 TTATTGCATTCTTCCTAGTGGTATCTCTGTGTCTTTAGTATGTGTAATAAAACGCTCTTA

481 TTGTTTGTAAGAACTGTTTTGGAGTTCTTGATTTATTTCGTATGTAGTTTTGGTGGTGCA


## 6. Primers for "3-hydroxy-3-methylglutaryl coenzyme A reductase(HMGR)" GENE

Different sequences taken:

U14624.1|AAU14624 Artemisia annua 3-hydroxy-3-methylglutaryl coenzyme A reductase (AHM1) mRNA, partial cds

AAU14625 Artemisia annua 3-hydroxy-3-methylglutaryl coenzyme A reductase (AHM4) mRNA, complete cds

| Oligo | Start | Length | Tm | GC% | Sequence | Prod.size |
|---|---|---|---|---|---|---|
| Left primer | 37 | 20 | 60.10 | 50.00 | GCCTCAGACGCATTACCATT | 484 |
| Right primer | 520 | 20 | 59.89 | 60.00 | CCTCCCTCCCTACAATCCTC | |

1 TCACACCGCTCACATTCACCAACACCAATCCCAAAA***GCCTCAGACGCATTACCATTACCA***

***61 TTATACCTAACCAACACCTTCTTCTTCACCTTATTCTTCTCAGTCGCTTACTATCTCCTT***

***121 CACAGATGGCGCGACAAGATCCGTTCCGGCACACCGTTGCACGTCGTCACGTTAACTGAA***

***181 TTATCCGCTATTGTTTTACTCATTGCTTCCTTTATTTATTTGTTAGGTTTCTTTGGTATT***

***241 GATTTTGTCCAGTCGTTTATTTCGCGCGAAAACGAACAATTGAATAATGATGATCATAAT***

***301 GTTGTTAGTACTAATAATGTGTTGTCTGATAGAAGGCTTGTTTATGATTATGGATTTGAT***

***361 GTCACTGGTGATAATGATGATGGTAATGATGATGATGTGATTGTTAAGAGTGTTGTTAGT***

***421 GGTGAGGTGAATTCGTATTCGTTAGAGGCGAGTTTAGGTGATTGTTATAGAGCGGCTAAG***

***481ATACGTAGACGTGCGGTTGAGAGGATTGTAGGGAGGGAGG***TTTTAGGGTTAGGGTTTGAG


## 7. Primers for "Psb A"

Different sequences taken:

AJ401617.1| Artemisia rupestris chloroplast psbA-tRNA-His intergenic region

DQ006143.1| Artemisia annua voucher Wells 4535 US PsbA (psbA) gene, partial cds

FJ418749.1| Artemisia annua PsbA (psbA) gene, partial cds

FJ418750.1| Artemisia capillaris PsbA (psbA) gene, partial cds

FJ418751.1| Artemisia carvifolia PsbA (psbA) gene, partial cds

**No Acceptable Primers Were Found.**

## 8. Primers for "sesquiterpene"

Different sequences taken:

1. AF156854.1|AF156854 Artemisia annua sesquiterpene synthase mRNA, partial cds
2. AF304444.2|AF304444 Artemisia annua sesquiterpene cyclase mRNA, complete cds
3. AJ249561.1| Artemisia annua mRNA for putative sesquiterpene cyclase
4. AJ271792.1| Artemisia annua mRNA for putative sesquiterpene cyclase (cASC125 gene
5. AJ271793.1| Artemisia annua mRNA for putative sesquiterpene cyclase (cASC34 gene)
6. AJ276412.1| Artemisia annua partial gASC21 gene for putative sesquiterpene cyclase, exons 1-3
7. AJ276413.1| Artemisia annua partial gASC22 gene for putative sesquiterpene cyclase, exons 1-3
8. AJ276414.1| Artemisia annua partial gASC23 gene for putative sesquiterpene cyclase, exons 1-3
9. AJ276415.1| Artemisia annua partial gASC24 gene for putative sesquiterpene cyclase, exons 1-3
10. EU252034.1| Artemisia annua sesquiterpene cyclase mRNA, complete cds

| Oligo | Start | Length | Tm | GC% | Sequence | Prod.size |
|---|---|---|---|---|---|---|
| Left primer | 77 | 20 | 60.22 | 50.00 | CAGACCACTTC GGAAAAGGA | 411 |
| Right primer | 487 | 20 | 59.55 | 50.00 | TCTGGAAGTGA GTCCAAGCA | |

1 AAACTCACCTTGGAAACATAGCAAAGGATCCTTCTCAGAATGCTTCTTTAAGAACCAAAA

61 TAGAACAAGCACTAAA***CAGACCACTTCGGAAAAGGATGCCAAGGCTAGAGGCACTGCATT***

***121 ACATACCTAAATACCAACAAGAAGCTTCTCATGACGAGACCTTGTTAAATCTTGCAAAGT***

***181 TGGATTATAACATGCTTCAATCAATACACAAAAGGGAGATTAGTGAAATCTGCAAATGGT***

***241 GGAAGGATTTGGATTTCTCAAACAAACTACCGCATGTTCGAGATAGATTGGTTGAAATAT***

***301 ATTTTTGGGTATTGGGGGTCTATTTTGAACCTCAACATTATCGTTCAAGGATGTTTCTCA***

***361 CCAAAACGAGTATGTGGTTAATTGTTCTTGATGACACATATGATAATTATGGTACTTACG***

***421 AAGAACTTAAGATCTTTACGGAAGCTGTTCAGAGATGGTCCTTAAGCTGCTTGGACTCAC***

***481 TTCCAGAA***TACATGAAATTAATATATCGAGAACTTATAAATCATTACCAGGAAATGGAAG

541 TCAGTTGAAA


## 9. Primers for "Squalene synthase"

Different sequences taken:

1. AF181557.1|AF181557 Artemisia annua squalene synthase (SQS1) mRNA, partial cds
2. AF302464.2|AF302464 Artemisia annua squalene synthase mRNA, complete cds
3. AF405310 Artemisia annua squalene synthase gene, complete cds
4. AY445505.1| Artemisia annua squalene synthase gene, partial cds
5. AY445506.1| Artemisia annua squalene synthase mRNA, complete cds

| Oligo | Start | Length | Tm | Gc% | Sequence | Prod.size |
|---|---|---|---|---|---|---|
| Left primer | 94 | 20 | 59.84 | 55.00 | GCCTCAGACGCATTACC ATT | 404 |
| Right primer | 497 | 20 | 59.40 | 40.00 | CCTCCCTCCCTACAATC CTC | |

1 AGTTTGAAAGCAGTATTGAAACACCCAGATGACTTTTATCCATTATTGAAGTTGAAAATG

61 GCTGCAAAGAAAGCCGAAAAACAGATCCCATCA***CAACCTCACTGGGCTTTCTCTTATTCC***

***121 ATGCTTCATAAAGTTTCTAGAAGTTTCGCTCTTGTTATTCAACAACTTAATCCCCAGCT***

***181 CGTGATGCTGTATGCTTTTTCTTCTTTCTTTAGATCCATCCATTCACTTTTTTCTTTTTT***

***241 CTAGTAATTATCGCGTAACCTTGCAATTATTTGTTGGATGTGTTGACCAGATGAGTTTTG***

***301 TATATTTAATGGCATTTGTATGTCAAAATAATTAAAATTAAGTATGATGATTAGAGTTGT***

***361 AGGTGATGTTCCTATATAGGTGTATTAGATCCCTGAATTATTACTCATACGACTTCGTAT***

***421 TCGATAGCCCTTTTTATGATACCTATCATATAGGGTCTTTCTTGGACCAGGAATTAGTTG***

***481 ACAGAAAGCGGATTTCA***ACTATAACCAACTGTACTAGAGTTGCAATTATTACCGGAAAAT

541 AAATAAGCTGACTAGTCGAAAATTATAAAGAAACGAAAGTTCATTATTAATCAGCTGGTG

601 C

## 2.5 PLANT MATERIALS

A total of 80 samples of the plant from 2 regions of varying altitudes was collected in the following manner:

**Table 4: Sources From Where Plants Were Obtained:**

| Source | No. of samples | Altitude |
|---|---|---|
| Leh Valley | 40 | ~11500ft |
| Nubra Valley | 40 | ~10500ft |

## 2.6 GENOMIC DNA EXTRACTION

Young leaves were frozen in liquid nitrogen and stored at -80 $^0$C prior to DNA isolation. Plant leaves (0.5gram-1gram) were taken and grinded with liquid nitrogen in mortal and pestle kept at -80$^0$C. (Mortal pestle is sterilized by oven for 1 hour and then kept in fridge at -80$^0$C). The powder was transferred to 2 ml eppindorf. Then, 700µl of pre-warmed extraction buffer was added and incubated at 60$^0$C for 1 hour.

**TABLE 5: Composition for extraction buffer:**

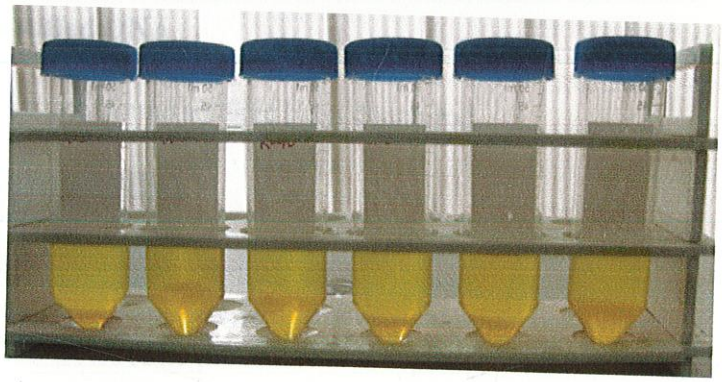
| BUFFER | CONCENTRATION | STOCK CONC | WORKING VOLUME(100ml) |
|---|---|---|---|
| TRIS | 100mM | 1M | 10ml |
| Nacl | 1.4M | 5M | 28ml |
| EDTA | 20mM | 0.5M | 4ml |
| CTAB | 2% | | 2gm |
| PVPP | 1% | | 1gm |
| WATER | | | 58ml |

Next, 24:1 chloroform: isoamyl alcohol (700µl) was added. The supernatant was taken and spun at 10,000 rpm for 10 minutes. Steps involving treatment with chloroform and isoamyl alcohol followed by spinning of supernatant, were repeated twice. Aqueous

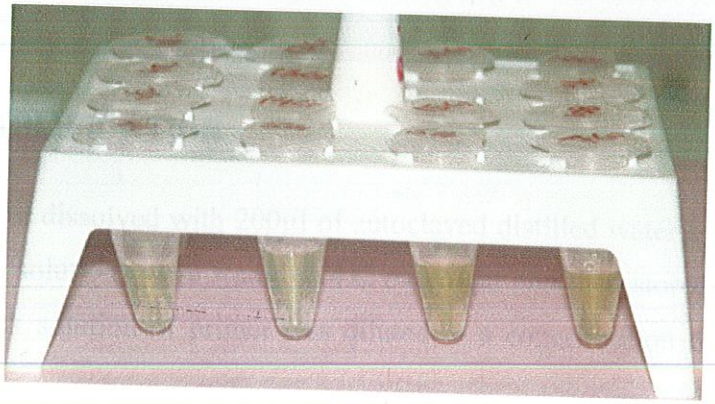
phase was transferred to fresh centrifuge tube with wide pore pipette and added 0.6 Volume (or 500 µl, whichever is minimum) of chilled isopropanol by quick & gentle inversion. It was incubated for 1 hour at -20$^0$C. DNA was then, rotated for 10 minutes at 10,000 rpm. The precipitated DNA was pulled out using disposable pipette and was washed with 70% ethanol (500 µl of ethanol was added and centrifuged at 10,000 rpm for 1 minute). This step was repeated one more time. The pellet was air dried and the precipitated DNA was dissolved in 200 µl of TE buffer. (Tris:EDTA::10:1).The DNA was then incubated at 4$^0$C overnight. RNA was removed by RNase treatment. DNA was quantified by comparing with known quantity of uncut λ DNA on the agarose gel, diluted to 12.5ng.µl$^{-1}$ and used in PCR.

The protocol was revised a few times to get the best possible results. Earlier the extraction buffer was added without warming it. Also, no incubation time was given after isopropanol treatment followed by a 10 min rotation at 10,000 rpm.


**After The Addition Of Isopropanol**



**Finally Obtained DNA after Dissolving in TE Buffer**



## 2.7Quantification of extracted DNA

## 2.7 QUANTIFICATION OF EXTRACTED DNA

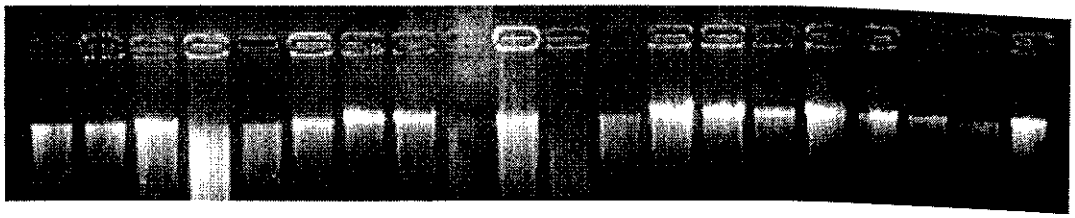
Checking the quality of the extracted DNA is very important as the product of DNA extraction will be used in subsequent experiments and poor quality DNA will not perform well in PCR.

For quantification 2ul of loading dye was added to 3ul of purified DNA. The samples were then loaded in 0.8% agarose gel. Lambda DNA was loaded in one well to compare the unknown DNA sample. The gel was run at 100V for 1hr. Then staining was done by using Etbr followed by de-staining and then the gel was observed under UV light.

**Results of Quantified DNA**

1. The ladder was found to have been denatured

2. **Quantified DNA** (The procedure was repeated with a fresh ladder)



## 2.8 PCR AMPLIFICATION

The primers were dissolved with 200µl of autoclaved distilled water by vortexing for 30 sec. The primer solutions were stored at 4℃ overnight and then stored at -20℃ till their usage. The stock solution of primer was diluted to a concentration of 50 pM for PCR amplification.

The following formula was used:

50pM X 100ul = WRITTEN CONC . X

» 50pM=conc. of primer sol. Required

» 100ul = the total working volume

» Written concentration = concentration of primers in pM on vial

» Unknown = amount of primer to be taken

» Water was added to make final working solution of primers = 100ul – unknown

## 2.8.1 Dissolution of primers :

**TABLE 6:**

| PRIMER ID | WRITTEN VOL | UNKNOWN | AUTOCLAVED DW |
|---|---|---|---|
| 12763 | 108.88 | 45.922(45) | 54.078(54) |
| 12764 | 115.515 | 43.248(43) | 56.716(57) |
| 12765 | 93.055 | 53.731(54) | 46.269(46) |
| 12766 | 113.43 | 44.080(44) | 55.920(56) |
| 12767 | 121.3 | 41.220(41) | 58.780(59) |
| 12768 | 116.205 | 43.027(43) | 56.973(57) |
| 12769 | 94.71 | 52.792(53) | 47.208(47) |
| 12770 | 90.115 | 55.484(55) | 44.516(44.5) |
| 12771 | 102.85 | 48.614(49) | 51.386(51) |
| 12772 | 101.74 | 49.144(49) | 50.856(51) |
| 12773 | 97.49 | 51.287(51) | 48.713(49) |
| 12774 | 120.355 | 41.543(41.5) | 58.457(58) |
| 12775 | 95.84 | 52.170(52) | 47.830(48) |
| 12776 | 94.27 | 53.039(53) | 46.961(47) |
| 12777 | 116.36 | 42.970(43) | 57.030(57) |
| 12778 | 103.71 | 48.211(48) | 51.789(52) |

**TABLE 7: For amplification of DNA the following materials were used:**

| MATERIALS USED | STOCK | REQUIRED | REQ. AMOUNT |
|---|---|---|---|
| Autoclaved DW | | | 7.8ul |
| DNA CONC. | 100ng/5ul | 40mg | 2ul |
| PRIMER{F} | 50pM | 5pM | 1.5ul |
| PRIMER{R} | 50pM | 5pM | 1.5ul |
| dNTPS | 2mM | 0.2mM | 1.5ul |
| Mgcl2 | 25mM | 1.8mM | 1ul |
| PCR buffer | 10X | 1X | 1.5ul |
| Taq polymerase | 5units | 0.5units | 0.2ul |
| Total volume: | | | 15ul |

**TABLE 8: Before amplication was done, the average melting temperature for each pair of forward and reverse primers was calculated:**

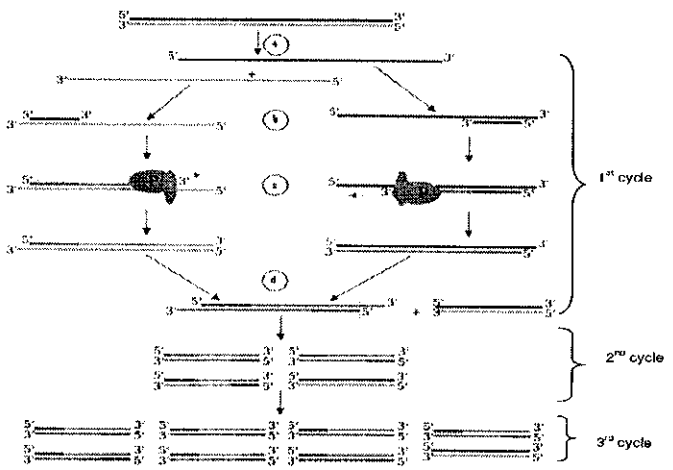
| PRIMER ID | Tm | avg |
|---|---|---|
| 12763 | 51,F | |
| 12764 | 49,R | 50 |
| 12765 | 51,F | |
| 12766 | 51,R | 51 |
| 12767 | 51,F | |
| 12768 | 51,R | 51 |
| 12769 | 49,F | |
| 12770 | 55,R | 52 |
| 12771 | 51,F | |
| 12772 | 51,R | 51 |
| 12773 | 51,F | |
| 12774 | 55,R | 53 |
| 12775 | 51,F | |
| 12776 | 51,R | 51 |
| 12777 | 53,F | |
| 12778 | 47,R | 50 |

**Polymerase Chain Reaction{PCR}**

8 primer pairs were used for the study of genetic diversity among the different genotypes of *Artemisia annua*. All the 8 primer pairs showed the amplification but none of the primer pairs were actually showing polymorphism. Amplification reactions were performed in volumes of 11 µl containing 3.9µl autoclaved distilled, 0.5µl(each) forward and reverse primers (1.6pM), 0.3µl dNTPs (0.2mM), 2µl $MgCl_2$ (37.5mM), 2.5µl PCR Buffer (1.X), 0.3µl Sigma Taq polymerase (0.5 units) and 50ng/ul of DNA. DNA amplification was performed using a Gene Cycler.

The amplification was done using Touchdown PCR (model). The first cycle consisted of denaturation of template DNA at 94 $^0$C for 4 min. The denaturation was reduced to 1 min for the next 40 cycles during which the primer annealing was done at a fall of ang Tm - 4.7 $^0$C for 1 min. Elongation was then done for 2 mins at 72$^0$C. Then, final extension was done at 72 $^0$C for 5 min and storage of the PCR product at 4 $^0$C for infinity.

The amplification products were resolved in 1.5% agarose gel (100 ml) was prepared by Adding 1.5 grams of Agarose in 1X TAE buffer(100ml). Wells were loaded with mixture of 2 µl of loading dye and 10 µl amplicon after the gel had solidified. DNA ladder of 100 kb was loaded along side the amplicons. Gel was run at 70 Volts for 2 hours. It was placed in EtBr (Ethidium Bromide) solution for 20 min in order to stain the gel. Followed by visualization in UV light for photography which was performed three times and the results were found reproducible.

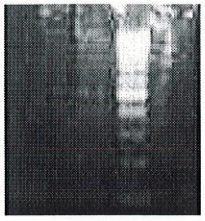


## 2.9 ANALYSIS OF AMPLIFIED DNA

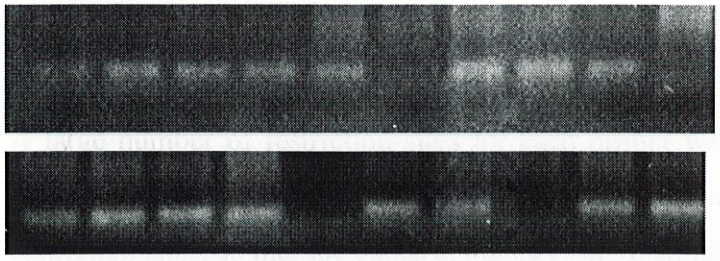
**Gradient PCR Result**

First of all to check the correct annealing temperature for the primer pairs gradient PCR was run. Gradient PCR is used to detect the amplification of the product at a particular temperature by providing range of temperatures during PCR. It was observed that annealing temperature was kept exactly 4.7°C lower than the average $T_m$ of the primer pair. Also water concentration has to be kept low in order to obtain a significant amplification.

**Result of Gradient PCR**



**Results of PCR For 20 Samples**

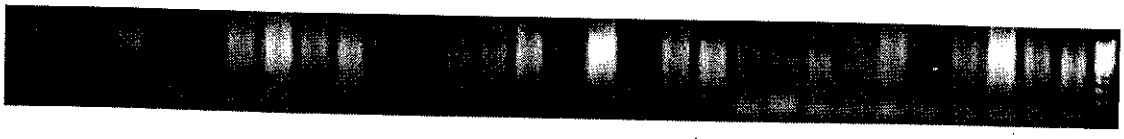


**Discussion:**

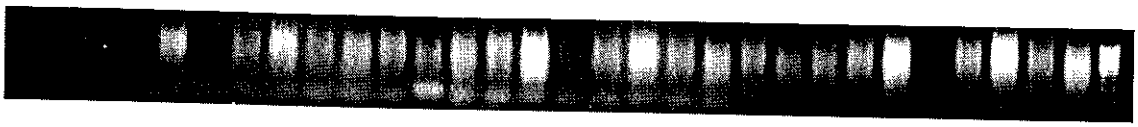
For checking band formation in PCR first single primer pair was used to standardize the protocol. Here, primers from gene "ads" were used and run with 20 samples. After the PCR it was found that there were no bands due to dimerization. Dimerization is a result of either selfing of the primers or due to the increased concentration of dimmers used to do the PCR.

To solve this problem, the concentration of the primers was decreased.

**Samples from Leh Valley:**



**Samples from Partapur:**



**Discussion:**

After decreasing the concentration of primers, finally the results of PCR were obtained. The figure depicts ladder on its extreme right wells. Starting from there on eight sets of primer pairs were added consecutively in all the samples. The prominent and thick band formation was seen corresponding to some of the primer pairs. Only that pair of primers and samples were selected for restriction digestions which were found to produce a thick band in PCR.

## 2.10 DETERMINATION OF RESTRICTION SITES

The first step was to find out the restriction sites for each primer in silico by using the software DNAstar (MapDraw).

A large number of restriction sites for each primer were found for different restriction enzymes. Those restriction enzymes were chosen which generally did not have many restriction sites, so that the bands could be easily distinguished.

**Results of MapDraw:**

```
Sequence : squalene.seq ( 1 > 404 )          64 Cut Sites
Enzymes  : All 242 enzymes (No Filter), 42 Enzymes Cut
Settings : Linear, Certain Sites Only, Standard Genetic Code

Enzyme              Freq        Position(s)

MnlI                  1     :     14
                    ‡
   CCTCNNNNNN N             :
   GGAGNNNNNN N             :
              †             :
```

```
Sequence : sesquiterpene.seq ( 1 > 411 )          75 Cut Sites
Enzymes  : All 242 enzymes (No Filter), 49 Enzymes Cut
Settings  : Linear, Certain Sites Only, Standard Genetic Code

Enzyme              Freq        Position(s)

HindIII               1      :     66
    ‡                        :
   A AGCT T                  :
   T TCGA A                  :
        †                    :

HinfI                 1      :    398
    ‡                        :
   G ANT C                   :
   C TNA G                   :
       †                     :

MnlI                  2      :     25     264
             ‡               :
   CCTCNNNNNN N              :
   GGAGNNNNNN N              :
            †                :
```

```
Sequence : fps.seq ( 1 > 401 )          75 Cut Sites
Enzymes  : All 242 enzymes (No Filter), 53 Enzymes Cut
Settings  : Linear, Certain Sites Only, Standard Genetic Code

Enzyme              Freq        Position(s)

Cac8I                 1      :    384
    ‡                        :
   GCN NGC                   :
   CGN NCG                   :
      †                      :

C
HindIII               1      :    217
    ‡                        :
   A AGCT T                  :
   T TCGA A                  :
        †                    :

HinfI                 1      :    339
    ‡                        :
   G ANT C                   :
   C TNA G                   :
       †                     :

MnlI                  4      :     78     192     290     372
             ‡               :
   CCTCNNNNNN N              :
   GGAGNNNNNN N              :
            †                :
```

```
Sequence : hmg.seq ( 1 > 484 )          97 Cut Sites
Enzymes  : All 242 enzymes (No Filter), 54 Enzymes Cut
Settings  : Linear, Certain Sites Only, Standard Genetic Code

Enzyme              Freq        Position(s)
EcoRI                 1      :    393
    ‡                        :
   G AATT C                  :
   C TTAA G                  :
        †                    :

MnlI                  6      :     12     381     402     458     470     474
             ‡               :
   CCTCNNNNNN N              :
   GGAGNNNNNN N              :
            †                :
```

## 2.11 RESTRICTION DIGESTION OF AMPLIFIED DNA BY RESTRICTION ENDONUCLEASES

Once all the desired information from the dry lab had been obtained , the procedure for standardizing the protocol for carrying out restriction digestion in the lab was performed.

**TABLE 9: The Standardized Protocol That Gave The Results Was As Follows:**

| Materials used | Volume(µl) |
|---|---|
| DNA | 10 |
| Restriction Buffer | 2 |
| Restriction enzyme | 1 (10 units) |
| Distilled water | 7 |
| Note: for some enzymes BSA was also added along with the restriction buffer. | |

Everything was kept in the ice bucket to avoid degradation. The above mentioned components were added in the PCR tubes and mixed gently. Then the samples were immediately kept for incubation at 37°C for 5 hours in a pre-set water bath. Except for the mixture containing Bsl I restriction enzyme, which was incubated at 55 °C. After the incubation was completed the mixture was spun at 3000 rpm for 15 seconds followed by heat inactivation (65°C/ 80°C) depending on the enzyme used and the temperature it required to be inactivated for about 10 minutes or so. Then 1% agarose (1gm in 100 ml 1X TAE buffer + 3µl Etbr) was prepared for running the mixture to check for digestion. The dye to mixture ratio was 1:5 (in µl).

**TABLE 10: List of Restriction Enzymes Used:**

| Restriction Enzyme | Buffer | BSA | Inactivation Temperature(°C) |
|---|---|---|---|
| Bam HI | 3 | Added | 65 |
| Bfa I | 4 | NA | 80 |
| Bsl I | 3 | NA | 80 |
| Cac 8I | 4 | NA | 65 |
| Eco RI | 2 | NA | 65 |
| Hind III | 2 | NA | 65 |
| Hin fI | 4 | NA | 65 |
| Mnl I | 4 | Added | 65 |

## TABLE 11: Components of Each Buffer Used:

### TABLE 11.1: Buffer 2: (pH 7.9 at 25°C)

| Components | Concentration (mM) |
|---|---|
| Nacl | 50 |
| Tris-Hcl | 10 |
| $MgCl_2$ | 10 |
| DTT | 1 |

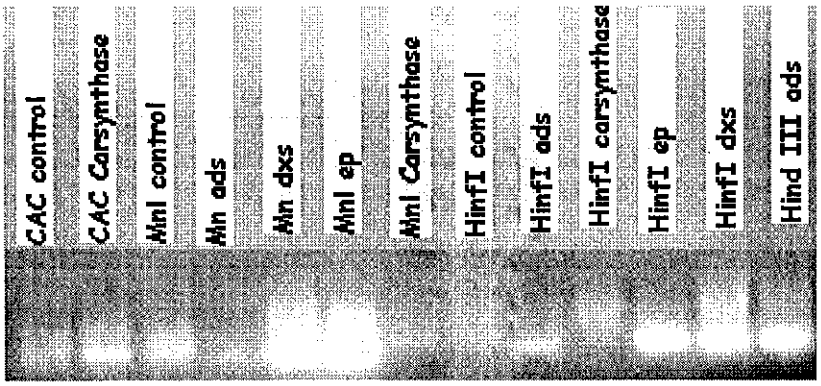
### TABLE 11.2: Buffer 3: (pH 7.9 at 25°C)

| Components | Concentration (mM) |
|---|---|
| Nacl | 100 |
| Tris-Hcl | 50 |
| $MgCl_2$ | 10 |
| DTT | 1 |

### TABLE 11.3: Buffer 4: (1X NE Buffer 4) (pH 7.9 at 25°C)

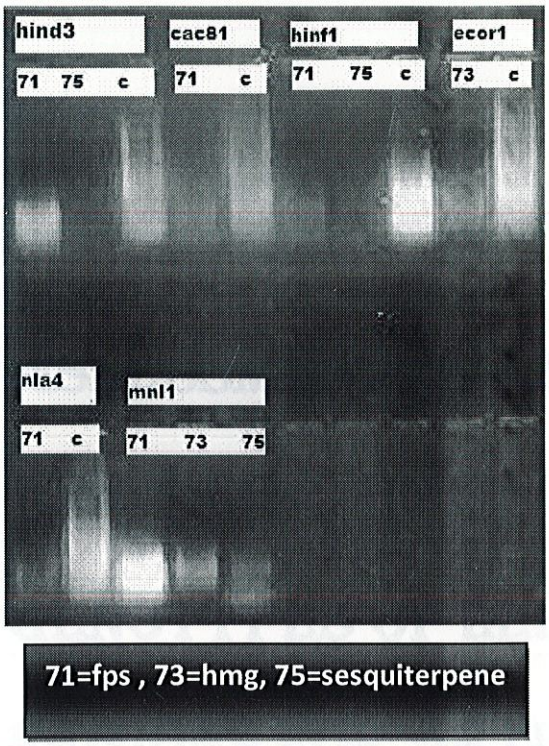
| Components | Concentration (mM) |
|---|---|
| Pottassium acetate | 50 |
| Tris acetate | 20 |
| Magnesium acetate | 10 |
| Dithiothreitol | 1 |

## 2.12 RESULTS




**Discussion:**

This figure depicts partial digestion of the sample by various restriction enzymes corresponding to their controls. Starting from extreme left, there are wells for cac8I control followed by cac8I used in digestion of caryophyllene synthase amplified pcr product. No restriction digestion was found in this. Then MnlI control followed by MnlI digestion of ads, dxs, epicedrol and caryophyllene synthase amplified pcr products is seen where only dxs and epicedrol showed partial digestion. Next hinf1 control followed by hinf1 digestion of ads, caryophyllene synthase, epicedrol and dxs amplified pcr products is seen, where caryophyllene synthase and dxs showed partial digestion.




71=fps , 73=hmg, 75=sesquiterpene

**Discussion:**

This figure depicts partial digestion of the sample by various restriction enzymes corresponding to their controls. Starting from extreme left upper row, there are wells for restriction digestion by hindIII enzyme on fps and sesquiterpene amplified pcr products followed by its control. Partial digestion was found in fps containing amplicon. Next restriction digestion on cac8I enzyme on fps and control was seen and no digestion was found at all. Then restriction digestion by hinfI showed no digestion at all. After that, using EcorI on amplified product by hmg primers showed partial digestion when compared to its control.

Coming on to the second row, Nla4 enzyme showed partial digestion on amplicon containing fps when compared to the control. And finally, MnlI enzyme showed partial digestion on amplicons containing fps, hmg and sesquiterpene.

# CHAPTER 3

## ASSESSMENT OF GENETIC DIVERSITY AMONG ARTEMISIA ANNUA GENOTYPES OF LEH AND PARTAPPUR REGIONS FOR ARTEMISIN PRODUCTION USING CAPS MOLECULAR MARKERS.

## 3.1 INTRODUCTION

Artemisinin is isolated from the aerial parts of *Artemisia annua* L. It is a promising and potent antimalarial drug which has a remarkable activity against chloroquineresistant and chloroquine-sensitive strains of Plasmodium falciparum, and is useful in treatment of cerebral malaria.

Because the low content (0.01–1 %) of artemisinin in *A.annua* is a limitation to the commercial production of the drug, many research groups have been focusing their researches on enhancing the production of artemisinin in tissue culture or in the whole plant of *A. annua*.

Biosynthesis of artemisinin has been reported to accumulate in the leaves, small green stems, buds, flowers, and seeds of *A.annua*. Its content varies with the plant developmental stages. There are mainly two suggestions on the stage of the highest content of artemisinin in plant development: one is that the highest content of artemisinin is reached before plant flowering, while the other is that it is reached in the full flowering period.

## 3.2 ANALYSIS OF PRESENCE OF ARTEMISININ BY SPECTROPHOTOMETRIC AND GAS-CHROMATOGRAPHIC TECHNIQUES {GC-FID}

### 4.2.1 Spectrophotometric Determination of Artemisinin (ART)

A simple and rapid method for determination of ART is described. The method is based on the reaction of $H_2O_2$ generated by cleavage of endoperoxide linkage of ART and its reaction with potassium iodide (KI) to liberate iodine (I). Liberated I bleaches red colored safranine O to colorless species and is measured at 521nm. Beer's law is obeyed in the range of 16-112 µg/ml for both ART & DHA. The molar absorptivity, Sandell's sensitivity, detection limit and quantitation limit for ART were found $0.3401\times10^4$ l/mol/em, $1.43\times10^{-2}$ µg/cm$^2$, 0.0625 µg/ml and 0.2075 µg/ml. The optimum reaction

conditions and other parameters were evaluated. The statistical evaluation of the method was examined by determining intraday and inters day precision.

**Experimental Procedure:**

Apparatus: spectrophotometer

Reagents: HCl - 5M

KI – 2%

CH3COONa – 2M

Safranine O – 0.01 % (0.01g dissolved in 50% ethanol and making up the volume to 100ml)

Standard ART solution: stock - 1000µg/ml (dissolve 0.1g ART in ethanol and raise the volume to 100ml), working solution – stock solution was diluted appropriately to get the working solution.

**Method of Determination of ART:**

1. Take 10ml calibrated flask.

2. Transfer different aliquots of ART in a series (16.0-112.0µg/ml)

3. Add 1ml of KI and 1ml of HCl to each flask.

4. Gently shake the mixture until the appearance of yellow color due to liberation of iodine.

5. Shake the reaction mixture for 5 minutes.

6. Contents were diluted with distilled water and mixed well.

7. The absorbance of each solution was measured at 521nm against corresponding reagent blank (made by replacing ART by distilled water).

8. Absorbance corresponding to the bleached color, which in turn corresponds to analyte ART concentration, was obtained by: ABSORBANCE OF BLANK- ABSORBANCE OF TEST SOLUTION

**Results and Discussion:**

The method involves reaction of $H_2O_2$, generated by cleavage of endoperoxide linkage of corresponding ART solution in acidic medium, with KI to liberate iodine. This liberation of iodine bleaches the red colored safranine O which were measured at 521nm. The decrease in absorbance is proportional to Artemisinin concentration.

**Analytical Data:**

1. Adherence to BEER'S LAW: Studied by measuring absorbance values at maximum absorbance and concentration ranges, this must show linear relation.
2. Regression analysis of BEER'S LAW data: LEAST SQUARES METHOD was made to evaluate the slopes, intercept and correlation coefficients for each system of Artemisinin.

The accuracy of the method was established by analyzing the pure drug at three levels and the precision as ascertained by calculating the relative standard deviation on the same solution.

## 3.3 STATISTICAL ANALYSIS

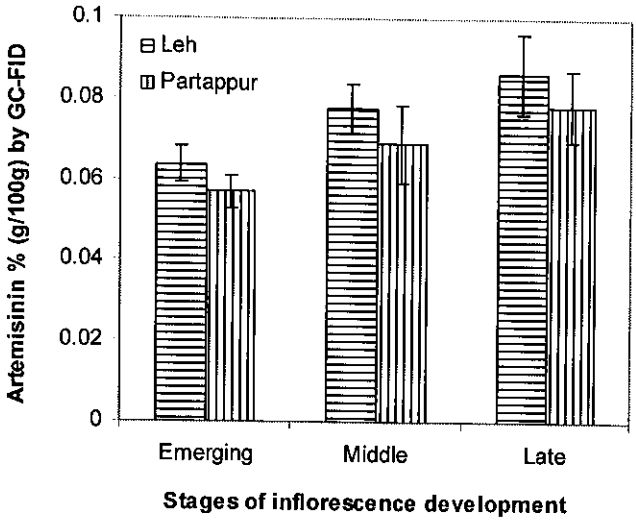



Figure. Artemisinin content (w/w %) of inflorescences analyzed by both (a) GC-FID and (b) Spectrophotometric methods at 3 stages of development of *A. annua*. The values are means of 10 replicates. Vertical bar represent ± standard deviation. Samples were obtained from 2 different sites Partappur and Leh from Ladhak region.

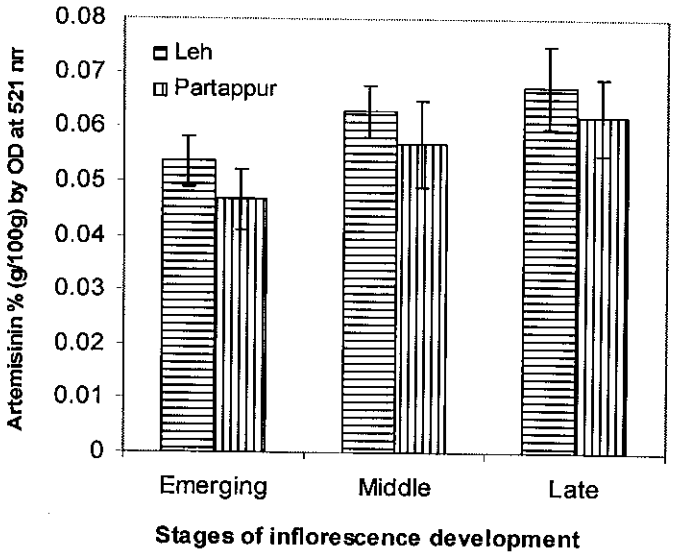
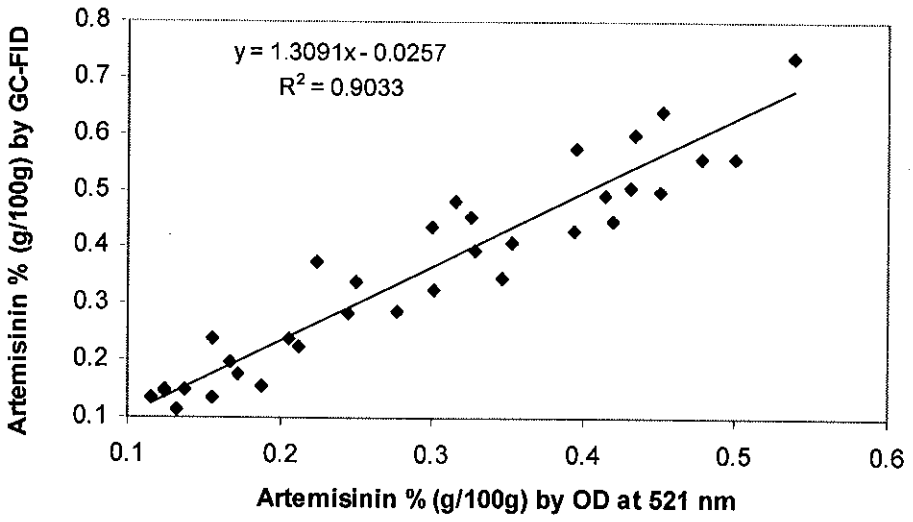



Figure. Artemisinin content (w/w %) of inflorescences analyzed by both (a) GC-FID and (b) Spectrophotometric methods at 3 stages of development of *A. annua*. The values are means of 10 replicates. Vertical bar represent ± standard deviation. Samples were obtained from 2 different sites Partappur and Leh from Ladhak region.

**Discussion:**

The Artemisinin content present during 3different stages of inflorescences, when analyzed by both GC-FID and spectrophotometric methods, the three different stages are emerging (budding stage), middle (flowering stage) and late (later stage). The values in the bar graph are means of ten replicates and vertical bars represent the standard deviation. Individual bars for different regions have been plotted. The artemisinin content was found to be more in samples from the Leh region as with those compared to the Partappur samples in both spectrophotometric and GC FID analysis. It was found that the artemisin yield was higher in samples from higher altitude i.e. Leh Valley. Hence, altitude affects artemisinin yield and they are directly proportional (higher the altitude, higher the yield). It was also observed that the artemesinin content was found to be very high in the later stages (Late) of inflorescence than the emerging and the middle stages.

(a) **Partappur**




(b) **Leh**

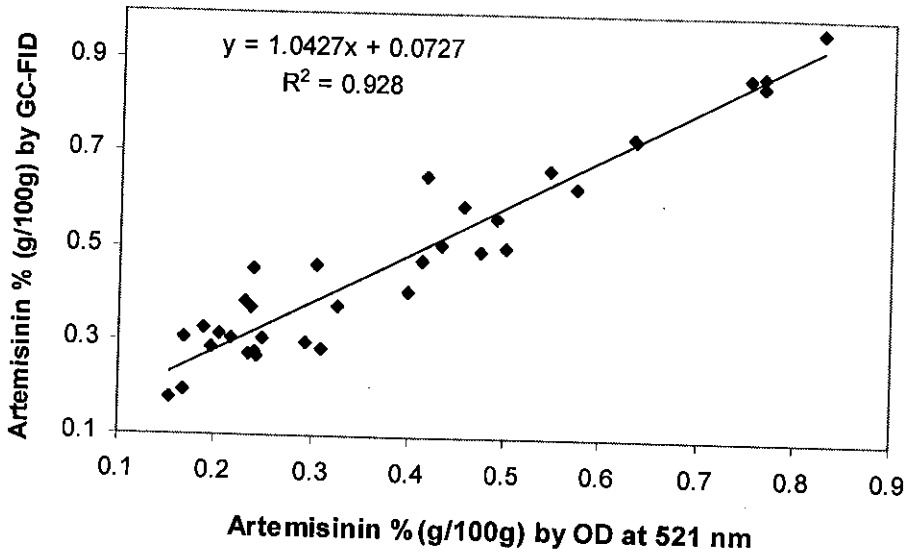



Figure. Correlation of artemisinin content (w/w %) of 35 plant materials analyzed by both GC-FID and Spectrophotometric methods. These plant materials consisted of leaves from different parts of 35 cultivars of *A. annua*. Samples were collected from 2 different sites (a) Partappur and (b) Leh from Ladhak region.

**Discussion:**

This result is the estimation of artemisinin from 35 different cultivars of *Artemisia annua*. The regression ($R^2$) is very high which implies a positive correlation between the values obtained from GC-FID and SPECTROPHOTOMETRIC methods. The graph here shows a linear increase in atremesinin content obtained by using these methods.

## 3.4 RECORDING OF DATA

**TABLE 12: Quantification of artemisinin from the different stages of inflorescence development of *A. annua* collected from Leh and Partappur from Ladhak region.**

| | Emergence | Middle | Late | F-value | P-value |
|---|---|---|---|---|---|
| | Mean ± SEM | Mean ± SEM | Mean ± SEM | | |
| **(a) Artemisinin % (g/100g) by GC-FID method** | | | | | |
| **Leh** | 0.064 ± 0.008 | 0.078 ± 0.002 | 0.086 ± 0.003 | F1 = 13.20 | < 0.001 |
| **Partappur** | 0.057 ± 0.004 | 0.069 ± 0.003 | 0.078 ± 0.002 | F2 = 5.00 | 0.030 |
| **(b) Artemisinin % (g/100g) by OD at 521nm** | | | | | |
| | 0.054 ± 0.006 | 0.063 ± 0.001 | 0.067 ± 0.002 | F1 = 8.71 | 0.001 |
| | 0.047 ± 0.004 | 0.057 ± 0.002 | 0.062 ± 0.007 | F2 = 4.55 | 0.038 |

**Discussion:**

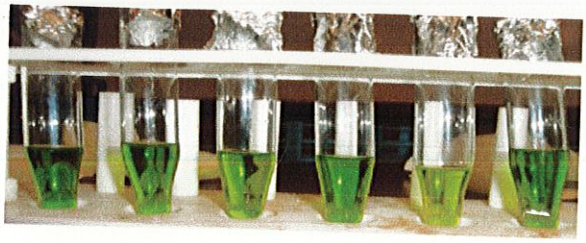
F1 and F2 are the F-value between the stages of development and site of sample collection. The F-value between the interaction of sites and stages of inflorescence development is 0.03 and 0.02 respectively using both the estimation methods and are not statistically significant. The statistical test used for the analysis is 2-way anova. The p-value is the observed significance level of a hypothesis test which equals the probability of making a Type I error. As the p-value is very also is nearly all the cases, null hypothesis is rejected. The result in our case is statistically significant because the F-values in all the cases are greater than p-values.

## 3.4EXTRACTION OF ARTEMISININ AND QUANTIFICATION

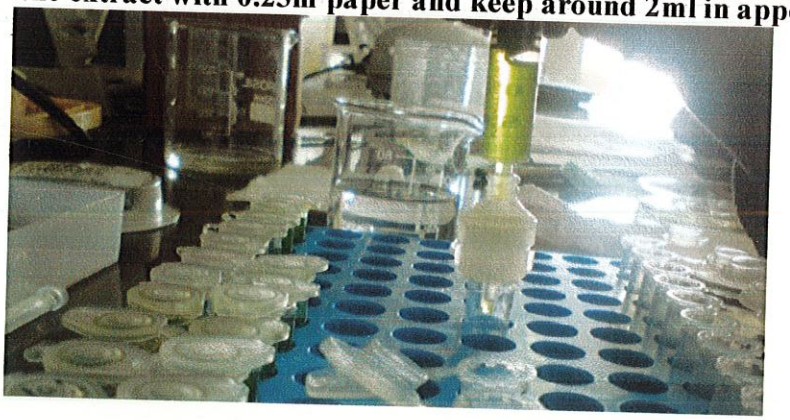
Dried leaves and inflorescences were ground to a powder in a pestle and mortar. Artemisinin was extracted following the procedure of Broomhead et al., (1990). The extract was filtered through Whattman filter paper No.1. Second, third and fourth extractions of the same samples were done with 10 ml ethanol for 10 minutes under condition mentioned above. All the extracts were pooled and ethanol evaporated to dryness in a water bath shaker at 60 $^{0}$C. The resultant residue was dissolved in 10 ml acetonitrile (HPLC grade) and filtered with 0.22 µm durapore membrane filter

**3. Add 50ml of ethanol to the residue and heat at 30°C for 2 hours**

**4.Filter the soln. and pool into the first test tube. Measure the volume and note.**



**5.Filter the extract with 0.25m paper and keep around 2ml in appendorf**



HPLC analysis was carried out using Nova Pack C18 cartridge column (250 x 4.6 mm) in HPLC system (Water). Acetonitrile: water: methanol (37:58:5) was used as a mobile phase with a flow rate of 1.0 ml. $min^{-1}$. Crude extract (20 µl) was used for injection into the HPLC system. Artemisinin was detected at 230 nm (490 E multi Wavelength Detector, Waters). Artemisinin (0.1 $g.l^{-1}$; Sigma, P-4405) was used as a standard for calculating Artemisinin content in the samples on the basis of peak heights. All the experiments on extraction of Artemisinin and HPLC analysis were repeated three times.

# CONCLUSION

DNA extraction protocol was standardized, which was a must as quality of DNA was very bad because of the presence of polyphnolic compounds, the extracted DNA was used for amplification of regions of Artemisia annua genes which are responsible for production of secondary metabolite, Artemisinin. These genes were of those enzymes which play a key role in the formation of Artemisinin. The primers designed for amplification were designed after a thorough study of DOXP pathway for Artemisinin production. Restriction digestion was also check for polymorphism, although no polymorphism was observed. The samples collected from partapur and leh valley of ladakh region. These samples were analyzed and quantified, results of which state that Artemisinin content increases with the stage of flower, that is, its low in early stage (budding stage) whereas comparatively higher in late stages, also, content of Artemisinin in samples from Leh region were higher than those of Partapur region i.e. the content was higher is samples from higher altitude. Results of this research could be very helpful in selection of genotype of Artemisia annua for commercial production.

# BIBLIOGRAPHY:


[Cancer Research 65:(23).Dec 1, 2005]

(JLP/2007/134907) Jay Keasling's work for Synthetic Biology at the University of California, Berkeley

General concepts for PCR primer design.
C W Dieffenbach, T M Lowe and G S Dveksler
*Genome Res.* 1993 3: S30-S37

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Molecular markers in Chinese medical materials

(wo/2007/112596) nucleotide sequences encoding enzymes in biosynthesis of dihydroartemisinic acid

- http://www.keygene.com/

- Zabeau, M and P. Vos. 1993. Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Office, publication 0 534 858 A1, bulletin 93/13.

Vos P, Hogers R, Bleeker M, *et al.* (November 1995). "AFLP: a new technique for DNA fingerprinting". *Nucleic Acids Res.* 23 (21): 4407–14. PMID 7501463. PMC: 307397. http://nar.oxfordjournals.org/cgi/pmidlookup?view=long&pmid=7501463.

Mueller UG, Wolfenbarger LL (October 1999). "AFLP genotyping and fingerprinting". *Trends Ecol. Evol. (Amst.)* 14 (10): 389–394. PMID 10481200. http://linkinghub.elsevier.com/retrieve/pii/S0169534799016596.

Meudt HM, Clarke AC (March 2007). "Almost forgotten or latest practice? AFLP applications, analyses and advances". *Trends Plant Sci.* 12 (3): 106–17. doi:10.1016/j.tplants.2007.02.001. PMID 17303467

Watson, L. E. et al. (2002). "Molecular phylogeny of subtribe Artemisiinae (Asteraceae), including Artemisia and its allied and segregate genera". *BioMed Central Evolutionary Biology* 2 (17). Available online.

Neff MM, Neff JD, Chory J, Pepper AE. dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. *Plant J. 1998 May;14(3):387-92.* PMID: 9628033

African Journal of Biotechnology Vol. 5 (25) pp. 2540-2568 29 December, 2006 Available online at http://www.academicjournals.org/AJB ISSN 1684–5315 © 2006 Academic Journals