Conservation and Assessment of Physico-chemical and Molecular diversity of the genetic resources of the genus *Aloe* occurring in Uttaranchal, India (Kumaon and Garhwal region)

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BY

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DEDICATED TO MY WIFE

AND WELL WISHERS

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "Conservation and Assessment of Physico-chemical and Molecular diversity of the genetic resources of the genus *Aloe* occurring in Uttaranchal, India (Kumaon and Garhwal region)" submitted by Mr. Rijwan N. Pinjari was carried out by the candidate under my supervision at the Plant Tissue Culture Division, National Chemical Laboratory, Pune.

Any material obtained from other sources has been duly acknowledged in the thesis.

(Dr. Mrs. S. R. Thengane)
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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled "Conservation and Assessment of Physico-chemical and Molecular diversity of the genetic resources of the genus *Aloe* occurring in Uttaranchal, India (Kumaon and Garhwal region)" submitted for Ph. D. degree to the University of Pune has not been submitted by me to any other university for a degree or diploma.

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KEY TO ABBREVIATIONS

^{0}C-	Degree Celsius.
2-D TLC –	2 Dimensional Thin Layer Chromatography.
AAA –	Amino Acid Analysis
AOAC-	Association of Official Analytical Chemists.
CAM –	Crassulecean Acid Metabolism
cm-	Centi meter
DARL –	Defenec Agricultural Research Laboratory
DRDO –	Defence Research and Development Organisation
EDTA –	Ethylene Diamine Tetra Acetic acid.
FACE-	Fluorophore-Assisted Carbohydrate Electrophoresis
FPLC –	Fats Performance/ Protein Liquid chromatography.
GAP-	Good Agricultural Practise.
GC- MS –	Gas Chrpmatography Mass Specroscopy
GLC –	Gas Liquid Chromatography.
ICP/AES –	Inductively Coupled Plasma Atomic Emission Spectrphotometry.
IEF –	Iso- Electric Focussing
IR –	Infra Red
Mb-	Milibar
Min –	Minutes
MORA-	Mandibular Orthopedic Repositioning Appliance.
MP –	Morse Potential
MSL –	Main Sea Level.
NCIM-	National Collection of Industrial Microorganisms.
NMR –	Nuclear Megnetic Resonance.
OD –	Optical Density
ORAC –	Oxygen Radical Absorbance Capacity.
PAGE –	Polyacrylaminde Gel Electrophoresis.
PVP-	Polyvinyl pyrrolidone
RAPD-	Rapid Amplified Polymorphic DNA.

RNase-	Ribonuclease.
Rpm-	Rotations Per Minute.
SCF	Supercritical Fluid Extraction
SDS-	Sodium Dodesyl Sulphate
SOD –	Sulphur Oxide Dismnutase
TBA –	Tertiary Butyl Alcohol.
TBE –	Tris Borate EDTA
TE-	Tris EDTA
TLC-	Thin Layer Chromatography
μ mole –	Micro mole
USD-	United States Dollar
UV-	Ultra violet.
WHO-	World Health Organisation
λ-	Wavelength

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ABSTRACT

Rationale of the study:

Ours is a vast country where wide variation in climate, soil, altitude and latitude is available. Nature has bestowed on us a very rich botanical wealth and a large number of diverse types of plants grow wild in different parts of the country. Our knowledge of medicinal plants has mostly been inherited traditionally.

Spreading and preserving the knowledge on medicinal plants and their uses has become important for human existence. There is a growing tendency all over the world to shift from synthetic to natural based product including medicinal plants. In modern medicines, plants still occupy a very significant place as a raw material for some important drugs and neutraceuticals, although synthetic drugs and antibiotics brought about a revolution in controlling different diseases.

In India use of different parts of several medicinal plants to cure specific ailments has been practiced from ancient times. Nearly 70 % of our population residing in the villages is dependent upon the indigenous systems of medicines like Ayurvedic, Siddha, Unani etc., which has been in existence for several years.

The genus *Aloe* consists of perennial, drought resisting, succulent plants belonging to Liliaceae family. The true *Aloe* is called as *Aloe barbadensis* (Mill.) and about 300 or more species are found around the world. It is also known by several names like The wands of Heaven, Heaven's Blessing, The Silent Healer etc. (Coats 1979).

The *Aloe* plant being a cactus plant contains about 98 to 99 % water with an average pH of 4.5. The remaining solid material contains about 246 different ingredients, which are responsible for the medicinal properties of *Aloe* (Joshi 1998, Xiao 2000). Among which some important ingredients are phenolics (anthraquinones) (Okamura 1996), vitamins (Coats 1979), minerals (Shelton 1991), enzymes (Sabeh 1996), sugars i.e. carbohydrates (Hirat and Suga 1983), sterols (Coats 1979), amino acids and salicylic acid (Sabeh 1996). The constituents of prime importance are aloin, aloe-emodin, barbaloin and acemannan as they are of high pharmaceutical and clinical importance.

The research on *Aloe* is mainly concentrated on the species *Aloe barbadensis* (Mill.) and *Aloe perryi*, which are of tremendous pharmaceutical, nutritional and economic importance.

This research work is undertaken to assess the amount of different phytochemicals, their potential use as prebiotics and antimicrobials in *Aloe* species/varieties collected from different regions of Uttaranchal (Kumaon and Garhwal). Identification of *Aloe* species by molecular marker techniques can also be established to identify species differences. The research work is confined to the six ecotypes of *Aloe barbadensis* (Mill.) found in this region and *Aloe perryi.*

Objectives:

1. Screening of Uttaranchal (Kumaon and Garhwal region) for collection of different *Aloe* species/varieties.
2. Authentification of these collected species/varieties.
3. To maintain these collected germplasm in polyhouses and fields with agro technological developments.
4. To study morphological features of collected species/varieties.
5. To evaluate the phytochemicals of nutritional importance.
6. Analysis of pharmaceutically important aloin content of these plants by HPLC methods.
7. *In vitro* evaluation of prebiotic and synbiotic potential of these plants on selected probiotics.
8. Assessment of anti microbial activity of these plants on human pathogens.
9. Genetic diversity analysis of these genotypes by ISSR markers.

Brief outline of the thesis is as follows.

Chapter No. 1:- Introduction

This chapter includes information about the purpose and need of research, survey of literature, details about the region i.e. Uttaranchal and its purpose of selection; geographical, climatic, soil and nutritional status of the region, distribution of *Aloe* and its importance from nutritional point of view and as a need of DRDO and its use as neutraceuticals, food and food supplements, products etc.

Chapter No. 2:- Collection, Conservation, Authentication, and Maintenance

This chapter covers conservation aspect, agricultural practices like plants' spacing, watering, weeding etc. standardized for the mass cultivation at different altitudes. It will also cover the maintenance of the plants in polyhouses, glass houses and in open fields. No. of plants collected and cultivated and authentication of the collected plants from the authorized organization, determination of physiological parameters will also be included in this chapter.

Chapter No. 3:- Neutraceutical Assessment

This chapter will include assessment of biochemical composition like carbohydrates, fats, proteins, vitamins, minerals etc. among all ecotypes and also grown at four different locations of Uttaranchal at different altitudes. Assessment of aloin, a pharmaceutically important compound, by HPLC among all ecotypes and at these four different altitudes along with the applications will be added.

Chapter No. 4:- *In vitro* evaluation of prebiotic and synbiotic potential

Content of this chapter will be use of gel from these ecotypes as potential source of prebiotics, their role in growth of specific probiotics and possibilities of these prebiotic for the manufacture of novel and tailored oligosaccharides. Their role in development of functional foods and targeted disease resistant capabilities will also be included.

Chapter No. 5:- Assessment for antimicrobial activity of the extracts

This chapter consists of the study of these plants for the activity against the common human and food pathogens like *E. coli, Staphylococcus* spp. and *Salmonella* spp. Water, hexane and methanol extracts from the oven dried leaves of these ecotypes were assessed for their anti microbial activity by bore well method and paper disc method.

Chapter No. 6:- Genetic diversity analysis by ISSR

This chapter will deal with genetic diversity analysis of the ecotypes by PCR based molecular technique using ISSR. Similarity or the differences in the genetic make up and basis for morphological and compositional differences on the basis of this genetic make up will also be discussed in this chapter.

Chapter No. 7:- Summary and Future Directions

This will be the summarization of all chapters. Application of the research for defense persons; especially the socioeconomic development and improvement of nutritional status of people in Uttaranchal region will be highlighted. The use of knowledge for generation of employment opportunities through mass and commercial cultivation, establishing cottage industries will also be discussed.

Chapter 1

Introduction

Aloe gets dubbed with emotive terms such as "The Silent Healer" and "Panacea" because of its fundamental actions to improve the vitally important systems of the body

1.1 INTRODUCTION:

In the modern world, the herbal medicines have become the need of today and tomorrow. The reason being the growing tendency of shift from synthetic to natural based products. However, in advanced medicines like nutraceuticals, cosmeceuticals and food therapy plants, still occupy a very significant place as a raw material. Since pre-historic times man has been in a way to search the cures and relief from physical and mental illness by using numerous plants and plant-derived products (Ballabh and Chaursiya, 2007). Use of different parts of several medicinal plants to cure specific ailments has been practiced from ancient times. In the United States, the market for medicinal herbs is worth more than $3 billion. Medicinal plants are distributed all over the world in diverse habitats but major share is in the tropical rain forest. According to the WHO (World Health Organisation) over 60% of the world's population and 80% in developing countries depends directly on plants for their medical purposes (Farnsworth *et al.,* 1985; Shrestha and Dhillion, 2003). Apart from this, the plant derivatives share 25% of modern medicinal system.

In the past, quantities needed to meet demand were relatively low; however, increasing commercial demand is fast outpacing the supply. Currently, between 4,000 and 10,000 medicinal plants are on the endangered species list and this number is expected to increase (Canter *et al.,* 2005). In India, utilization of plants for medicinal purposes has been documented long back in ancient Indian literature like Ramcharitmanas of Tulsidas (1631 Samvat); Charak Samhita (Sastri *et al*., 1996) etc. However, organized studies in this direction were initiated in 1956 (Rao, 1996) and off late such studies are gaining recognition and popularity due to revival of interest in herbal and traditional medicines especially where modern medicines has limitations in curing some of the ailments. Hence spreading and preserving the knowledge on medicinal plants and their uses has become important for human existence.

1.2 Biodiversity: The Indian Scenario:

The most striking feature of the earth is the existence of life, and the most striking feature of life is its diversity (Tilman, 2000). Topography, soil, climate and geographical location of a region influence the vegetational diversity of a particular ecosystem. India is a vast country with wide variation in climate, soil, altitude and latitude. Nature has bestowed a very rich botanical wealth and large number of diverse types of plants grows wild in different parts of the country.

In India knowledge of medicinal plants has been mostly inherited traditionally. Nearly 70 % of Indian population residing in the villages is dependent on the indigenous systems of medicines like Ayurvedic, Siddha, Unani and Tibetian etc., which are in existence since historic times.

Out of the total 4,20,000 flowering plants reported from the world (Govaerts, 2001) more than 50,000 are used for medicinal purposes (Schippmann *et al.,* 2002). In India, the total land area is 2.4% of the total geographical area of the world and it comprise of 8% of total global biodiversity with around 49000 species of plants. Among these 4900 are endemic in nature (Kumar and Asija, 2000). Approximately 8000 species are of medicinal plants. Around 43% of the total Indian flowering plants are reported to be of medicinal importance (Pushpangadan, 1995). Among these medicinal plants one third are trees, an equal proportion are of shrubs and the remaining one third are herbs, grasses and climbers. Very small portion of medicinal plants are lower plants like lichens, ferns, algae etc. Majority of the medicinal plants are higher plants (Maiti, 2000). In India, around 386 families and 2200 genera of the medicinal plants are reported. Some of these are from *Asteraceae, Euphorbiaceae, Laminaceae, Fabaceae, Rubiaceae, Poaceae, Acanthaceae, Rosaceae* and *Apiaceae*. Among these the largest contribution is of *Asteraceae* having 419 species. The major part of the medicinal plants i.e. 90% used by pharmaceutical industries, is collected from wild.

The ecosystems of Himalayas, the Khasi and Mizo hills of north eastern India, the Vindhya and Satpura ranges of northern peninsular India and the western ghats contains nearly 90% of the Indian higher plant species. Hence these ecosystems have the special importance in traditional medicinal practices. Among these, peninsular Indian forest and Western ghats have the varietal richness (Parrola, 2001).

Peninsular India extending downwards from the states of Gujrat, Madhya Pradesh and Southern Bihar are dominated by a continuum of tropical forests namely thorn forest, dry deciduous forest, moist deciduous forest, dry evergreen forest, wet evergreen forest and semi evergreen forest. The complexity with respect to soil, topography and climate has created an exceptional variety of biomass and specialized habitats within this region. The ecosystem of southern peninsular India with the Southern Western Ghats contains more than 6000 species of higher plants including an estimated 2000 endemic species. Of these 2500 species representing over 1000 genera and 250 families have been used in Indian system of medicine (Jain, 1991).

1.2.1 Mega- Biodiversity hotspots: -

The varietal richness in flora and fauna of a particular region is called as the Biodiversity and the region with maximum biodiversity is called as Biodiversity Hotspot. On earth 25 biodiversity hot spots are reported and out of these India has two mega-biodiversity centers - The Eastern Himalayas and the Western Ghats.

Eastern Himalaya, which covers parts of Nepal, Bhutan, the northeast Indian states of West Bengal, Sikkim, Assam, and Arunachal Pradesh, southeast Tibet (China), and northern Myanmar. It is the major source of plant taxa- *Rhododendron, Primula and Pediculari.*

The Western Ghats runs north to south along the western edge of the Deccan Plateau, and separates the plateau from a narrow coastal plain along the Arabian Sea. The range starts near the border of Gujarat and Maharashtra, south of the River Tapti, and runs approximately 1600 km through the states of Maharashtra, Goa, Karnataka, Tamil Nadu and Kerala ending at Kanyakumari, at the southern tip of India. About sixty percent of the Western Ghats are located in the state of Karnataka. These hills cover 60,000 km² area, average elevation is around 1,200 m and has over 5000 species of flowering plants. At least 325 globally threatened species occur in the Western Ghats.

1.2.2 Trade of herbal drugs in India: -

Over 70% collection of the plants involves destructive harvesting because of use of plant parts like bark, roots, stem, wood and whole plant in case of herbs. But only less

than 20 species are under cultivation. This is a definite threat to the genetic diversity and stocks. The continuing decline of plant diversity will have a greater impact on human society than any other type of biodiversity loss. It is imperative, therefore, that efforts are increased to assess the conservation status of plants for the IUCN Red List of Threatened Species, the de facto baseline reference for many conservation decisions (Schatz, 2009).

Of the 960 traded medicinal plant species, 178 species are consumed in volumes exceeding 100 MT (Metric Tonnes) per year, with their consolidated consumption accounting for about 80% of the total industrial demand of all botanicals in the country. Analysis of above 178 species by their major sources of supply reveals that 21 species (12%) are obtained from temperate forests, 70 species (40%) are obtained from tropical forests, 36 species (20%) are obtained largely or wholly from cultivations/plantations, 46 species (25%) are obtained largely from road sides and other degraded land use elements and the remaining 5 species (3%) are imported from other countries. The annual demand of herbal raw drugs in the country has been estimated at 3,19,500 MT for the year 2005-06. An annual trade value for these herbal raw drugs in was 1,0691 crores (Ved and Goraya, 2007).

1.3 The Himalayas:

Indian Himalaya (27^{O} 50' – 37^{O} 06' N and 72^{O} 30' – 97^{O} 25' E) includes the parts of trans, northwest, west, central and eastern Himalaya. Himalayan ranges are one of the richest sources of several valuable and economically important medicinal and aromatic plants. The reason being the diversified topography, soil and microclimatic zones. A total geographical area of Indian Himalaya is about 594427 sq. km, which is about 18% of the total area of the country. Length is about 2400km and width is around 240-320 km. Over 51 million people (6% of the total population of India) reside in Indian Himalayan Region (Anonymous, 1992). The vegetation varies from subtropical, temperate, and subalpine to alpine types. It harbors about 8000 species (47.06 % of the total flowering plants of India) of which 30 % are endemics, 10.2 % trees, 8.44 % wild edibles and over 15 % medicinal (McGinley, 2008).

The Himalaya is divided in three main regions viz.

- **Eastern Himalayas** which includes Assam, Darjeeling, Sikkim Himalaya- The Eastern Himalaya is also a center of diversity for several distributed plant taxa, such as *Rhododendron, Primula and Pedicularis.*
- **Central Himalayas** which includes Nepal Himalaya- Central Himalayan biodiversity is used variously for fodder, fuel wood, timber, leaf litter for manuring crop fields, construction, industrial raw material and several non-timber forest produce
- **Western Himalayas** which includes Kashmir, Himachal Pradesh, Kumaon and Garhwal Himalayas.

The Himalayan forest vegetation ranges from tropical dry deciduous forests in the foothills to alpine meadows above timberline (Singh and Singh, 1992). Of the estimated 8,000 species of plants in the Himalaya hotspot, about 3160 are endemic with 71 genera. Further more, five plant families are endemic to the region, the Tetracentraceae, Hamamelidaceae, Circaesteraceae, Butomaceae and Stachyuraceae. The largest family of flowering plants in the hot spot is the Orchidacea with 750 species. It indicates that further exploration will probably reveal a much higher degree of plant endemism.

In Himalaya, a zone of permanent rock and ice begins at about 5500 to 6000 meters. However the records of occurrence of vascular plants have been found at some of the highest elevation on earth. Certain plants were recorded at the height of more than 6100 meters and high altitude scree plants in the mustard family are recorded at 6300 meters on the slopes of Mount Kamet in the north western Himalayas.

In India this immense mountain range, which covers nearly 750,000 km^2, has been divided into two regions: the Eastern Himalaya, which covers parts of Nepal, Bhutan, the northeast Indian states of West Bengal, Sikkim, Assam, and Arunachal Pradesh, southeast Tibet (China), and northern Myanmar; and the Western Himalaya, covering the Kumaon-Garhwal, northwest Kashmir, and northern Pakistan. While these divisions are largely artificial, the deep defile carved by the antecedent Kali Gandaki River between the Annapurna and Dhaulagiri mountains has been an effective dispersal barrier to many Kanchenjunga-Singalila Complex.

The Kanchenjunga-Singalila Complex is one of the five prioritized landscapes of the Eastern Himalayas and possesses globally significant populations of landscape species. The complex stretches from Kanchenjunga Conservation Area (KCA) in Nepal, which is contiguous with Khanchendzonga Biosphere Reserve in Sikkim, India, to the forest patches in south and southwest of KCA in Ilam, Panchthar and Jhapa districts. KCA along with its lowlands Panchthar, Ilam and Jhapa districts is floristically rich with over 2000 species of flowering plants of which, several are found to be endemic to the Himalayan region. It is designated as one of the WWF 'Global 2000' eco-regions and is declared as a 'Gift to the Earth'

1.3.1 Western Himalaya: -

It is in the west of Nepal having total geographical area of 329032 sq. km. (Joshi, 1987). 67.5% of this area is in Kashmir and about 17% area is in Himachal Pradesh. While hilly district of Uttaranchal state i.e. Kumaon and Garhwal region covering a total area of about 51125 km^2. Western Himalaya (28^O 05' – 31^O 25' N and 77^O 45' – 45' – 81^O E) includes the parts of Kumaon and Garhwal regions. In the east, it is bound by Nepal, in west by Himachal Pradesh, in north by Tibet and south by plains of Uttar Pradesh. The major part of the area is mountainous with undulating topography and is characterized mainly by snow capped mountains, hills, deep and vast valleys. It has large altitudinal range (300 – 7,817 MSL) and vegetation along the altitudinal gradient (Samant and Dhar, 1997). Climatic zones of Western Himalayas are divided into following types (Table No. 1/1).

Table No. 1/1: Climatic zones and altitudes of Western Himalayas

Sr. No.	Climatic zone	Altitude (MSL) in meters
1.	Tropical	Up to 1000
2.	Warm temperate	1000-2000
3.	Cold temperate	2000-3000
4.	Alpine temperate	3000-4000
5.	Glacial temperate	4000-5000

Western Himalaya has very rich heritage of medicinal and aromatic plants (Nautiyal *et al.,* 2002). About 5200 plant species are being utilized in different systems of medicine; more than 1750 herbal species are native of Indian Himalayan region, in which western Himalaya has a share of about 1000 species which are still in use. Since ancient times the local people of the Western Himalayas have used several herbs for their everyday use and medicament such as food supplements, dyes and tonic as well as drugs and purgatives (Pandey *et al.,* 2004). The natural environment of this region has affected to a great extent due to over grazing, cultivation on slopes, sub marginal lands, deforestation, change in weather pattern and unplanned development activities like construction of tourist resorts, roads, building etc. Indiscriminate and severe exploitation has disturbed ecological balance to a greater extent.

This rich and varied diversity of the plant species are now seriously threatened.

1.4 Uttaranchal (Uttarakhand):

Uttaranchal is a part of Western Himalayan ranges, starting from Shivalik foothills to the Greater Himalayas with Tibet at its northeastern border. It is located in between latitude $29^0$5'-$31^0$25' N and longitude $77^0$45'-81^0 E and from 28 º 43 'N to 31 º 27' N longitudes and 77º 34' east to 81º 02' E latitude. Its total geographical area is 53483 sq. kms out of which 35394 sq. kms is forest area i.e. approximately 64% and 93% is mountainous regions (www.uttara.in). It is surrounded by Nepal in the East, China in the North, Himachal Pradesh in the west and Uttar Pradesh in the South. In the northwestern corner of the Uttaranchal are two states viz. Himachal Pradesh and Haryana. Geographically and administratively it is divided in to two regions Kumaon and Garhwal, comprised of total 13 districts (Fig. 1/1).

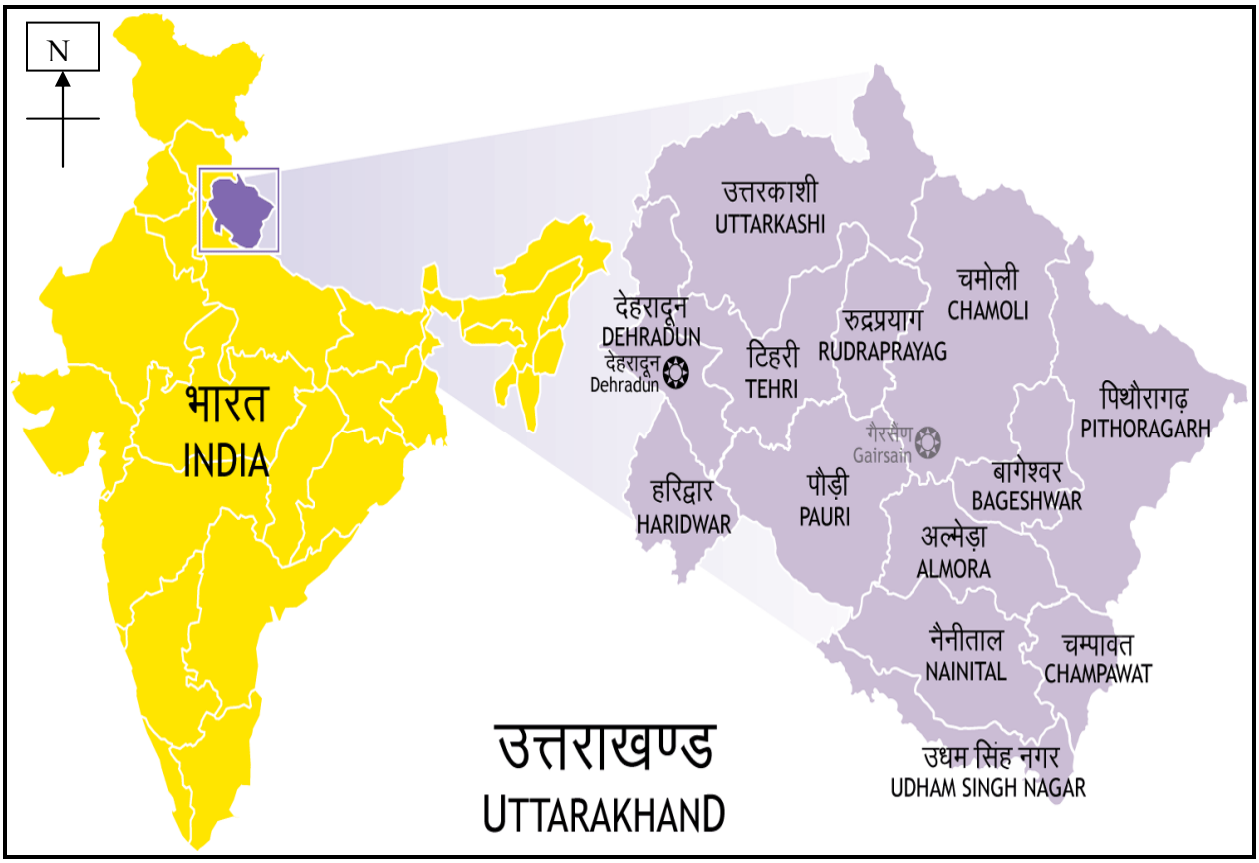


Figure 1/1: Geographical map of Uttaranchal (www.bestmapsofindia.com)**.**

Uttaranchal is largely a rugged, mountainous region where the altitude ranges from 371 to 7817 MSL. This altitudinal variation has resulted in the complex diversity of topography, meteorology, flora and fauna, with diverse agro climatic conditions. The region is sparsely populated, communication is difficult and many areas are inaccessible. Natural catastrophes like drought and landslides (Pascual, 2001) are common. The region lags behind in the agro industrial development and the level of poverty is high.

Earlier studies indicate that health of the residents in this region is poor. Hilly terrain imposes the heavy burden on the health of the people and aggravates the problem of undernutrition. Even though agriculture is the occupation of the 70.6% of the population only 13.21% area is under cultivation. About 2 lakh soil samples have been analyzed during last 5 years to identify the status of primary nutrients in the soil. It has been observed that the level of organic carbon is very low in Udhamsingh Nagar district, whereas it is medium in the remaining 12 districts. Phosphorus content is low all over the

State. Potassium elements have been found to be rich in soils of district Nainital, Bageshwer and Tehri but medium in Uttarkashi, Pauri, Chamoli, Rudraprayag, Haridwar, Udham Singh Nagar, Almora, Pithoragarh and Champawat (Dutta and Pant, 2003).

Study by Rao *et al.* (2008) revealed that due to excessive use of chemical fertilizers on cereal crops and by regular rotation of Paddy-Wheat crop, organic carbon content in the region has been declining every year and the same situation is occurring in entire plain area. Out of the total food grain production of the State, 60% comes from the extensively cultivated area of the plains. Use of compost, green manure, crop residues and bio-fertilizer to make-up the level of organic carbon contents at optimum level is necessary.

The state is the major supplier of the temperate fruit and off season vegetables. The forest sources include timber, fuel wood, fodder, medicinal plants, resins and a number of other forest products. In spite of many tourist attractions in the state, unfortunately due attention has not been given to the development of the state. Disruption of the ecosystem and the scarcity of the water, food, fodder and fuel have made it difficult for the people to maintain the subsistence existence (Dutta, 1998). Traditional arrangements have slowly changed due to the increased physical, administrative and economic integration of mountain areas into wider socioeconomic systems extending beyond the mountains. The result is the marginalization of the both traditional resources, used practices and the indigenous coping mechanisms. This has increased pressure on mountain resources and led to external intervention to extract natural wealth from the mountains. Resource use is demand driven rather than supply driven. The final consequence is the emergences and the accentuation of the cumulative environmental change leading to unsustainability of previously sustainable subsistence systems (Jodha, 2001).

A study by International Center for Integrated Mountain Development (ICIMOD) revealed a large number of negative indicators, suggesting that mountains are in a state of unsustainability (Singh and Sharma, 1998). In some districts of Uttaranchal as many as 25% of the children suffers from severe undernutrition (Pant *et al.,* 1997). The health status of the people in Garhwal Himalayas is generally poor as compared to the population in the rest of the country with the children being most affected (Datta and

Kumar, 1997). According to Dutta (1998) 80% children are in malnourished category. The children suffer from higher level of poor health as compared to preadolescent, adolescent and teenagers (Carlson and Wardlaw, 1990) that indicates certain specific factors may have adverse effects on health as children grow older (Dutta and Kumar, 1997). Limited information exists on the determinants of growth during this critical period of life (Ferguson et al 1997). To the large extent ethnic communities in the state of Uttaranchal rely on native plant species for sustenance of their traditional healthcare system, both logistically and economically.

A government project was carried out between April and July 2000 to assess the state of undernutrition in the people of Garhwal Himalayas of Uttaranchal. About 854 people were tested in three agro climatic situation, high hills, mid hills and low hills as subtropical (250-1200 m), sub temperate (1200-1700 m) and temperate (1700-3500 m) as well as in rural and urban settings. The study revealed that above 30% of the population suffers from undernutrition this is higher than the average of 20% reported by Wardlaw (2000). However, the gender did not appear to affect the level of undernourishment. The agro climatic situation had the maximum negative impact on the nutritional status of the population. Rural people were found to be more affected as compared to urban population (Bhattarai, 1992; Cordio, 1997). It may be thus concluded that the people residing in the high hills and the rural population, on whom developmental activities should be focused, are relatively undernourished.

The degraded environment is directly related to the reduced productivity and depletion of other natural resources that have been mainstay of the hill population from the centuries. This decrease of indigenous wealth automatically lowers the standard of living of the local people and their nutritional status is the first casualty (Dutta, 1998).

1.5 The genus *Aloe*:

Until recently, *Aloe* was placed in the Lily family (Liliaceae), but according to Reynolds (1966), it has now been assigned to its own family, known as Aloaceae. Nonetheless, it is related to the lily family plants, such as garlic, onion, and asparagus, all of which are known to have medicinal properties (Urch, 1999; Lawless and Allan, 2000).

Reynolds (1950, 1966) described 314 species in his classic monographs on *Aloe,* but now over 360 species are accepted (Harding, 1979).

The genus *Aloe* consists of perennial, drought resisting, succulent plants. The Arabic word "alloeh" means shining and bitter, and likely refers to the bitter-tasting *Aloe* latex. These plants are adaptable to habitats with low or erratic water availability, characterized by the capacity to store large volumes of water in their tissue, and able to utilize crassulacean acid metabolism (CAM), which is an adaptation to the photosynthetic pathway in hot climates that involves the formation of malic acid (Takahashi *et al.,* 2005; Ni and Tizard, 2004). They are native to the subtropical regions of Africa (Grisebach 1864; Polunin, 1969; Bianchini and Corbetta, 1985) and Mediterranean (Britton and Millspaugh, 1962; Fahn and Zohary, 1981) especially South Africas' Cape Province and the mountains of tropical Africa. But now are indigenous to dry sub-tropical and tropical climates (Holmboe, 1914; Post, 1932; Britton and Millspaugh, 1962; Polunin, 1969, Fahn and Zohary, 1981; Bianchini and Corbetta, 1985; Honychurch, 1989; Reynolds, 1985), including the southern United States (Grindlay and Reynolds, 1986; Viljoen and Wyk, 2000). They occupy many different kinds of natural habitats, from forest to exposed rock surfaces, but they are absent from moist lowland forests of mainland Africa.

In India 22 species of the *Aloe*are prevalent out of which four main species have medicinal properties: *Aloe barbadensis* (Miller), *Aloe perryi* (Baker), *Aloe ferox*, and *Aloe arborescens* (Urch, 1999; Atherton, 1998). Most *Aloe* species are non-toxic, but few are extremely poisonous, containing a hemlock-like substance (Atherton, 1998). *Aloe* species are stiff and rugged, consisting mainly a rosette of large, thick, fleshy leaves. Many common species are seemingly stemless, with the rosette growing directly at ground level; other species may have a branched or un-branched stem from which the fleshy leaves spring. The leaves are generally lance-shaped with a sharp apex and a spiny margin. They vary in color from grey to bright green and are sometimes striped or mottled. *Aloe* flowers are small, tubular, and yellow or red and are borne on densely clustered, simple or branched leafless stems. The plants are cultivated as ornamental plants, especially in public buildings and gardens.

1.6 *Aloe barbadensis* (Mill.):

Aloe barbadensis (Mill.) or *Aloe vera* (L.) is called as the true Aloe. It is also known by several names like 'The wands of Heaven', 'Heaven's Blessing', 'The Silent Healer' etc. This species is perennial characterized by long spear-like leaves. It may grow to a height of four feet. The leaves contain a clear thick gel-like substance. The leaves also produce a bitter yellow juice which, when dried, becomes aloe latex. Systematic position of the *Aloe barbadensis* (Mill.) is given below.

Aloe barbadensis is a most popular herbal remedy till date and has enjoyed a long history of lay acceptance (Davis *et al*., 1994; Visuthikosol *et al.*, 1995).

1.6.1 Systematic position of *Aloe barbadensis* (Mill.) or *Aloe vera* (L.):-

Kingdom	Plantae –	Plants
Subkingdom	Tracheobionta –	Vascular plants
Super division	Spermatophyta –	Seed plants
Division	Magnoliphyta –	Flowering plants
Class	Liliopsida–	Monocotyledons
Subclass	Liliidae	
Order	Liliales	
Family	Aloeaceae-	*Aloe*family
Genus	*Aloe L.*	aloe

In fact, in a self-reporting survey that examined herbal use in clinical patients and U.S. residents, *Aloe barbadensis* was the most frequently cited, accounting for 41.2% of the respondents who used herbs (Vogelzang, 2001; Mary and Frederick, 2006).

In the food industry, *Aloe barbadensis* is used as a functional food, especially for the preparation of health food drinks and other beverages, including tea. The amount of gel that finds its application in the pharmaceutical industry is not negligible as far as the manufacturing of topical ointments, gel preparations, and tablets and capsules are concerned. The gel also finds its application in the cosmetic and toiletry industries, where

it is used as a base material for the preparation of creams, lotions, soaps, shampoos, and facial cleansers (Kojo and Qian, 2004).

Commercial exploitation of *Aloe* gel has been carried out for at least last 50 years. Various companies in US act as primary growers and processors of the plant and manufacture bulk supplies of the gel for domestic and export market. Many other companies are secondary processors of the *Aloe* products for cosmetic uses and chain stores often buy the gel for incorporation into their own brand name product (Grindlay and Reynolds, 1986). A cultivation of *Aloe barbadensis* has acquired a great commercial importance for medicinal products and cosmetic processing but information is scarce about agronomic management of this crop.

In Western societies, especially in the U.S., *Aloe barbadensis* has been grown mainly to supply the latex component of the leaf to the pharmaceutical industry (Lee *et al*., 2000). However, over the last decade, *Aloe barbadensis* has gained wide popularity as a therapeutic botanical and, consequently, a large industry has developed (Reynolds and Dweck, 1999).

1.6.2 Chemical composition of *Aloe barbadensis* (Mill.): -

The 246 different ingredients, which are responsible for the medicinal properties of *Aloe barbadensis,* are distributed in different parts of the plant like leaf, transparent gel, sap and skin.

Aloes have in common green fleshy leaves covered by a thick cuticle or rind and an inner clear pulp (Figure 1/2 and 1/3). The rind of the *Aloe barbadensis* leaf accounts for approximately 20– 30% by weight of the whole plant leaf, and the pulp represents approximately 65–80% (Femenia *et al*., 1999). The rind lends turgidity to the leaf and consists of multiple layers of cells interspersed with chloroplasts, where the constituents (carbohydrates, fats, and proteins) are synthesized. The vascular bundles are located within the leaf pulp, but are just beneath and adjacent to the thick rind. The numbers of these bundles vary, depending on the size of the leaves and the age of the plant (Ni *et al*., 2004). The vascular bundles are composed of three types of tubular structures: the xylem—transports water and minerals from the roots to the leaves; the phloem—transports synthesized materials to the roots; and the pericyclic tubules—store and

transport the *Aloe barbadensis* latex along the margin of the leaf. The *Aloe barbadensis* latex is also commonly referred to as "*Aloe* juice," "*Aloe* sap," or simply "*Aloe*." The leaf pulp, the major part of the leaf by volume, is the innermost portion of the leaf and is composed of large thin-walled parenchyma cells that contain *Aloe barbadensis* gel. The gel is clear mucilaginous aqueous extract of the leaf pulp (Yaron, 1993). Carbohydrates synthesized in excess are transported by the phloem to the cells in the leaf pulp for storage (Femenia *et al*. 1999). Water, minerals, and malic acid, a small organic acid formed by CAM, are also transported to the leaf pulp. Thus, the *Aloe barbadensis* gel serves as the water and energy storage component of the plant (Paez *et al*. 2000; Mary *et al*., 2006).

Many of the health benefits associated with *Aloe barbadensis* have been attributed to the polysaccharides contained in the gel of the leaves. These biological activities include promotion of wound healing, antifungal activity, hypoglycemic or antidiabetic activity antiinflammatory, anticancer, immunomodulatory and gastroprotective properties. The recently discovered effects and applications of the leaf gel include the potential of whole leaf or inner fillet gel liquid preparations of *Aloe barbadensis* to enhance the intestinal absorption and bioavailability of co-administered compounds as well as enhancement of skin permeation. In addition, important pharmaceutical applications such as, the use of the dried gel powder as an excipient in sustained release pharmaceutical dosage forms (Hamman, 2008) is also reported.

Whole plant of *A. barbadensis* has the pharmaceutically and nutritionally important constituents. These are mainly distributed in (Fig. 1/2 and Fig.1/3) leaf, pulp, sap, and skin. The major components distributed in these plant parts identified with the modern techniques are given below-

Figure 1/2: Leaf, gel and sap of *Aloe barbadensis* (Mill.)

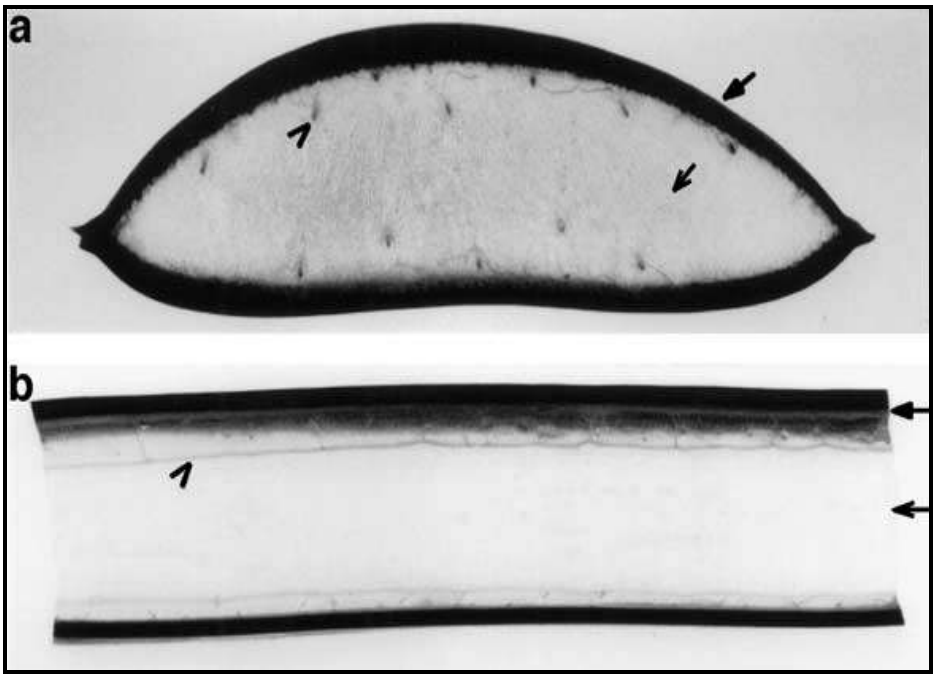


Figure 1/3: Gross view of *A. barbadensis* (Mill.) leaf sections.

(a) Cross section and (b) longitudinal section. Closed arrows indicate the rind; open arrow indicates the pulp; open arrowheads indicate the vascular bundle (Ni *et al*., 2004).

A. Leaf: - Leaves of plant are yellowish green to dark green in color. They are present in rosette like structures. The *A. barbadensis* var. chinensis has small white spots on the leaf. Major components are present in this part of the plant. Some of them are enlisted in Table No. 1/2.

Table No. 1/2: Chemical composition of whole leaf *of Aloe barbadensis*

Compound	Result	Analytical method	Reference
FREE MONOSACCHARIDES	Glucose, mannose	TLC, GC-MS	Waller *et al*., 1978
SOLUBLE CARBOHYDRATES			
Mono- and polysaccharides	25% of solid fraction	N.R.	Atherton, 1998
AMINO ACIDS			
20 out of 22		TLC	Atherton, 1998
7 out of 8 essential		TLC	*WHO Mono-graphs,* 1999
Arginine> asparagine> glutamic acid, aspartic acid, serine (and 12 more)		2-D TLC, AAA	Waller *et al*., 1978
PROTEINS			
6-9% of dry mass		Pearson method	Femenia *et al*., 1999
Amylase, lipase, carboxy peptidase		N.R.	Atherton, 1998
LIPIDS			
ß-Sitosterol	148 µmole/100g	MP, IR, GC-MS	Waller *et al.,* 1978
Lupeol	66 µmole/100g	TLC, MP, IR	
Campesterol	12 µmole/100g	NMR, GC-MS	
Cholesterol	11 µmole/100g		
VITAMINS	A, C, E, B	N.R.	Atherton, 1998

ANTIOXIDANTS			
Flavonoids	4-5 g/kg	Colorimetric assay	Hu *et al.,* 2003
	Peroxidase activity	Spectrophotometry, IEF	Esteban *et al., 2000*
Total antioxidants	Total antioxidants	DPPH	
Total antioxidants	2% µmole TE/g fresh plant	ORAC	Zheng and Wang, 2001
VOLATILE COMPOUNDS	123 compounds	GLC, GC-MS	Umano *et al.,* 1999
MINERALS	Na, Ca, Mg	Flame photometer, AAS	Atherton, 1998

B. Pulp/Gel: - The pulp, the major part of the leaf by volume, appears to be clear and mucilaginous. It is the part of plant that has been most widely used for therapeutic purposes and contains maximum amount of constituents (Table No. 1/3).

Table No. 1/3: Chemical composition of gel of *Aloe barbadensis*

Compound	**Result**	**Analytical method**	**Reference**
Water	98.5-99.5%	Dehydration	Femenia *et al.,* 1999; Waller *et al.,* 1978
CARBOHYDRATES			
SUGAR COMPOSITION			
Glucose	31 µ mole/g dry weight	Hydrolysis, TLC, GLC	Waller *et al.,* 1978
Mannose	39 µ mole/g dry weight		
Xylose, Arabinose, galactose	Around 4 µ mole/ g dry weight		

In cell walls: Galacturonic acid Mannose Glucose	% of dry weight: 38% 22% 12%	FACE	Ni and Tizard, 2004
In organelles: Galacturonic acid Mannose Glucose	% of dry weight: 40% 32% 20%		
In liquid gel: Galacturonic acid Mannose Glucose	% of dry weight: 63% 13% 24%		
TOTAL CARBOHYDRATES			
Cell wall	93% w/w	Anthrone assay	Ni and Tizard, 2004
Cellular organelles	< 50% w/w		
Liquid gel	0.22% w/v		
FREE MONOSACCHARIDES			
Filet	16 % dry weight	HPLC (Wilson)	Femenia *et al.,* 1999
Gel	26 % dry weight		
Glucose	95 % of total soluble sugars		
Glucose	655 umole/g dry weight	GC-MS	Paez *et al.,* 2000
Fructose	288 umole/g dry weight		
Galactose	162 umole/g dry weight		
SOLUBLE POLYSACCHARIDES			
Acemannan	97 % mannose	GC-MS	Manna and McAnalley, 1993

Acemannan structure	Beta 1-4 acetylated mannan	Methylation analysis	Mandal and Das, 1980
	1-4 linked glucomannan backbone	NMR, Methylation analysis	Tai-Nin Chow *et al.,* 2005
	4 partially acetylated glucomannan	N.R.	Gowda *et al., 1979*
PECTINS	Up to 40-50 % in cell walls	N.R.	Ni and Tizard, 2004; Femenia *et al.,* 2003
POLYSACCHARIDES COMPOSITION			
Mannose: Glucose: Galactose	93%: 3%: 3%	Enzymatic hydrolysis, GC-MS	Talmadge *et al.,* 2004
	Filet/gel (mol %)	Hydrolysis, GC, colorimetric assay	Femenia *et al.,* 1999
Mannose	46%/52%		
Glucose	27%/27%		
Galactose	5%/3%		
Glucose/Mannose	37%/47% of dry mass Total-15%	Hydrolysis, TLC and GLC	Waller *et al*., 1978
Xylose, Arabinose (or fucose), Galactose, Rhamnose			
INSOLUBLE POLYSACCHARIDES			
Fibre	Filet 58% Gel 35%	Klason	Femenia *et al.,* 1999
Cellulose	In cell walls	N.R.	Femenia *et. al*., 2003
Glucuronic acid containg polysaccharide	In cell walls (pulp)	N.R.	Ni and Tizard, 2004

AMINO ACIDS	18 detected (major glutamic and aspartic acid)	N.R.	Gjerstad, 1971
	90% of essential	N.R.	Khan, 1983
	7 out of 8 essential	AAA	Atherton, 1997; Khan, 1983
PROTEINS			
Filet	7%	Pearson	Femenia *et al*., 1999
Gel	9%		
In Acemannan	3-4%	N.R.	Talmadge *et al.,* 2004
Glycoproteins	29000 Da	N.R.	Reynolds and Dweck, 1999
Glycoproteins	55000 Da	Chromatography, spectroscopy, electrophoresis	Choi *et al.,* 2001
Lectins	Aloctin I and Aloctin II with 5% neutral sugar	N.R.	Akev and Can, 1999
LIPIDS			
Fatty acids	N.R.	GC-MS	Yamaguchi *et al.,* 1993
Fillet	4%	Gravimetry	Femenia *et al.,* 1999
Gel	5%		
	Beta sitosterol	GC-MS	Yamaguchi *et al.,* 1993
	Beta sitosterol	NMR	Moon *et al.,* 1999
HYDROCARBONS	N.R.	GC-MS	Yamaguchi *et al.,* 1993

ORGANIC ACIDS			
Mallic acid	409 µmole/g dry weight	GC-MS	Paez *et al.,* 2000
Mallic acid (in acemannan)	< 7%	N.R.	Talmadge *et al.,* 2004
Salicylic acid		N.R.	Atherton, 1998
Dehydroabietic acid derivative	N.R.	GC-MS	Yamaguchi *et al.,* 1993
ANTIOXIDENTS			
Total antioxidants	Total antioxidants activity	SCF (CO_2 extraction), ethanol extraction	Hu *et al.,* 2005
GTH	Se containing enzyme	FPLC	Sabeh *et al.,* 1993
SOD	7 SOD enzyme	SDS-PAGE	Sabeh *et al.,* 1996
Potent chromone derivative	Supresses peroxidation	TBA, In-vivo	Lee *et al.,* 2000
ANTHRAQUINONES			
Aloin B, Aloerisin A, Aloesin	N.D	GC-MS	Paez *et al.,* 2000
MINERALS			
Ca	35400 ppm	ICP/P-5200	Yamaguchi *et al.,* 1993
K	7200 ppm		
Mg	3210 ppm		
P	1720 ppm		
Sr, B, Ba, Al, Fe	4-86 ppm		
	Filet/gel (% of dry matter)	ICP/AES	Femenia *et al.,* 1999
Ca	5%/3%		
K	3%/4%		

Mg	1%/1%	ICP/AES	Femenia *et al.,* 1999
Na	2%/3.5%		
Fe, Cu, Zn P	0.01%-0.1%		
ASH			
Fillet	15	Gravimetry	Femenia *et al.,* 1999
Gel	23		
N.R.=Not reported , N.D.=Not detected			

C. Sap: - It is the yellowish sticky fluid oozes out from the leaf after a cut. The major compounds of the sap are anthraquinones and the constituents are aloin and barbaloin. Comparatively less amount of constituents are found in sap and they are given below- (Table No. 1/4)

Table No. 1/4: Chemical composition of sap of *Aloe barbadensis*

Compound	Result	Analytical method	Reference
FREE MONOSACCHARIDES			
Glucose	95% of total soluble sugars	HPLC, Wilson	Femenia *et al.,* 1999
Glucose	77 µmole/g dry weight	GC-MS	Paez *et al.,* 2000
Fructose	8 µmole/g dry weight		
Arabinose, Sucrose	4 µmole/g dry weight		
	1.1-0.3 µmole/g dry weight		
MALLIC ACID	N.D.	GC-MS	Paez *et al.,* 2000
ANTHRAQUINONES			
Anthraquinone	28%	TLC, Colori	WHO Monographs,

derivatives (barbaloin)		metric assay	1999
Aloin A (alsol, barbaloin)	2100 µmole/g dry weight	GC-MS	Paez *et al.,* 2000
Aloin B	24% peak area	HPLC-MS	Rebecca *et al.,* 2003
Aloenin B	25% peak area		
Aloin A	30% peak area		
	Aloin A, aloin B, aloerisin A, aloesin, isoaloeresin, aloe-emodin	HPLC	Saccu *et al.,* 2001
VOLATILE COMPOUNDS	Over 60 compounds	GC-MS	Saccu *et al.,* 2001
ASH	<2%	N.R.	www.who.int, 1998
N.R.=Not reported, N.D.=Not detected			

D. Skin: - It is the greener portion of the leaf after removal of the pulp. It is also called as the peel. It is found to be rich in pharmaceutically and nutritionally rich compounds (Table No. 1/5). The major portion is of starch, cellulose and fibre.

Table No. 1/5: Chemical composition of skin of *Aloe barbadensis*

Compound	Result	Analytical method	Reference
Water	90- 98%	N.R.	Femenia *et al.,* 1999
FREE MONOSACCHARIDES			
Glucose	11% of total dry mass 95% of total soluble sugars	HPLC, Wilson	Femenia *et al.,* 1999

SOLUBLE POLYSACCHARIDES			
Composition:	(mol %)	Hydrolysis, GC, colorimetric assay	Femenia *et al.,* 1999
Mannose	30%		
Glucose	25%		
Xylose	12%		
Uronic acid	14%		
Galactose	8%		
Arabinose	6%		
FIBERS			
Lignin	20% of dry weight	Klason	Femenia *et al.,* 1999
LIPIDS	3% of dry weight	Gravimetry	Femenia *et al.,* 1999
ASH	13% of dry weight	N.R.	Femenia *et al.,* 1999
ANTIOXIDANTS			
Total antioxidants	Total antioxidant activity	SCF (CO_2 extraction), ethanol extraction	Hu *et al.,* 2005
	2.6 Trolox equivalents	ABTS	
	40% inhibition	DPPH	
Glyoxalases	Glyoxalase I and glyoxalase II	SDS-PAGE, IEF	Norton *et al.,* 1990
SOD	7 enzymes	SDS-PAGE	Sabeh *et al.,* 1996
MINERALS			
	% of dry weight	ICP/AES	Femenia *et al.,* 1999
Ca	4%		

K	2%	ICP/AES	Femenia *et al.,* 1999
Mg	1%		
Na	2%		
Fe, Cu, Zn, P	0.01% -0.1%		
N.R.=Not reported, N.D.=Not detected			

1.7 Uses of *Aloe* species:

Aloe contains a wide rage of chemicals, which are responsible for its clinical and pharmaceutical importance. It contains around 246 different types of components. Till date *Aloe* has mostly been used for the various clinical, pharmaceutical and nutritional purposes. Some of the major uses are,

1. Abortifacient
2. Adjuvant
3. Alkalinizing
4. Analgesic
5. Anesthetic (local)
6. Angiogenic
7. Anti asthmatic
8. Anti microbial
9. Anti burn effect
10. Anti diabetic
11. Anti fertility effect
12. Anti hyperglycemic
13. Anti inflammatory
14. Anti leshmaniasis
15. Anti mycobacterial
16. Anti pyretic
17. Anti tumor
18. Anti ulcer
21. Cardiac depressant
22. CNS depressant
23. Cytotoxic
24. Death
25. Estrogenic
26. Free radical scavenging
27. Hemagglutinin
28. Hypolipemic
29. Imunostimulant
30. Mutagenic
31. Ovulation inhibition
32. Oxygen radical inhibition
33. Peptidyl transferase inhibition
34. Phagocytosis inhibition and stimulation
35. Prevention of hair loss and hair conditioner
36. Protein synthesis inhibition
37. Toxic
38. Wound healing acceleration

19. Anti viral
20. Cell attachment enhancement

1. Abortifacient effect: - Female rats were tested for use of *Aloe*as an abortifacient. 95% Ethanol, water and petroleum ether were used for the extraction of abortifacient compound from fresh leaves of aloe. These extracts were administered orally and doses of ethanol, water and petroleum ether in the concentration 150.0 and 100.0 mg/kg of rat respectively were found inactive for abortifacient activity (Reynolds and Dweck, 1999).

2. Adjuvant activity: - Juice of freeze dried leaves of *Aloe* was used for the experiment. This juice was found to be effective when administered intraperitoneally to rat at variable dosage levels (Saito *et al.,* 1989)

3. Anti- oxidant and Pro- oxidant activity: - The anti-oxidant activities of anthraquinone and anthrones of *Aloe barbadensis* have been evaluated using different model systems (Lee *et al.,* 2000; Yen *et al.,* 2000). Aloe-emodin was also shown to have some protective effects against carbon tetrachloride-induced lipid peroxidation in rat liver (Arosio *et al.,* 2000).

4. Analgesic activity: - 95% ethanolic extract of aerial parts administered intragastrically to mice at a dose of 500 mg/kg was found to be active versus hot plate method. 0.125 % concentration of fresh leaf gel formulated into toothpaste, which already contained sodium fluoride, was equivocal. Active results were found when patients with acute and chronic athletic injuries were treated with fresh leaf pulp. Water extract of dried leaf juice administered intraperitonially to rats at a dose of 250 mg/kg was active versus tail flick response to radiant heat. Water extract of fresh leaf juice administered subcutaneously to mice at a dose of 100 mg/kg was found to be active (Reynolds and Dweck, 1999).

5. Anesthetic activity (local): - Fresh leaf juice was found to be active as an anesthetic for insect stings on human adults. The biological activity has been patented (Panda, 2009).

6. Angiogenic activity: - Dichloromethane extract of gel by portioning between hexane and 90% aqueous methanol increased proliferation of CPAE cells. It also enhanced mRNA expression of urokinase type MMP in CPAE cells whereas the expression of plasminogen activator inhibitor -1 mRNA was not changed (Lee *et al.,* 1998).

7. Anti asthmatic activity: - Fresh leaf juice administered orally to human adult patients was found to be active (Panda, 2009).

8. Antimicrobial activity: - Fresh leaf components extracted in water, hexane and methanol were proved to be active against some common food pathogens like *salmonella, E. coli, streptococcus*, *staphylococcus* and other bacteria (Heggers *et al.,* 1979).

Antimicrobial activity of the *Aloe barbadensis* juice against Gram-positive bacteria (*Mycobacterium smegmatis, Staphylococcus aureus, Enterococcus faecalis, Micrococcus luteus* and *Bacillus sphericus*), Gram-negative bacteria (*Pseudomonas aeruginosa, Klebsiella pneumoniae, Escherichia coli* and *Salmonella typhimurium*) and *Candida albicans* was also determined *in vitro* (Alemdar and Agaoglu, 2009).

9. Anti burn effect: - Water extract of dried leaves, applied externally to third degree burns induced by X rays on rats at a concentration of 10% was found active. The inner rind of leaf was dried before being extracted. Inhibitors of cyclooxygenase, such as indomethacin, decrease sunburn redness and increase tissue perfusion when applied to sunburn skin (Penneys, 1982).

10. Anti diabetic activity: - Use of *Aloe barbadensis* L. extract in preventing and curing diabetes has been known worldwide (Cheng and Wan, 2005). In streptozotocin-induced diabetic rats fed with *A. vera* (300 mg/kg body wt), the fasting plasma glucose levels were reduced to normal and body weight was found to be increased. Excess proliferation of epithelium in the small intestine observed in diabetic rats, was reduced after *A. vera* feeding. In diabetic rats and diabetic rats fed with *A. vera*, no change was noticed in the kidney and stomach (Noor *et al.,* 2008).

Oral administration of *Aloe barbadensis* gel extract at a dose of 300 mg/kg bodyweight per day to streptozotocin induced diabetic rats for a period of 21 days resulted in a significant reduction in fasting blood glucose, hepatic transaminases (aspartate aminotransferase and alanine aminotransferase), plasma and tissue (liver and kidney) cholesterol, triglycerides, free fatty acids and phospholipids and a significant improvement in plasma insulin (Rajasekaran *et al.,* 2006).

11. Anti fertility effect: - 95% ethanol and petroleum ether extract of leaves was proved to be active when administered orally to female mice. Positive data was reported but they

are of questionable significance to fertility regulations. Water extract was found to be inactive. 95% ethanol, water and petroleum ether extracts of root administered orally to female mice was found to inactive (Panda, 2009).

12. Anti hyperglycemic activity: - Leaf pulp and extract were ineffective for non-diabetic rats but leaf pulp was proved effective against diet-induced insulin-dependent diabetes mellitus (IDDM) and diet-induced non-insulin-dependent diabetes mellitus (NIDDM) rats. The effectiveness was enhanced for type II diabetes as compared to glibenclamide (Okyar *et al.,* 2001).

The effects of Processed *Aloe barbadensis* gel (PAG) on the NIDDM were studied in C57BL/6J mice. Oral administration of PAG for 8 weeks reduced circulating blood glucose concentrations to a normal level and also decreased plasma insulin (Kim *et al.,* 1999).

13. Anti inflammatory activity: - Several models of inflammation (adjuvant arthritis and hind paw oedema in rats, ear swelling in mice and rabbits, synovial pouch and burn in mice, rats and guinea pigs) and phlogogen (carrageenan, kaolin, albumin, dextran, gelatin, mustard, croton oil, streptozocin etc.) have been used for this study (Davis *et al.,* 1986; Davis *et al.,* 1989; Vazquez *et al.,* 1996). *Aloe* gel contains small amount of salicylic acid, emodin, emoline and barbaloin, which shows anti-inflammatory activity (Robson *et al.,* 1982). The anti inflammatory activity of *Aloe*gel could be due to the inhibition of prostanoids (Penneys, 1982; Hiroko *et al.,* 1989) and C-glucosyl chromone (Hutter *et al.,* 1996).

Alternative mechanisms are inhibition of polymorphonuclear (PMN) leucocyte infiltration (Davis and Maro, 1989) or destruction of bradykinase or inhibition of bradykinin activity (Fujita *et al.,* 1976). However it has been observed that the vehicles used in commercial *Aloe* gel preparations (mineral oil, petrolatum and aquaphor) may inhibit prostanoid production themselves (Penneys, 1982).

An *Aloe*gel containing anthraquinone is more effective than anthraquinone free gel (Davis *et al.,* 1986). Integrins play a role in inflammation, permitting Inflammatory cells to leave the blood stream and enter damaged tissues (Capasso *et al.,* 1998). *Aloe* gel and hydrocortisone seem to inhibit the inflammatory process in an additive, dose dependant manner when given concurrently (Davis *et al.,* 1991).

14. Anti leshmaniasis: - Oral administration of *Aloe vera* in mice is effective to reduce the incidence of tumors and *Leishmania parasitemia* by >90% in the liver, spleen, and bone marrow (Feily and Namazi, 2009).

The presence of alkaloids, triterpenes, cyanidines, proanthocyanidines, tannins, and saponins in AVL were proved to be effective in four *Leishmania donovani* strains in promastigotes (IC_{50} ranged from 70-115 µg/ml) and amastigotes (IC_{50} ranged from 3.1-11.4 µg/ml) (Dutta *et al.,* 2008)

15. Anti mycobacterial activity: - 95% ethanol and water extract of fresh leaves were proved to be active for *Mycobacterium tuberculosis*. It was found that these extracts were effective only when used in broth cultures and found to be inactive on agar plate while ethyl acetate extract of dried leaves was proved to be active on agar plate (Reynolds and Dweck, 1999).

16. Anti pyretic activity: - 95% ethanolic extract of aerial parts administered intragastrically to mice at a dose of 500 mg/kg was active versus yeast induced pyrexia (Reynolds and Dweck, 1999).

17. Anti tumor activity: - The exposure of aloe-emodin to two liver cancer cell lines that differed in p53 expression, however, suggested alternative mechanisms for the differing anti-proliferative activities of aloe-emodin. In human liver cancer cells that express p53, aloe-emodin induced a p53-dependent pathway that was accompanied with enhanced expression of p21 and resulted in cell cycle arrest. In human liver cancer cells that were p-53 deficient, aloe-emodin was shown to induce a p21-dependent pathway that did not cause cell cycle arrest, but rather promoted apoptosis (Kuo *et al.,* 2002). In cell-based ELISA and Western blot analysis, aloe-emodin was shown to abolish cisplatin-triggered activation of extracellular signal-regulated kinase (ERK) in rat glioma and murine fibroscarma cells (Mijatovik *et al.,* 2005).

18. Anti ulcer activity: - An aqueous extract of *Aloe barbadensis* leaf gel administered orally to ethanol induced ulcer rats was proved to be active. Decrease in number of gastric lesions was observed suggesting cytoprotective and acid regulating properties of the leaf gel (Sivagnanam *et al.,* 2003).

19. Anti viral activity: - 95% ethanolic extract of dried leaves in cell culture was proved to be active against the plant pathogens like distortion ring spot, wild mosaic and ringspot viruses.

Fresh leaf juice containing anthraquinones in cell culture was proved to be active against human pathogens like herpes simplex virus (HSV) 1 and 2 (Kahlon *et al.,* 1991).

20. Cell attachment enhancement effect: - Fresh leaf homogenate in cell culture was active versus human embryonic lung cells and inactive versus CA-ME-180 and human embryonic lung cells. Fresh leaves were active on human lung cells and CA-cervical squamous cells. Leaf homogenate in cell culture was active on CA-ME-180 and human embryonic lung cells. Leaf juice (commercial sample) was active on human lung cells and CA-cervical squamous cells (Panda, 2009).

21. Cardiac depressant activity: - Tincture of leaf juice produced weak activity in rabbit heart perfusion (Panda, 2009).

22. CNS depressant activity: - Hot water extract of leaves administered intraperitonially to rabbits at a dose of 10 mg/kg was proved to be active (Panda, 2009).

23. Cytotoxic activity: - Aloe-emodin was shown to have specific dose-dependent cytotoxic effects on non-epithelial tumors, in particular neuroblastoma cells; however, human epithelial tumors, blood-derived tumors, and normal fibroblasts were almost refractory to the aloe-emodin treatments (Pecere *et al.,* 2000)**.**

24. Death: - 95% ethanolic extract of aerial parts administered intragastrically to mice at a dose of 3 gm/kg was found to be inactive. Higher dose of this extract or different solvent found to be lethal (Panda, 2009).

25. Estrogenic effect: - Leaf juice administered orally to immature rats at a dose of 10ml/ kg produced weak activity (Panda, 2009).

26. Free radical scavenging activity: - The DPPH radical and superoxide anion scavenging activities were determined. As one of the most potent components, isorabaichromone together with feruloylaloesin and p-coumaroylaloesin showed potent DPPH radical and superoxide anion scavenging activities (Yagi *et al.,* 2002).

27. Hemagglutinin activity: - A commercial sample of leaf juice, chromatographic fraction of fresh leaf gel, fresh leaf homogenate was proved to be active (Panda, 2009).

28. Hypolipemic activity: - Hot water extract of dried leaf juice administered intragastrically to rats at a dose of 0.5 gm/ kg for seven days was proved to have hypolipemic activity. The effect was tested by a mixture of *Nigella sativa, Commiphora, Ferula assafoetida, Aloe barbadensis* and *Boswellia serrata* versus streptozotocin induced hyperglycemia (Kwanghee *et al.,* 2009).

29. Imunostimulant activity: - *Aloe* gel directly stimulates the immune system (Womble and Helderman, 1988) through its active ingredient acemannan. It increases the lymphocyte response to alloantigen and it activates macrophages to produce nitric oxide (Karaca *et al.,* 1995) and cytokines (Shida *et al.,* 1995)

Aloeride, a high molecular weight polysaccharide increases NF kappa beta directed luciferase expression in THP-1 human monocytic cells to 50%. It also induces the expression of mRNAs encoding IL-1 beta and TNF alpha (Pugh *et al.,* 2001).

Lyophilized extract of leaves applied externally on mice at a concentration of 1.6 7% was proved to be active versus UV irradiation induced suppression of contact hypersensitivity (Crowell *et al.,* 1989).

30. Mutagenic activity: - Aqueous extract was found to be active on *Salmonella typhimurium* TA98 and *Salmonella typhimurium* TA102 at a concentration of 50 µg/plate with inhibition rate of 84% and 90% respectively (Lee *et al.,* 2000).

95 % ethanolic extract of dried plant juice on agar plate at a concentration of 10 mg/plate was found to be inactive on *Salmonella typhimurium* TA98 and weak activity was observed on *Salmonella typhimurium* TA102.

31. Ovulation inhibition effect: - Ethanol: water (1:1) extract of leaves administered orally to female rabbits at a dose of 100 mg/ kg was equivocal versus copper acetate induced ovulation (Panda, 2009).

32. Oxygen radical inhibition: - Water extract of fresh leaf juice in cell culture was proved to be active on polymorphonuclear leukocytes versus PMA stimulated release. It was antagonized by Ca^{+2} ionophore (Panda, 2009).

33. Peptidyl transferase inhibition: - Dried leaves at a concentration of 10 mg were proved to be active (Panda, 2009).

34. Phagocytosis inhibition and stimulation: - Aqueous (dialyzed) fraction of fresh leaves at a concentration of 0.5 mg/ ml was found to be inactive on polymorphonuclear

leukocytes but fresh leaf juice at a concentration of 4 mg/ml was found to be stimulant of phagocytosis (Panda, 2009).

35. Prevention of hair loss and hair conditioner: - Water extract of dried leaves applied externally on human adults at a concentration of 86.6 % was proved to be effective hair conditioner.

Fresh leaf gel applied externally on human adults at a concentration of 6.8 ml/ day was proved to inhibit the loss of hairs (Panda, 2009).

36. Protein synthesis inhibition: - Chromatographic fraction of fresh leaf gel in broth culture was proved to be active on *Bacillus subtilis*. Dried leaves at a concentration of 1 mg were also found active. Incorporation of leucine into protein was inhibited as well as was elongation factors EF-1 and EF-2 (Panda, 2009).

37. Toxic effect (general): - 95% ethanolic extract of dried aerial parts in the drinking water of mice, at a dose of 100 mg/kg for three months was proved to be toxic. Effect of toxicity included alopecia, degeneration and putrefaction of the sex organs, sperm damage, and decrease of RBC levels (Boudreau and Beland, 2006).

38. Wound healing acceleration: - Wound healing process was investigated in 63 male rats with microscopic and cell count methods. The wound surface and healing were assessed on days 4, 7, and 14, and then a sample from the wound was prepared and investigated microscopically. The results also showed that the number of neutrophil, macrophage, and fibroblast cells and the wound thickness in the control group were statistically different from the experimental groups. It was found that the wound diameter thickness in the experimental group was greatly lower due to twice administration of gel and the power of wound healing was more than other groups (Davis *et al.,* 1986; Takzare *et al.,* 2009)

Water extract showed the presence of saponin with high polarity and maximum percentage yield 21.8 % of dried leaf weight, which are supposed to facilitate the healing process. Wound healing activities were noticed within 4-8 days of granulation of wound and maximum 82.29 % maturation of collagen was observed in albino rats (Rajput *et al.,* 2009).

Some of the other uses of *Aloes* are enlisted below-

1. Alkalinizing
2. Anti compliment
3. Anti crustacean
4. Anti fungal
5. Anti hypercholesterolemic
6. Anti implantation
7. Anti leukopenic
8. Arachidonate metabolism inhibition
9. ATPase (Na/K) inhibition
10. Bradykinin antagonist
11. Bronchodilator activity
12. Cell proliferation stimulation
13. Cell transformation inhibition
14. Conditioned taste aversion
15. Cosmetic
16. DNA synthesis inhibition
17. Embryo toxic
18. Emollient
19. Glucose-6- phosphate dehydrogenase inhibition
20. Hair loss stimulant
21. Histamine release inhibition
22. Hypotensive
23. Irritant
24. Lectin
25. Leukocyte migration inhibition
26. Mitogenic
27. Molluscicidal
28. Phorbol ester antagonist
29. Plant growth inhibitor
30. Polymorphonuclear leukocyte activation inhibition
31. Protein kinase inhibition
32. Smooth muscle stimulant
33. Stability study
34. Teratogenic
35. Toxicity assessment (quantitative)
36. Uterine stimulant
37. Peptidyl transferase inhibition

1.8 Biotechnology of medicinal plants:

With the current pace, global population will rise drastically in next few decades and so do the need for food, fibre, fuel and drugs. Now it has become of an immense importance to look towards advanced technologies to help in meeting the needs of this burgeoning population. There is also a rising demand for utilization of natural resources. But these can be utilized in a sustainable manner without harming the present habitat and

ecology. In addition some aggressive strategies should be implemented to overcome the already existing problems of depletion of medicinal plants and their conservation.

There is a need to blend the traditional knowledge with frontier technologies. This can be achieved through the use of advanced biotechnological tools and techniques. Besides numerous advantages, biotechnology can be used for enhancing the formation and accumulation of desirable natural products, with possible product modification in medicinal plants (Julsing *et al.,* 2007). Plant Tissue Culture techniques are important for multiplication and genetic enhancement of the medicinal plants by adopting techniques such as *in-vitro* regeneration and genetic transformations (Tripathi and Tripathi, 2003). It can also be harnessed for production of secondary metabolites using plants as bioreactors with the help of various technologies (Abdin *et al.,* 2007). Advancement in tissue culture combined with the genetic engineering techniques specifically transformation technique have opened up new avenues for production of pharmaceutical, nutraceutical and other beneficial substances (Khan *et al.,* 2009). Thus, the approaches of medicinal plant biotechnology currently focus more on distinct natural products and biosynthetic pathways (Matthys *et al.,* 2007). Some of the major useful biotechnological approaches for the said purposes are -

1. Plant Tissue Culture: - Plant tissue culture refers to the growing and multiplication of cells, tissues and organs of plant on defined solid or liquid media under aseptic and controlled conditions. The most common tissue culture techniques used for the conservation, secondary metabolite production and regeneration of medicinal plants are micropropagation, organ culture (root, shoot flower etc.), cell culture (callus and cell suspension culture), etc. (Khan and Khanum, 1998; Khan *et al.,* 2009).

2. Genetic transformation: - This technology has been proved to be a powerful tool for the production of plants with the desirable traits. Initially it was used for the agricultural crops. Now it is widely accepted for medicinal plants. It promises to overcome substantial agronomic and environmental problems which can not be solved by the conventional methods.

Genetic transformation can be achieved by agrobacterium mediated like *Taxus* (Han *et al.,* 1994), *Digitalis* (Sales *et al.,* 2003) or non-agrobacterium mediated transfer like *Rhizobium* sp. (Broothaerts *et al.,* 2005), direct gene transfer by particle bombardment like *Hyoscyamus muticus* (Leo *et al.,* 2000) etc. Transgenic medicinal plants can also be obtained by electroporation e.g. *Solanium dulcamara* (Chand *et al.,* 1988), chloroplast transformation e.g. *Chlamydomonus reinhardtii* etc. (Boynton *et al.,* 1988) (Tripathi and Tripathi, 2003).

3. Combinatorial biosynthesis: - It is one of the most recent biotechnological developments in the field of medicinal plants. Combinatorial biosynthesis is the combination of the metabolic pathways of the different organisms at genetic level. In this method genes from the different microorganisms are combined for the new and interesting plant secondary metabolites. Metabolic pathways like hydroxylation and glyoxylation are important from pharmaceutical point of view, and can be performed by this method. The yeast *Candida utilis* was engineered for the production of β-carotene, lycopene and astaxantene (Miura *et al.,* 1998). Many secondary metabolites like terpenoids, alkaloids, carotenoids have been reported to be produced on large scale by this method (Khan *et al.,* 2009).

4. Bioengineering: - the bioengineering techniques are mainly used for the transfer of the gene of interest or synthesis of the specific gene and its insertion and expression in the targeted plant. By this method metabolic pathways can be altered at the genetic level for the enhanced production of secondary metabolites and expression and synthesis of new molecule in the plant. It is a proved tool for the production of medicinal plants with the specific agronomical traits like herbicides, pesticides resistance etc. (Saito, 1992 and Paola *et al.,* 2001).

5. Bioanalytics and Metabolomics: - These are the blending of analytical and metabolic engineering techniques viz. coupling of LC with NMR for separation and structural elucidation of a compound. MS (Mass Spectroscopy) is useful for the determination of molecular mass. DNA microarray (genes of *Centella asiatica*), Expressed Sequence Tags

(ESTs) e.g. monoterpene indole alkaloid pathway in *Catharanthus roseus,* are the methods used for the medicinal plants. All these techniques fall under this category. Mapping of transcriptome of the plant for the functional and application genomics is the new emerging trend.

All these biotechnological techniques (Table No. 1/6) can be used for the conservation, secondary metabolite enhancing and utilisation, genetic improvements of the *Aloe* plants.

Table No. 1/6: *In vitro* and biotechnological work carried out on *Aloe* species

Sr. No.	Techniques	Species	Reference
1.	*In vitro* regeneration and micropropagation	*Aloe vera L.*	Roy and Sarkar, 1991
2.	Tetrahydroanthracene glucosides from callus culture	*Aloe barbadensis*	Yagi *et al.,* 1998
3.	Tissue culture and rapid propagation.	*Aloe arborescens*	Zeng and Peng, 2000
4.	Micropropagation	*Aloe vera L. var. chinensis (Haw.)*	Liao *et al.,* 2004
5.	Somatic organogenesis	*Aloe arborescens*	Velcheva *et al.,* 2005
6.	Short chain fatty acid production	*Aloe vera*	Progribna *et al.,* 2008
7.	Anti microbial activity	*Aloe vera*	Alemdar and Agaoglu, 2009

1.8.1 Future frontiers: -

Role of biotechnology of medicinal plants for nutraceutical, cosmeceutical and in Food Drug Therapy are an emerging fields. Food Biotechnology has already in existence since few years and proved to be acceptable widely. Medicinal plants have rooted their existence in this trend also. The research in functional genomics and proteomics has revolutionised the area. Remote sensing and Geomatics technologies have demonstrated the potential for assisting in the management of precious resources of medicinal plants. Information and communication technology; space technology; geographical information systems (GIS) are the tools of frontier technologies which would help in creating medicinal plant management systems, making plans for sustainable cultivation of medicinal plants and bringing new areas (like through development of wastelands) into productive cultivation and conservation.

1.9 Genesis of thesis:

Due to irregular, unscientific and overexploitation of herbs from natural habitat, there is depletion of high value medicinal herbs, to the stage of extinction. In past naturally available medicinal herbs were enough to meet the requirement of the masses. But with rapidly growing population and increasing awareness towards the herbal product, there is imbalance on production and consumption of these herbs. If the proper strategy is not initiated at right time, the stage may come when many important herbal species will become extinct. So the development of agro-technology for high demand medicinal plants' commercial scale cultivation is the need of hour.

Himalayas have a fragile ecosystem. Attempts to exploit the natural resources to meet the basic needs of the population may lead to the irreversible damage as explained in ICIMOD newsletter (Pascual, 2001), where poverty, death and environmental degradation are considered as reasons of pressure on resources. Hence, to develop awareness, the year 2002 was declared as "The International Year of Mountains."

In recent years such reports has increased awareness on increasing environmental fragility within mountainous regions, suggesting that prerequisite to a healthy environment is a healthy population. Undernutrition leads to mental impairment among children and low work output among the adults. Chronic undernutrition also precipitates

nutrition related disorders like goiter, anemia and night blindness. Undernutrition further accentuates chronic diseases in adult such as diabetes, heart disease, cancer and hypertension (Wardlaw, 2000; Swaminathan, 2003)

FAO estimates that more than half of the mountain population in developing and CSI countries (250-370 million people) is vulnerable to food insecurity. (This estimate of vulnerability is not to be confused with FAO's estimate of the undernourished population. Typically about half of those identified as vulnerable are actually undernourished). As noted above, mountain environment differs according to altitude, latitude and terrain. These differences influence both livelihood opportunities and sources of vulnerability. Cultural traditions in mountain regions are often strong and resilient. Yet lack of crop diversity and limited access to current information and knowledge about good nutrition and health care practices expose mountain people to high rate of malnutrition and disease. Traditional attitudes and beliefs may also lead people to continue land use practices that are no longer suitable to evolving conditions in mountain environment. In many places, traditional livelihood strategies are no longer sustainable because of mounting demographic pressure, rapid deforestation, erosion and loss of soil quality. Mountain cities offer economic opportunities by tourism but it brings pollution and increased need for cash with them, which results in weakening indigenous highland institution (Dutta and Pant, 2003).

The state policies and planning for Uttaranchal have clearly identified medicinal plants as a potential source for uplifting state economy and generating employment for the unemployed youth. In this context medicinal plant cultivation is being cited as a need for mountain development. Medicinal plants are viewed as a possible bridge between sustainable economic development, affordable healthcare and conservation of vital biodiversity.

As seen earlier the Uttaranchal regions (Kumaon and Garhwal) are nutrient deficient regions. The lack of information, natural disasters and complete ignorance towards the forest and its useful resources (like medicinal plants' cultivation and conservation), poor economic status, illiteracy has worsened the condition. High altitude has its own impact on the normal human life like sun burns, frost bites, high land slopes, nutrient deficient soil, its erosion and many more. The chemical analysis has already

proved that the *Aloe* plant is of immense importance in terms of nutrition, clinics and pharmaceutics. Availability of *Aloe* plants in this region irrespective of its natural habitat made the task easier to fight with the situation and ease the human life with the medicinal and nutritional properties of the plant. Hence research has been undertaken at DRDO to fulfill the nutritional, pharmaceutical and clinical requirements of the troops and people in this hilly region. It was also kept in mind that in addition, cultivation of these plants, setting up large and cottage scale industries or harvesting and transporting the raw material to these industries, processing of the raw material in this region itself will provide means of employment to the local people who in turn will improve their socioeconomic status.

The basic research interest of DRDO (Defence Research and Development Organisation) in medicinal plants was developed after the cases of sunburn and nutritional deficiency syndromes of the troops posted at these high altitude regions. A survey was carried out by the DRDO to find out the potential plant sources to overcome such health obstacles. DRDO works under the Ministry of Defence, Government of India.

The project was sanctioned under the scheme Charak by the Directorate of Life Sciences, DRDO. It is well known that the area is blessed with the rich heritage of medicinal plants. To utilize the local resources of the medicinal systems survey was carried out and around 300 medicinal plants were collected out of which 110 endangered plants are still under cultivation at DARL *was* found among the various medicinal plants.

Aloe species was the focus in the project entitled "Development of Agrotechnology and extraction of active ingredients for the development of herbal products." Defence Agricultural Research Laboratory (DARL), Pithoragarh, Uttaranchal was the coordinator laboratory. Other collaborators were DIPAS (Defence Institute of Physiology and Allied Sciences) New Delhi and DRDE (Defence Research and Development Establishment), Gwalior. The success came in the form of development of six products out of which 3 get patented and technology was transferred for the public benefit. Remaining three is under research and development process.

NCL (National Chemical Laboratory), Pune was one of the collaborators of the project with world-class facilities for the biotechnology research. Hence it was decided to carry out the biotechnological part in NCL.

The objectives of the present study were,

1. Screening of Uttaranchal (Kumaon and Garhwal region) for collection of different *Aloe* species/varieties.
2. Authentification of these collected species/varieties.
3. To maintain these collected germplasm in polyhouses and fields with agro technological developments.
4. To study morphological features of collected species/varieties.
5. To evaluate the phytochemicals of nutritional importance.
6. Analysis of pharmaceutically important aloin content of these plants by HPLC methods.
7. *In vitro* evaluation of prebiotic and synbiotic potential of these plants on selected probiotics.
8. Assessment of anti microbial activity of these plants on human pathogens.
9. Genetic diversity analysis of these genotypes by ISSR markers.

Chapter 2

Collection, Conservation, Authentication and Maintenance

In scientific work, those who refuse to go beyond fact;

rarely get as far as fact

2.1 INTRODUCTION:

Around 80% of the world's population currently uses herbal medicines directly as raw, decocts or extracts with easily accessible liquids such as water, milk or alcohol (Farnsworth, 1990). Traditional knowledge helps scientists to target plants that may be medicinally useful (Cox and Balick, 1994). Already an estimated 122 drugs from 94 plant species have been discovered through ethnobotanical leads (Fabricant and Farnsworth, 2001; Fennell *et al*., 2004). The medicinal plants thus are of great interest to the pharmaceutical industries because of their defined and undefined natural products (Ameenah, 2006). In most of the countries these medicinally important plants are collected from the wild harvest. This wild harvesting has immensely pressurized the natural resources and so do the habitats and ecosystem.

Harvesting from the wild is causing loss of genetic diversity and habitat destruction. Domestic cultivation is a viable alternative and offers the opportunity to overcome the problems that are inherent in herbal extracts such as misidentification, genetic and phenotypic variability, extract variability and instability, toxic components and contaminants. The use of controlled environments can overcome cultivation difficulties and could be a means to overcome variation in bioactive compounds and toxins (Canter *et al.,* 2005).

Aloe barbadensis (Mill.) are among the major medicinal plants harvested for their wide range of uses. The major contribution of this collected material comes from the wild e.g. in Bangladesh the wild collection of *Aloe barbadensis* is around 90% (Dixie et. al. 2003). Not only this species but also some other species of *Aloe* from Madagascar and South Africa are of medicinal importance e.g. *Aloe ferox, Aloe chinensis, Aloe variegate* etc. These are also collected from wild and now listed as endangered species.

Main threats to natural ecosystems are due to -

A. Over-collection of plants: -

Some species of Madagascar and South Africa are over-collected by people and supplied to the nursery traders (Newton and Chan, 1998; Oldfield, 1997). The rising popularity of 'field trip holidays' by amateur growers as a new kind of tourism also

results in some collection of wild plants. An individual might collect only one or two plants of each species and may feel that little or no harm is being done to the population. However, as the number of such travelers grow the result can be a major depletion of well-known populations of plants to which access is easy.

B. Destruction of plants due to harvesting of leaf exudates: -

There is also a lucrative trade in leaf exudates, required mainly for medicinal and cosmetic purposes, and these are frequently harvested from wild plants. Much of this activity is well organized, but there is also a large unofficial exploitation of wild plants (Newton, 1994). In South Africa '*Aloe* tapping' is a well established industry for over 200 years using *Aloe ferox* as the main species, which is being exported since 1761. The species is widespread in Western Cape and Eastern Cape Provinces. Newton and Vaughan (1996) estimated that a total of about 700 tons of crystalline bitter is harvested each year from about 17 million plants, 95% of which are from wild. Much of this harvesting and export is illegal or undocumented. However, they concluded that the harvesting is carried out on a sustainable basis because of traditions established in the communities of aloe tapers. With no traditional or other controls in place, various species may be harvested without regard to chemical composition. In some areas this harvesting might be done on a sustainable basis (Newton, 1994) but cases are known where whole populations are destroyed in the process.

C. Destruction of natural habitats: -

One of the major problems for destruction of natural habitat is overgrazing. Many people in arid areas have herds of domestic animals in numbers far greater than the carrying capacity of the land, and the land becomes increasingly denuded of vegetation. In many countries where aloes are native, the rise in human population resulted in an increased demand for agricultural land or construction of houses. This has led to wholesale clearing of natural vegetation. In some areas, the continued expansion of human populations is forcing people to move into arid areas, where many aloes occur.

Apart form these; several medicinal plant species have slow growth rates, low population densities, and narrow geographic ranges which adversely affect their

population (Kala, 1998; Nautiyal *et al.,* 2002). Well-known species threatened by wild harvesting include *Arcostaphylos uva-ursa* (bearberry), *Piper methysticum* (kava), and *Glycyrrhiza glabra* (liquorice) (Vines, 2004). Between 4000 and 10000 medicinal species might be now endangered (Edward, 2004).

Cultivation of medicinal plants with the recommended agricultural practices is thus the need of time. Currently only 10% of the total medicinal plants are cultivated. Hence, WHO (World Health Organisation) has recommended the standardized agricultural practices for the cultivation of the medicinal plants. These practices are known as General Agricultural Practices (GAP) for medicinal plants.

Cultivation of *Aloe* as a minor crop is increasing day by day as it provides quick and regular income to farmers. However, farmers are not using any recommended practices for cultivation of this plant which results in poor yield and quality. Good and standardized cultivation practices and their management in field may be one of the strategies for boosting up the yield and quality of *Aloe* plants.

Conservation: -

Conservation attempts to protect aloes, as endangered species have been made at two levels – national and international. Many countries have signed various international agreements on the conservation of biodiversity, though the will to act, which is implied by the signature, is not always translated into action. Most countries have national legislation aimed at protecting endangered species, animals and plants and at preserving habitats in selected localities, such as in national and reserved parks. Unfortunately, enforcement of the legislation is poor and needs to be implemented strictly in most African countries. However, there is a problem of lack of enforcement personnel and concentrated national effort for solving economic and social problems. Only South Africa has strong enforcement activity, but still much illegal activity is known to occur (Newton and Chan, 1998).

The most effective conservation attempt at the international level is provided by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) which started in 1976. It aims at controlling the movement of endangered species and derivatives between countries, prohibiting trade in some species (listed in

CITES Appendix I) and requiring official documentation for numerous other species (listed in CITES Appendix II). Currently, 22 species of *Aloe* (mostly Madagascan) are listed in CITES Appendix I and the rest in Appendix II, with the sole exception of *Aloe barbadensis*. The documentation requirement provides an opportunity for monitoring the international trade in the listed plants and the legal trade. Data for the period 1983–1989 suggest that the most heavily traded aloes are not the species regarded as threatened in the wild, and much artificially propagated material is involved (Oldfield, 1997). However, there is concern about the demand for rarer species of Madagascar and South Africa. To meet the demand in the horticultural trade many nurserymen and individual growers propagate their stock plants by seeds or by vegetative means. This is a positive action to reduce the pressure on wild populations. There are also attempts to re-introduce some rare species of succulent plants to their natural habitats, using material propagated in cultivation. One such scheme involves plans to plant out seedlings of Madagascar's largest aloe, *Aloe suzannae*, raised in South Africa from field-collected seeds (Smith and Swartz, 1997 and 1999).

The World health Organization (WHO) guidelines for good agricultural practices in vices over the past two and half decade global interest in traditional systems of medicines including herbal medicines. It has increased not only in developing countries but also in developed countries. Global and national markets for medicinal herbs have been rapidly growing. Global sales of herbal medicines are touching the heights of 70,000 billion USD. Obvious economic benefits are being realized from the tremendous expansion of alternative herbal products market (Mander, 1998). As a consequence quality and safety of the herbal products for traditional therapies have become important concerns for both authorities and public. These includes inadvertent use of wrong species, poor quality of material, variation of quality of finished products- genetic (intrinsic) or environmental (extrinsic), cultivation methods, harvest, post harvest processing, transport and storage practices, microbial and biochemical contamination etc.

The most promising development for the conservation of medicinal plants is the use of tissue culture techniques, by means of which thousands of plants can be produced within a few months. Relatively few aloes have been propagated by tissue culture, but there are reports of success (Barringer *et al.*, 1996; Fay and Gratton, 1992; Fay *et al.*,

1995). In terms of limitations of this technique in the form of cost effectiveness, field trials and expression of natural characters, propagation by the cultivation is the best option. Globally, information on the propagation of medicinal plants is available for less than 10% and agro-technology is available only for 1% of the total known plants (Lozoya, 1994, Khan and Khanum, 1998). This trend shows that developing agro-technology should be one of the thrust areas for research. Furthermore, in order to meet the escalating demand of medicinal plants, farming of these plant species is imperative. Apart from meeting the present demand, farming may conserve the wild genetic diversity of medicinal plants. Also, rapid decline of plant resources due to their conventional use needs *ex-situ* and *in-situ* conservation, training of the community regarding collection of medicinal plants and their marketing (Khan and Syed, 2003).

It is clear from the literature that the main source of the medicinal plants used by the large population of the world including the industries and folklore is collected from the wild. In India many plants are documented as the resources of medicines (Kirtikar and Basu, 1984; Jain, 1991; Joshi, 1995; Katewa and Guria, 1997; Singh and Pandey, 1998; Katewa *et al.*, 2001 A, B and 2003; Katewa and Jain, 2003; Jain *et al.,* 2004). So the proper care should be taken regarding the harvesting and authenticity of the collected material as collectors don't have scientific knowledge of the plant's identification, its contents and uses. The authentication of material collected is important as it provides the good quality and the quantity of the component (Report submitted to Government of Andhra Pradesh, India).

Hence, in view of pharmaceutical, nutraceutical and cosmeceutical importance of *Aloe*, the present study was carried. Other purpose of the study was to develop appropriate agro-technology, which will ensure a steady, affordable and sustainable supply of quality source of *Aloe.* The complete Uttaranchal region, which comprises of the Kumaon and Garhwal (Fig. 2/4) region was thoroughly screened for wild collection of the *Aloe* and the available sources of *Aloe* were collected. Also the research was performed to develop GAP at Defence Agricultural Research Laboratory (DARL), Pithoragarh with following objectives:

1. To contribute the quality assurance of *Aloe* plants to improve the quality, safety and efficacy of finished herbal products.
2. To encourage and support the sustainable cultivation and quality of *Aloe* species in the ways that respects the environment and supports the conservation.
3. To provide technical guidance to establish national and regional GAP guidelines and related standard operating practices.
4. To find out the efficacy of the GAP by evaluation of the morphological parameters.

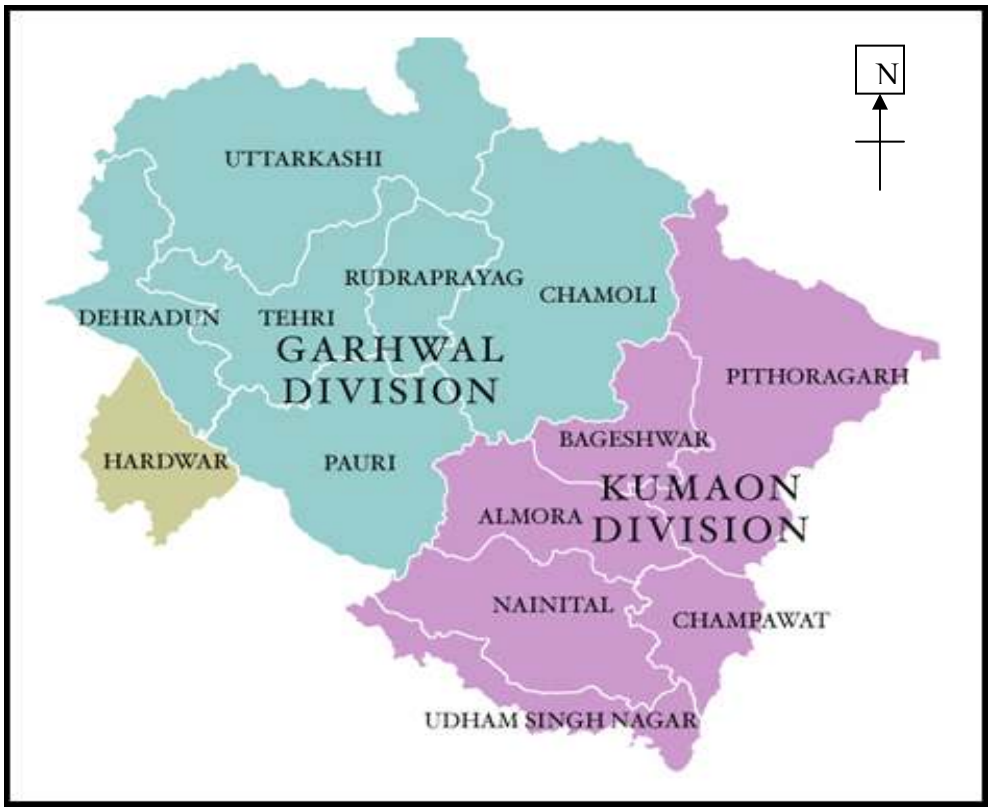


Fig. 2/4: Kumaon and Garhwal regions of Uttaranchal, India (www.bestmapsofindia.com).

2.2 MATERIALS and METHOD:

2.2.1 Collection: -

Materials:

- Normal saline solution (0.8% NaCl)
- Sucrose
- Water
- Cotton
- Root suckers
- Digging equipments
- Muslin cloths
- Sprinklers

Method:

Survey of different places of Uttaranchal was carried out for collection of *Aloe* species. As the locations are difficult to reach and climate does not favor in between June to February, summer season was selected for the collection i.e. March to May. The distance in between the locations and time of collection played very crucial role. Hence a very specific method was followed during the collection. The plantlets were dug out of the ground along with roots and soil without damaging the mother plant. It was then covered with the cotton wrapped by muslin cloth and water was sprinkled to maintain the moisture. In case of small plantlets, soil was removed and roots were packed with wet cotton. Also water was sprinkled to maintain the moisture. In case of the long distance, instead of water normal saline solution containing a pinch of cane sugar was sprinkled to maintain the microenvironment for survival of the plant.

2.2.2 Conservation: -

Materials:

- Collected plant materials

- Soil (Clay, sand and brick powder)
- FYM
- Fertilizers
- Trial beds

Method:

Initially the collected plantlets were transplanted in trial beds prepared for *Aloe* plants. The details of the preparation of bed are discussed in 2.2.1 under 'Development of Agro technology'. After one year newly grown suckers were re-transplanted in glass houses, poly houses and in an open field. Around 25 to 30 suckers were used for the purpose. The experiments for development of specific agro technological practices were done in accordance with WHO guidelines for cultivation of medicinal plants. And specific agricultural technique for conservation, mass cultivation and maintenance was developed. This GAP was used for conservation and multiplication for the rest of the period.

2.2.3 Authentication: -

Materials:

- Fresh leaf
- Flowers
- Knives
- Metal bench press
- Filter papers
- Muslin cloths
- Saline solution (0.8% NaCl)
- Ethanol (70%)

Method:

For authentication of these plants taxonomic literatures were surveyed. Herbaria were prepared and sent to Botanical Survey of India (BSI) Dehradun and Central

National Herbarium (CNH), Calcutta for authentication. For this purpose two plant parts viz flowers and leaf material were used.

Herbarium for flowers was prepared by washing and cleaning them under running tap water. The material was air dried and kept in the filter paper. Table top metal bench press was used for appropriate pressure. After three days paper was changed and changing was done regularly till material became complete dry. It was then maintained for further study.

Aloes being succulent needs specific method for preparation of herbarium. The leaf was cut from the bottom, cleaned of dust under running tap water and gel was removed by cutting the leaf in such a way that the thorns on the edges should not be disturbed. Every traces of gel were removed. The leaf was then washed with 0.8% saline solution and then with the anti fungal agent. The antifungal agent used here was ethyl alcohol (70%). It was then wrapped in four filter papers and pressed under metal bench press. The frequency of changing of filter paper was more at initial stages and depending upon the moisture content of the species. Same method was applied for the preparation of herbarium of *Aloe barbadensis* ecotypes and other species.

2.2.4 Development of Agro technology: -

Materials:

- Glass houses and poly houses (100 X 60 feet)
- Irrigation system through galvanized pipes
- Root suckers
- Sharp blades
- Knives
- Power tillers
- FYM
- N: P: K (20:20:20)
- Urea
- Equipments for irrigation
- Hoeing and ploughing instrument

- Aluminium plates for labeling
- Metal handouts
- Metal wiring
- Diathane M-45 (Local brand)
- Hot air oven (Lab India, Delhi)
- Grinder with motor (Local brand)
- Air tight plastic containers
- Polythene bags
- pH meter (AIMIL, Hyderabad)

Method:

As mentioned in collection, the propagation was done through root suckers or plantlets, occasionally by cutting and rarely by seeds.

For the cultivation of *Aloe barbadensis* soil was ploughed or digged deeply. Then manure and fertilizers were spread over the ploughed soil and mixed with 6' cut harrow or with power tiller or bullock plough in open cultivation, while for poly house and glass house it was done with the help of either power tillers or with prolonged spade or hoeing rakes. 400 quintal of decomposed Farm Yard Manure (FYM), 20:20:20 kg/hectare of N: P: K (Nitrogen: Phosphorous: Potassium) was applied as a basal dose for both, open and protected conditions. After the plants attained the age of 4 to 5 months 20 kg/hectare additional dose of Nitrogen was applied in the form of urea.

The drainage channels were prepared vertically and horizontally in prepared plots as per the requirement.

The plantlets of either two months old or with 10 to 12 cm of height were taken out from the vicinity of mother plant and transplanted. The spacing between row to row and plant to plant was 50 X 40 cm under protected condition of cultivation and 40 X 40 cm under open condition.

For transplantation of plantlets dish shaped bowels, called as Thaule locally, were prepared in ploughed soil and in these bowels plantlets were transplanted. The roots with stock were put 3 to 4 inch deep in the ground and then covered with soil. The time of

transplantation of plantlets under open conditions was in the first week of March and under protected conditions it was done in 2nd or 3rd week of February.

The irrigation under protected conditions needs to be done immediately after transplantation in the bowels either directly or with drip irrigation daily up to three days. Then there after followed every 3 days up to one month of crop growth.

In case of open conditions of cultivation if the plants are transplanted in small area, same schedule as above was followed. For the plants transplanted in larger areas flood irrigation was done immediately after transplantation followed by weekly interval up to one month. After these initial steps under both types of cultivations further irrigation was done as and when required basis.

I) Crop protection: -

Problem of weeds was not much. Some broad leaved weeds with shallow root system emerged after 3 to 4 weeks which were removed by hoeing.

Earthing of plants was done with the help of rigid support to keep it aligned and to facilitate emergence of root suckers.

No major insects, pests and diseases were observed during cultivation. The tips of plant leaves start drying after 5 to 7 months of growth in case of drought and low irrigation schedule. Sometimes appearance of black spot disease in mature leaves was observed when the conditions were hot and humid due to *Fusarium* attack. This was controlled by spraying 0.3 to 0.5% solution of Diathane M-45.

II) Mass cultivation and Maintenance: -

By following above mentioned practices plants were transplanted in open fields and glass houses. The transplanting conditions were same for both the sites i.e. glass houses and open fields. Same GAP was followed at DARL, Pithoragarh and Field Station Auli (Joshimath). Labeling was done to respective beds with painted metal hand outs.

III) Maturity index and harvesting: -

One year old plant was considered as a mature plant. Harvesting was done with the help of sharp knife starting from lower first and second rosette of leaves cutting from

adaxial side. The harvesting was done later on, in stages at an interval when upper leaves also attained average size as the one year old plant which was harvested initially.

IV) Post harvest processing and storage: -

Along with these GAP post harvest processing, preservation of leaves, gel and extraction of active ingredients were also optimized. It is as given below

Aloe barbadensis leaves though have a tough outer cuticle layer; it has delicate fleshy inner pulp. If does not subjected to immediate processing, the leaves starts decaying after 2 to 3 days after harvesting owing to excess moisture content. Therefore, immediate processing steps for chopping, drying, grinding and extraction of leaves should be undertaken.

The harvested leaves and plants as per the requirement were collected in plastic containers or stainless steel trays or in the buckets if aloin is to be extracted.

If the processing was to be done on small scale then the harvested leaves were wiped with the help of wet muslin cloth before chopping. If the plants were in bulk quantity leaves were washed under running tap water after collection of aloin and then wiped out with dry muslin cloth.

Chopping of leaves was done with sharp stainless steel blade knife into small pieces of size 1-2 mm thick and 4-5 mm long. The chopped fraction was collected in steel or aluminum drying trays.

For drying of leaves specific method was followed. First fresh leaves were collected and washed properly under running tap water. The water was wiped with clean cloth. They were then chopped into small pieces with the help of a knife and kept in an open air for about two hours. They were then kept in an oven at 40^0C for whole night. It was then taken out spread properly and temperature was raised to 60^0C. After five to six hours temperature was increased to 100^0C and maintained till all the traces of water has been removed. Then it was grind to fine powder in mill having stainless steel blades.

The finely grinded material was filled in air tight glass, sealed polythene bags or plastic containers and stored in cool and dry place. This powder was used for various experiments like estimations of different biochemical constituents, minerals etc. which are discussed in Chapter No. 3.

2.2.5 Morphological Parameters: -

Materials:

- ✓ Fresh leaf
- ✓ Plantlets
- ✓ Scales of different lengths
- ✓ Threads
- ✓ Weighing balance

Method:

The morphological parameters like leaf length and width (bottom, middle and young), weight of an individual leaf, height, number of leaves, number of plantlets i.e. suckers, moisture content etc. were observed in the cultivated plants of *Aloe* (Table No. 2/2 to 2/6). The study was carried out as per the standards of Association of Official Analytical Chemists (AOAC) methods. For these observations one year old plants were used as a maturity index. In these experiments thread was used and length of thread of particular reading was compared with the standard scale for the measurements.

For statistical analysis 30 plants were used. The plants collected from the actual sites were considered as control throughout the study. At higher altitude the Ecotype 1 was taken as a control which was used for comparative field evaluation.

Analysis of Variance (ANOVA) was carried out using 'Agrobase 99,' Agronomix Software, Inc., Manitoba, Canada (Mulitze, 1998).

2.2.6 Implementation of agro technology for mass cultivation at higher altitude: -

Materials:

- ⇒ Glass and poly houses (100 X 60 feet)
- ⇒ Fresh leaf
- ⇒ Plantlets
- ⇒ Scales of different lengths
- ⇒ Threads

⇒ Weighing balance

Method:

Mass cultivation of *Aloe barbadensis* Ecotype 1 was carried out at Auli. The same method of cultivation was followed with some modifications according to the soil condition and climatic requirements. For the experiments glass houses and poly-houses of 100 X 60 feet were constructed. Temperature was maintained 25^0C by using proper aeration and watering system. In open field as well as in glass house, soil beds having dimension of 1X1 meter were prepared. These beds were raised 10 m from land surface to prevent water logging during rainy season. In both conditions ridges were made prior to transplantation and soil was dug before forming the ridges. Total 1000 healthy plantlets of equal size were selected for transplantation in each experimental bed (Fig. 2/5 and 2/6). During transplantation row-to-row and plant-to-plant spacing was 50 cm X 50 cm. All the transplanted plants in glass house and in open field were provided with fertilizer like N, P and K in the ratio 150:100:100 Kg/ha respectively. Soil was irrigated at 15 days interval. Weeding was done every time before irrigation. No insecticide or pesticides were used during this period. The soil used in the glass house was same as that in the open field having a pH range from 5.5 - 6.0. Humidity in the glass house was maintained between 40 to 50 %.

The morphological parameters like length and width of leaves, number of leaves per plant, height of the plant, weight of an individual leaf and number of suckers per plant were measured by using the same techniques as mentioned in 2.2.3. The plants in the glass houses were used for the experimental evaluation. For all these experiments one year old plants were taken as a maturity index. The gel pH was also checked by pH meter as a measurement of gel quality. For this the upper rind of leaf was removed and the clear gel was diluted as 1:10 with de-ionized distilled water.

Figure 2/5: *Aloe barbadensis* cultivation in glass house condition

Figure 2/6: *Aloe barbadensis* cultivation in open field condition

2.3 RESULT:

2.3.1 Collection: -

During vigorous screening of the Uttaranchal region (Kumaon and Garhwal) the genus *Aloe* was found to be distributed all over the region. The plants were found only as wild patches in specific regions. Those were the only ecotypes and species of *Aloe* which were available in Uttaranchal. The locations are given in Table No. 2/7. Out of these places conservation and mass cultivation was carried out at two different locations and the work is still going on. These places are Defence Agricultural Research Laboratory (DARL), Pithoragarh and its field station at Auli (Joshimath).

From Table No. 2/7 it is depicted that the *Aloe barbadensis* ecotypes were distributed from 1082' MSL to 5414, MSL all over the Uttaranchal. While *Aloe saponaria* and *Aloe perryi* were distributed at comparatively lower elevations i.e. 1105' and 1082' MSL respectively. *Aloe humilis* and *Aloe zebrina* were collected from Joshimath (7983') and Tehri Garhwal (3786') respectively which are at higher altitude.

All the ecotypes of *Aloe barbadensis* were found in the Kumaon region except Ecotype 5. Among the other species, *Aloe perryi* was found in Kumaon region, while *Aloe saponaria, Aloe humilis* and *Aloe zebrina* were found in Garhwal region. *Aloe humilis* was found at the highest altitude of 7983' MSL i.e. Joshimath. Ecotype 6 and *Aloe perryi* were found in comparatively lowest altitude of 1082' MSL i.e. Haldwani.

During collection it was also observed that all the sites were sandy and rocky showing same type of surrounding vegetation. The introduced plants near the residential areas were avoided and only wild sources were considered.

2.3.2 Authentication: -

As per herbarium specimens available in the herbaria of Botanical survey of India (BSI), Dehradun and Central National Herbarium (CNH), Kolkata and along with the views of the taxonomists, it may be concluded that most of the plants collected belongs to *Aloe barbadensis* varieties which are called as ecotypes (Fig. 2/7). Six ecotypes found in Uttaranchal region were designated as Ecotype 1 to Ecotype 6. Other species authenticated were *Aloe perryi, Aloe saponaria, Aloe humilis* and *Aloe zebrina* (Fig. 2/8).

During the preparation of herbaria it was observed that the frequency of changing the blotting paper was more for the *Aloe barbadensis* ecotypes and *Aloe perryi* than other species due to high amount of moisture content i.e. approximately more than 90%. Hence, treatment with the antifungal agent was repeated very frequently to avoid fingal growth. It was dried at the earliest and other moisture should interfere was avoided. The winter season was also the limiting factor because of high moisture content in the air..

Table No. 2/7: Geographical details of locations in Uttatranchal for *Aloe barbadensis* ecotypes and other *Aloe* species

Sr. No.	Location	Latitude	Longitude	Altitude (MSL)	Plants Collected
1.	Pithoragarh	29^0 35' 00"	80 13' 00"	5397’	Ecotype 1
2.	Lamgarha	29^0 50' 45"	78 42' 39"	5375’	Ecotype 2
3.	Champawat	29^0 20' 00"	80 06' 00"	5414’	Ecotype 3
4.	Betalghat (Haldwani)	29^0 35’ 33"	79 53’03"	1257’	Ecotype 4
5.	Dehradun (Raiwala)	30^0 01' 00"	78 13' 00"	1105’	Ecotype 5 and *Aloe saponaria*
6.	Haldwani	29^0 13' 00"	79 31' 00"	1082’	Ecotype 6 and *Aloe perryi*
7.	Joshimath	30^0 32’ 00”	79^0 36’ 00”	7983’	*Aloe humilis*
8.	Tehri Garhwal	30^0 22’ 00”	79^0 36’ 00”	3786’	*Aloe zebrina*
9.	Auli (Joshimath)	30^0 32’ 00”	79^0 36’ 00”	9000’	Cultivation site for Ecotype 1

Figure 2/7: Ecotypes of *Aloe barbadensis* (Mill.) collected from Uttaranchal

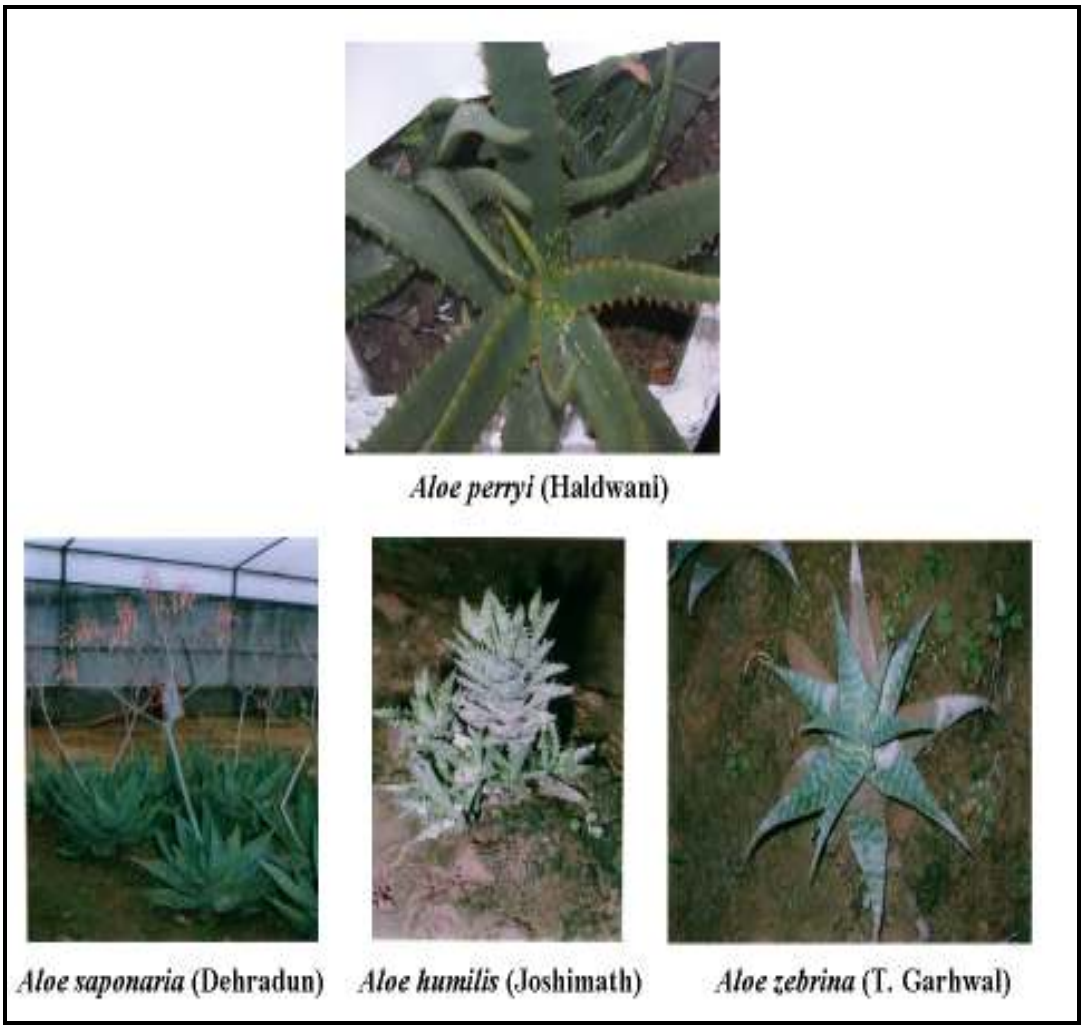


Figure 2/8: Other species of genus *Aloe* collected from Uttaranchal

2.3.3 Morphological Parameters of the *Aloe barbadensis* ecotypes and other *Aloe* species cultivated by using agro technology developed at DARL, Pithoragarh: -

To assess the efficacy of the GAP and the conservational practices various morphological parameters were studied as an indicator. The length and width of leaves plant height, number of leaves, weight of leaf and number of suckers per plant (Table No. 2/10 and 2/13) were compared with the control. The plants actually collected from the wild were considered as the control. Here also parameters of one year old plants were studied. All the cultivated plants showed the improved results as compared to the control ones. From the Table No. 2/10 it was observed that leaves of *Aloe barbadensis* ecotype 3 were very long (70.04 ± 0.71 cm) among all the ecotypes. But the significant increase in

length was found in the leaves of Ecotype 2 as compared to its control. Similar results were obtained for the width of leaves i.e. maximum width of leaves was found in Ecotype 3 which can be directly correlated with the quantity and quality of the pulp and the moisture content of the leaf.

Ecotype 4 was found to be the tallest among all the ecotypes (74.88 ± 5.46 cm) and the most significant enhancement in height was found in Ecotype 2 as compared to its control. Maximum number of leaves (13.66 ± 1.01) and weight of an individual leaf (610.3 ± 15.61 gm) was observed in Ecotypes 2. Significant increase in weight of an individual leaf was observed in Ecotype 3 which was around 31% (control- 525.67 ± 67.14 and cultivated- 610.3 ± 15.61). Maximum number of suckers per plant (15.97 ± 2.22) as compared (13.45 ± 1.47) to control was observed in Ecotype 4. Ecotypes 2, 3 and 4 performed well in all of these parameters. Among all the ecotypes, Ecotype 2 (5375') and Ecotype 3 (5414') are from equal altitude. Ecotype 4 was collected from the Haldwani (1257') which is very near to the plains.

Analysis of variance proved the significant differences among the cultivated ecotypes. Table 2/9 indicates that Ecotypes 4 was found to be tallest and showed maximum significant number of suckers among the cultivated ecotypes. While Ecotype 2 showed maximum significant number of leaves and weight of individual leaf among the cultivated ecotypes.

Different *Aloe* species showed tremendous morphological differences which can be seen in Fig. 2/8. The types of leaves, their colour, length and width, clustering of leaves can be differentiated visually. Among the species- *Aloe perryi, Aloe saponaria, Aloe humilis and Aloe zebrina,* the significant differences were observed in control and cultivated plants for the length and width of leaves. The most significant growth in length and width was observed in *Aloe perryi.* Number of leaves per plant (23.93 ± 1.95) was found to be maximum in *Aloe humilis* but height of plant was found to be the lowest (13.95 ± 1.56). *Aloe saponaria* showed the maximum weight of leaf (415.22 ± 14.91 gm) and maximum number of suckers per plant i.e. 8.04 ± 1.00 as compared to control.

Analysis of variance of these species of *Aloe* (Table No. 2/12) showed that *Aloe saponaria* was best among all the cultivated ecotypes in parameters – plant height, weight of individual leaf and number of suckers.

Table No. 2/8: Comparative morphological parameters of *Aloe barbadensis* ecotypes collected from Uttaranchal

Eco-types	Plant Height (cm)		No. of leaves per plant		Weight of leaf (gm)		No. of suckers per plant	
	Control	Cultivated	Control	Cultivated	Control	Cultivated	Control	Cultivated
1	55.41 ± 9.41	61.7 ± 5.00	12.22 ± 1.52	13.6 ± 0.89	524.11 ± 42.84	609.56 ± 24.18	5.14 ± 1.04	6.67 ± 2.11
2	51.34 ± 4.56	61.93 ± 3.51	12.09 ± 1.42	**13.66 ± 1.01**	525.67 ± 67.14	**610.3 ± 15.61**	4.98 ± 0.81	6.37 ± 1.77
3	54.29 ± 2.19	61.67 ± 1.98	8.99 ± 1.86	10.3 ± 1.86	456.20 ± 14.88	549.78 ± 84.62	5.72 ± 0.96	6.8 ± 1.14
4	68.45 ± 7.86	**74.88 ± 5.46**	12.00 ± 1.58	12.63 ± 1.08	364.44 ± 50.99	401.47 ± 13.17	13.45 ± 1.47	**15.97 ± 2.22**
5	50.91± 8.44	57.79 ± 3.74	9.07 ± 1.79	10.07 ± 0.91	294.27 ± 17.31	302.61± 80.97	9.14 ± 0.92	9.83 ± 1.37
6	65.84 ± 5.22	70.96 ± 3.76	11.56 ± 1.78	12.13 ± 1.18	325.61 ± 18.49	417.22 ± 94.54	10.77 ± 1.09	11.03 ± 2.50

[Values are mean of 30 replicates ± Standard Deviation (SD)]

Table No. 2/9: Analysis of variance for morphological parameters in *Aloe barbadensis* ecotypes

Trait (Morphological)	df (degree of freedom)	MS (Mean square)	P value	Rank
Plant height	5	1298.476	0.0000	4>6>2>3>1>5
No. of leaves	5	74.147	0.0000	2>1>4>6>3>5
Weight of leaf	5	478581.126	0.0000	2>1>3>6>4>5
No. of suckers	5	146.382	0.0000	4>6>5>3>1>2

(1- Ecotype 1; 2- Ecotype 2; 3- Ecotype 3, 4- Ecotype 4; 5- Ecotype 5; 6- Ecotype 6)

Table No. 2/10: Comparative data of length and width of leaves of *Aloe barbadensis* ecotypes collected from Uttaranchal

	Length (cm)		Width of Control (cm)			Width of Cultivated (cm)		
	Control	**Cultivated**	**Bottom**	**Middle**	**Tip**	**Bottom**	**Middle**	**Tip**
Ecotype 1								
Mature leaf	52.64 ± 5.11	58.22 ± 3.74	5.65 ± 1.21	3.32 ± 0.91	0.22 ± 0.56	9.12 ± 0.90	5.98 ± 0.50	0.38 ± 0.08
Middle leaf	35.61 ± 4.81	40.72 ± 3.45	2.96 ± 0.98	2.64 ± 0.91	0.28 ± 0.42	5.32 ± 0.58	3.16 ± 0.52	0.34 ± 0.05
Young leaf	16.34 ± 8.11	19.62 ± 6.59	0.97 ± 1.44	0.69 ± 0.81	0.10 ± 0.29	1.9 ± 0.91	1.1 ± 0.44	0.18 ± 0.13
Ecotype 2								
Mature leaf	59.85 ± 4.27	68.5 ± 2.39	5.34 ± 1.78	3.64 ± 0.56	0.05 ± 0.04	9.28 ± 0.28	5.08 ± 0.28	0.12 ± 0.04
Middle leaf	46.52 ± 1.74	51.22 ± 2.69	4.51 ± 0.68	2.91 ± 0.87	0.08 ± 0.04	6.22 ± 0.88	3.9 ± 0.66	0.12 ± 0.04
Young leaf	2.81 ± 5.66	25.06 ± 3.11	1.23 ± 0.72	0.29 ± 0.50	0.09 ± 0.05	1.6 ± 0.41	0.96 ± 0.30	0.14 ± 0.05
Ecotype 3								
Mature leaf	62.75 ± 1.70	**70.04 ± 0.71**	5.54 ± 0.78	4.22 ± 0.91	0.37 ± 0.17	**9.8 ± 0.60**	**6.82 ± 0.30**	**0.64 ± 0.17**
Middle leaf	40.37 ± 0.97	46.04 ± 0.59	5.51 ± 0.51	2.21 ± 0.93	0.1 ± 0.00	6.38 ± 0.33	4.36 ± 0.43	0.3 ± 0.00
Young leaf	29.62 ± 0.24	33.5 ± 0.90	1.42 ± 0.68	1.91 ± 0.64	0.1 ± 0.00	4.18 ± 0.19	3.14 ± 0.15	0.1 ± 0.00

[Values are mean of 30 replicates ± Standard Deviation (SD)]

Table No. 2/10: continued…..

	Length (cm)		Width of Control (cm)			Width of Cultivated (cm)		
	Control	**Cultivated**	**Bottom**	**Middle**	**Tip**	**Bottom**	**Middle**	**Tip**
Ecotype 4								
Mature leaf	53.91 ± 3.88	60.94 ± 4.68	4.65 ± 1.61	1.53 ± 0.91	0.26 ± 0.53	6.04 ± 0.63	3.6 ± 1.45	0.38 ± 0.04
Middle leaf	43.05 ± 2.42	47.46 ± 4.18	2.81 ± 0.64	3.06 ± 0.73	0.19 ± 1.31	4.88 ± 0.58	3.06 ± 0.38	0.26 ± 0.05
Young leaf	23.96 ± 5.11	24.86 ± 4.31	0.93 ± 1.00	0.64 ± 1.61	0.81 ± 0.53	2.6 ± 0.99	1.58 ± 0.68	0.14 ± 0.05
Ecotype 5								
Mature leaf	58.97 ± 0.77	65.7 ± 3.46	5.94 ± 1.01	2.37± 2.88	0.11 ± 0.08	8.14 ± 0.87	4.68 ± 1.05	0.28 ± 0.08
Middle leaf	41.57 ± 2.33	45.84 ± 4.01	2.86 ± 0.98	1.08 ± 0.67	0.09 ± 0.52	4.34 ± 0.21	2.88 ± 0.26	0.26 ± 0.09
Young leaf	22.09 ± 6.34	24.56 ± 3.69	1.18 ± 2.29	0.52 ± 4.55	0.09 ± 0.48	2.48 ± 0.95	1.46 ± 0.46	0.14 ± 0.05
Ecotype 6								
Mature leaf	62.44 ± 0.03	67.86 ± 0.77	4.94 ± 0.56	2.96 ± 2.56	0.2 ± 0.82	8.44 ± 0.94	4.52 ± 0.46	0.4 ± 0.00
Middle leaf	42.19 ± 2.56	46.34 ± 5.11	3.27 ± 2.94	1.69 ± 0.56	0.19 ± 0.43	5.4 ± 1.44	3.12 ± 0.05	0.26 ± 0.05
Young leaf	26.42 ± 1.58	29.8 ± 6.09	1.52 ± 0.64	0.1 ± 1.02	0.2 ± 0.94	2.28 ± 0.29	1.3 ± 0.38	0.2 ± 0.05

[Values are mean of 30 replicates ± Standard Deviation (SD)]

Table No. 2/11: Comparative morphological parameters of *Aloe* species collected from Uttaranchal

Aloe species	Plant Height (cm)		No. of leaves/ plant		Weight of leaf (gm)		No. of suckers per plant	
	Control	**Cultivated**	**Control**	**Cultivated**	**Control**	**Cultivated**	**Control**	**Cultivated**
Aloe perryi	35.28 ± 4.12	44.3 ± 3.47	13.07 ± 3.76	13.43 ± 3.76	114.36 ± 53.58	174.81 ± 19.99	2.48 ± 1.94	4.63 ± 1.38
Aloe saponaria	50.08 ± 6.41	**54.83 ± 2.33**	15.22 ± 4.06	17.64 ± 2.81	380.12 ± 37.41	**415.22 ± 14.91**	6.08 ± 1.53	**8.04 ± 1.00**
Aloe humilis	11.54 ± 3.51	13.95 ± 1.56	21.63 ± 3.81	**23.93 ± 1.95**	88.62 ± 29.64	102.00 ± 1.02	4.11 ± 0.37	6.04 ± 0.01
Aloe zebrina	22.61 ± 6.24	26.19 ± 2.11	10.03 ± 2.64	13.86 ± 0.96	285.00 ± 22.54	345.00 ± 32.99	3.05 ± 0.61	3.81 ± 0.00

[Values are mean of 30 replicates ± Standard Deviation (SD)]

Table No. 2/12: Analysis of variance for morphological parameters *Aloe* species collected from Uttaranchal

Trait (Morphological)	df (degree of freedom)	MS (Mean square)	P value	Rank
Plant height	3	12858.923	0.0000	2>1>4>3
No. of leaves	3	686.217	0.0000	3>2>4>1
Weight of leaf	3	772765.757	0.0000	2>4>1>3
No. of suckers	3	111.961	0.0000	2>4>3>1

(1- *Aloe perryi*; 2- *Aloe saponaria*; 3- *Aloe humilis*; 4- *Aloe zebrina*)

Table No. 2/13: Comparative data of length and width of leaves of different *Aloe* species collected from Uttaranchal

	Length (cm)		Width of Control (cm)			Width of Cultivated (cm)		
	Control	**Cultivated**	**Bottom**	**Middle**	**Tip**	**Bottom**	**Middle**	**Tip**
Aloe perryi								
Mature leaf	44.94 ± 9.81	51.68 ± 5.68	3.69 ± 1.08	2.37 ± 1.49	0.1 ± 0.13	5.6 ± 0.37	3.44 ± 0.29	0.4 ± 0.13
Middle leaf	25.63 ± 0.54	30.62 ± 2.26	1.09 ± 0.91	0.69 ± 0.88	0.1 ± 0.00	2.18 ± 0.16	1.4 ± 0.28	0.3 ± 0.00
Young leaf	15.06 ± 4.84	18.6 ± 6.44	0.91± 1.44	0.27 ± 0.43	0.1 ± 0.00	1.4 ± 0.4	0.9 ± 0.22	0.1 ± 0.00
Aloe saponaria								
Mature leaf	35.84 ± 3.64	39.42 ± 4.70	5.29 ± 2.03	4.63 ± 1.49	0.1 ± 0.13	6.84 ± 1.08	5.46 ± 0.29	0.4 ± 0.13
Middle leaf	27.81± 1.08	31.62 ± 1.26	4.12 ± 0.91	3.15 ± 0.88	0.1 ± 0.00	3.61 ± 0.16	4.09 ± 0.28	0.3 ± 0.00
Young leaf	22.06 ± 2.84	25.6 ± 2.44	2.16± 0.78	1.27 ± 0.43	0.1 ± 0.00	2.84 ± 0.4	2.00 ± 0.22	0.2 ± 0.00
Aloe humilis								
Mature leaf	8.73 ± 6.44	10.72 ± 3.94	5.07 ± 2.00	2.37 ± 1.49	0.1 ± 0.13	6.87 ± 0.87	3.44 ± 0.29	0.4 ± 0.13
Middle leaf	7.31± 2.18	9.23 ± 3.47	4.08 ± 1.16	0.69 ± 0.88	0.1 ± 0.00	5.24 ± 1.07	1.4 ± 0.28	0.3 ± 0.00
Young leaf	4.08 ± 3.19	6.88 ± 1.87	2.97 ± 2.33	0.27 ± 0.43	0.1 ± 0.00	3.34 ± 1.44	0.9 ± 0.22	0.1 ± 0.00
Aloe zebrina								
Mature leaf	34.94 ± 4.81	38.68 ± 1.68	4.69 ± 1.08	2.88 ± 1.64	0.1 ± 0.13	5.6 ± 0.37	3.44 ± 0.29	0.4 ± 0.13
Middle leaf	31.63 ± 0.54	33.62 ± 2.26	3.09 ± 0.91	1.69 ± 0.88	0.1 ± 0.00	2.18 ± 0.16	1.4 ± 0.28	0.3 ± 0.00
Young leaf	29.06 ± 3.84	31.6 ± 3.94	2.91± 1.44	0.99 ± 0.43	0.1 ± 0.00	1.45 ± 0.4	0.9 ± 0.22	0.1 ± 0.00

[Values are mean of 30 replicates ± Standard Deviation (SD)]

2.3.4 Performance in terms of morphological parameters of *Aloe barbadensis* Ecotype 1 at Auli using the developed agro technology: -

Mass cultivation study of *Aloe barbadensis* Ecotype 1 was carried out at the higher altitude at Auli (9000') and same parameters were assessed regarding performance at higher altitude.(Table No. 2/14 and 2/15). The data was recorded after one year. Gel pH was also checked initially and at the time interval of 50 days up to 150 days. The pH was found to be always in the range of 5.6 to 5.8. Plant height and number of leaves per plant were found to be reduced at higher altitude as compared to the control. However, significant increase in weight of leaf (524.11 ± 42.84 to 573.52 ± 24.18) and in formation of number of suckers (5.14 ± 1.04 to 7.31 ± 0.91) per plant was recorded. The enhancement in length, width in mature, middle and young leaves measured at bottom, middle and tip was also recorded (Table No. 2/15).

Table No. 2/14: Comparative morphological parameters of *Aloe barbadensis* Ecotype 1, cultivated at higher altitude (Auli, 9000' MSL)

Sr. No.	Parameter	Result	
		Control	Cultivated
1.	Plant height (cm)	55.41 ± 9.41	51.99 ± 3.41
2.	Number of leaves per plant	12.22 ± 1.52	10.51± 2.47
3.	Weight of leaf (gm)	524.11 ± 42.84	573.52 ± 24.18
4.	Number of suckers per plant	5.14 ± 1.04	7.31 ± 0.91

[Values are mean of 30 replicates ± Standard Deviation (SD)]

Table No. 2/15: Comparative length and width of *Aloe barbadensis* Ecotype 1 cultivated at higher altitude (Auli, 9000' MSL)

Ecotype 1 cultivated at (Auli)								
	Length (cm)		Width of control (cm)			Width of cultivated (cm)		
	Control	Cultivated	Bottom	Middle	Tip	Bottom	Middle	Tip
Mature leaf	52.64 ± 5.11	48.68 ± 3.88	9.12 ± 0.90	5.98 ± 0.50	0.38 ± 0.08	6.39 ± 2.81	4.01 ± 0.91	0.30 ± 0.51
Middle leaf	35.61 ± 4.81	32.55 ± 1.53	5.32 ± 0.58	3.16 ± 0.52	0.34 ± 0.05	3.96 ± 0.91	2.29 ± 0.52	0.28 ± 0.47
Young leaf	16.34 ± 8.11	14.92 ± 4.74	1.9 ± 0.91	1.1 ± 0.44	0.18 ± 0.13	0.99 ± 2.09	0.82 ± 1.00	0.10 ± 0.00

[Values are mean of 30 replicates ± Standard Deviation (SD)]

2.4 DISCUSSION:

Uttaranchal is divided into two parts geographically and culturally. These parts are Kumaon region that includes districts of Pithoragarh, Almora, Champawat, Bageshwar, Udham Singh Nagar and Nainital. While Garhwal regions includes districts of Chamoli, Pauri Garhwal, Haridwar, Rudra prayag, Uttar Kashi, Tehri Garhwal and Dehradun, the state capital.

It was decided to screen the whole Uttaranchal area for the collection of *Aloe*. The collection was exclusively carried out in the months of March to May as the rainy season starts from June till September. The rains in hills are very heavy which brings floods and land slides. The transport and communication becomes very difficult in those days. After September winter starts with chilling cold and most of the area get covered by the snow. The collection becomes impossible. Hence this schedule was followed for collection.

The screening revealed that *Aloe* plants were distributed all over Uttaranchal in the altitudinal range of 1082' to 7983' above the MSL. All the available species of genus *Aloe* were collected. Details of the location at which the *Aloe* plants were found are described in Table No. 2/7. It was also observed that the plants were found in wild patches in specific regions. These regions are generally far away from the residential areas. These patches were generally at the slopes without big trees and soil was mostly rocky and dry. The nearby vegetation was also found to be same in all the regions mostly of Agave spp. and pines. The plants transplanted near the residential areas were not collected as they may be introduced.

The collected plants were found to be very difficult to identify in terms of similarity and differences. Hence they were sent for the identification and authentication. Different agencies like Kew Garden, London, International Aloe Science Council (IASC) USA, South African Botanical Garden, South Africa were consulted. Lot of problems was faced for the preparation of herbarium of leaf as it contains about 90% of moisture. The initiations of fungal growth, blackening of leaf were very frequent obstacles. Hence saline solution and antifungal agents were used to overcome it. Frequency of treatment with antifungal agent was dependant upon the moisture content of the species. In the rainy and winter season atmospheric moisture content remains high which was also

considered and hence the preparations of herbaria were carried out strictly in summer season. As per the Botanical Survey of India (BSI), Dehradun and Central National Herbarium (CNH), Kolkata, among the collected plants, 6 were *Aloe barbadensis* and hence depending upon the site of collection they were named as Ecotypes of *Aloe barbadensis* (Ecotype1 to 6 for different locations). Other four were identified as - *Aloe perryi, Aloe saponaria, Aloe zebrina* and *Aloe humilis.*

In morphological parameters it was observed that the location of collection, types of soil and the nutrient content of the soil played a very crucial role. The plants at the slopes and the water deficient region showed more length and width of leaves which is the measure of the high quantity and quality of pulp and more moisture content. The low soil water potential reduces fresh leaf yield, plant growth rate, and the leaves production (Rodr´ıguez-Garc´ıa, 2007). In the present study Ecotypes 2, 3 and 4 were found to be acclimatized with these conditions. The locations of these ecotypes are of very high slopes and hence to retain the large amount of moisture leaves may have developed water retention capacity which can be correlated with the length, width of leaves and number of leaves. The soil type and nutrient content of the soil are comparatively improved as compared to the hilly and sloppy regions of other sites. Thus these ecotypes have superior phenotypic traits as compared to others. For the other species of *Aloe,* drastic variation among the morphological parameters is due to species difference.

ANOVA showed maximum height of plant and number of suckers in Ecotype 4. It was collected from plains and rich soil nutrient region. Ecotypes 2 showed high weight and number of leaves because due to harsh climate it may have developed water retension tendency. It can be mentined that the main reason for variation among the other species is species specific differences.

Among the genus *Aloe, Aloe barbadensis* is the most important species from pharmaceutical and medicinal point of view. Other species are not recommended for edible or other related purposes. Hence *Aloe barbadensis* was selected for the mass cultivation and the conservation and agro technology was developed for this. The guidelines recommended by World Health Organisation (WHO) for the cultivation of medicinal plants were followed. Initially the cultivation was carried out at Pithoragarh

(5387'). After the good response and due to the policy of DRDO the cultivational experiments were carried out at higher altitude at Auli (9000').

Up till now semiarid regions, the sandy soils, dry coastal areas and dunes were considered to be suitable places for the cultivation of *Aloe barbadensis*. Commercial cultivators of *Aloe barbadensis* have stressed the fact that low temperatures are devastating for the growth of plants. Later modern cultivation practices for commercial purpose were developed for places where the winter is mild and frost is infrequent like Florida, parts of Texas and the West Indies (Kent 1980; Haller 1990). In such places a protective cover was used when frost was forecasted (Kent 1980). It was found that *Aloe barbadensis* could be cultivated at an altitude of 310 m and 380 m characterized by low winter temperature and mild frost events (Saks and Gordon, 1995). In India, the states like Gujarat, Rajasthan, Madhya Pradesh and Maharashtra are suitable for cultivation (Maiti, 2002). In the present study suitability of climatic conditions and soil requirements for cultivation of *Aloe* at Pithoragarh (5397' MSL) and Auli (9000' MSL) were studied. Climate in Pithoragarh was very cold with very less amount of moisture in the air. In summer temperature is 15 to 30 ^{0}C with frequent showers and in winter -10 to 25 ^{0}C with snow fall at night. Soil type was loamy. Hence in the present studies to cope up with this soil conditions, sand and powdered bricks were added in the soil for proper drainage system.

It is also well known that *Aloe barbadensis* flourishes in climate of high temperature up to 45^{0}C to 48 ^{0}C and withstand drought. Although it is tropical plant roots survive freezing temperature. The leaves are damaged in 4 to 5 ^{0}C and new plants may emerge out again from the roots when temperature becomes normal (Maiti, 2002). In the present study similar results were obtained. It was also observed that the plants cultivated in open fields were badly affected by extreme cold conditions as compared to the plants cultivated in glass and polyhouses. The plants get dried at the tip of the leaves and colour of leaves became reddish green. It may be due to the alteration in the chlorophyll synthesis and its degradation as the sun remains covered in the clouds in rainy and winter season.

In nature *Aloe* grows on poor to well fertile soils and in deserts, but it should be free of water logging. The sandy loam to loamy sand soils or sandy clay soils are

recommended for commercial cultivation. It was reported that *Aloe* tolerates high pH (8.5) and high Na and K salts (Maiti, 2002).

Under hilly conditions the propagation is generally done by plantlets because the seed formation due to onset of winter at the time of maturity is not possible and also because the flowering and seed formation starts in the second year of plant growth. In the present study also same method of replantation of suckers was used for propagation and multiplication.

Aloe barbadensis is more responsive to nutrients. However, excess doses as well as improper sources of chemical nutrients can show negative effects on quality (Mirza *et. al.* 2008). Organic manures are more effective for *Aloe barbadensis* growth and yield as compared to chemical fertilizers (Saha *et. al.* 2005). It was also recommended that soil should be supplemented with ammonium nitrate (www.ikisan.com) and 5 to 10 tonnes of Farm Yard Manure (FYM) per hectare (www.hitayu.com). In the present study 400 quintal of decomposed FYM, 20:20:20 kg/hectare of N: P: K (Nitrogen: Phosphorous: Potassium) was applied as a basal dose for both, open and protected conditions. After the plants attained the age of 4 to 5 months 20 kg/hectare additional dose of Nitrogen was applied in the form of urea.

For transplantation of suckers line to line spacing and plant to plants spacing was kept as per the standards for the cultivation of the medicinal plants. Generally 15 cm deep pit and 60 X 60 cm plant spacing is recommended (Maiti, 2002). Considering this and depending upon the area root suckers were transplanted.

Aloe barbadensis requires constant irrigation to maintain proper growth, health and quality of pulp. The cultivation is generally rainfed or irrigated by sprinklers (www.hitayu.com). Hence standardized protocol was followed for irrigation. Beds were prepared above the soil for efficient channelizing of water. It has been observed by the experiments that irrigation if done in 2 to 3 weeks interval the pulp consistency and amount is better than the irrigation done on as and when required basis. It should keep in mind that water logging even for half an hour of irrigation must be avoided. The delayed irrigation retards quality as well as quantity of pulp.

In *Aloe* cultivation no major problem of weeding was recorded (Maiti, 2002, www.hitayu.com, www.ikisan.com). Still time to time weeding and hoeing was carried

out and proper care was taken for infection and pest control. As the *Aloe* does not have a stem to keep it in standing position and to avoid falling down to the earth, stick was tied for the support. It is called as an earthing. Earthing of crops is essential for two main reasons, one for keeping the plant straight. Otherwise plant drops to sides due to heavy weight of leaves gained in later stage of growth and another reason is by proper earthing plantlet emergence is more. It was observed in plants that were not subjected to earthing produced only 3 to 4 plantlets whereas plants which were given proper earthing produced 10 to 12 plantlets. The earthing is recommended by an interval of one month or after each hoeing and weeding. Earthing should be done at least up to 6 inch height of ground in several steps. The plantlets emerge out after 9 to 10 months of crop age. In case of protected cultivation, plantlets start emerging out in last week of December and 1st week of January, whereas in open conditions the plantlets emerge out in March. Therefore for transplantation of plantlets in open conditions they should be taken from the plants growing under protected conditions in the month of March.

In case of *Aloe barbadensis*, no proper index of maturity is available. Generally 18 to 24 months old plants are considered as mature (www.ikisan.com). It has not been worked out at which stage the plant should be harvested. However from the present study it was found that the growth of lower leaves may be taken as maturity index. In case of open condition of cultivation the leaf growth on an average becomes static when it attains 46 to 48 cm length from where further growth stops. This stage is achieved after 8 or 9 months of growth towards the onset of winter i.e. in November to December. At this stage the crop under open cultivation is recommended to be harvested. In case of protected cultivation the leaf size comes to be 62 to 65 cm of length after 9 to 10 months i.e. in December and January. This is the stage recommended as harvesting index for protected cultivation.

After nine to ten months of growth proper maturity index was observed. Very carefully optimized protocol for harvesting, cleaning, drying, removing of gel and its preservation was followed. All these methods have given the fruitful results and very good yield was obtained. Covered conditions i.e. glass and polyhouses gave more yield (2400 q/ha) as compared to open fields i.e. 1750 q/ha. In ideal conditions i.e. in natural

habitat of the *Aloe barbadensis* 525 quintals /hectare yield is reported (www.hitayu.com). In the present study the yield was three times greater than this.

For extraction of the ingredient the criteria selected was maturity index of leaf. It is depicted from the literature that the one year old plant is supposed to be healthy and mature enough to have entire ingredient at the full strength. Same criteria were followed for the rest of the research. After cutting the leaves were washed properly to remove foreign material and dust. They were then dried, wiped with the help of clean cloth and then send for chopping, drying in sunlight, drying in oven and then grinding**.** Drying of chopped material was done in a dryer under hot circulating air at 38 to 40 degree centigrade for 70 to 96 hours. The dryer was having an externally driven motor in which fans were mounted on one inner side of dryer having exhaust on the top.

It is reported that about 15-20 tonnes/ hectares of fresh leaves can be obtained from the field cultivation in Rajasthan, India (Maiti, 2002). This place is ideal for cultivation of *Aloe*. In our experiments the average yield of fresh leaves in protected conditions under above agronomical standards has been achieved to be 2400 q/ha and under open condition it was found to be 1750 q/ha which is higher than the reported. This means all the practices used for the cultivation of *Aloe* at higher altitude are appropriate and can be recommended of the mass cultivation in hilly regions of Uttaranchal.

During the experiments it was observed that all the morphological parameters in the cultivated ecotypes and species showed the good improvement and enhancement in all the evaluated morphological parameters as compared to the control, which was the result of the appropriate cultivational agro technology developed for the plants of genus *Aloe* collected from the Uttaranchal region of India. The technology proved beneficial not only for the *Aloe barbadensis* ecotypes but also for the other collected species viz *Aloe saponaria*, *Aloe humilis* and *Aloe zebrina*.

By using this technology mass cultivation at the various sites in Uttaranchal region can be carried out for *Aloe,* which will help in reducing the stress on wild collection of the plants.

The present study also shows that the *Aloe* plants can be cultivated at much higher altitudes. Controlled and closed cultivation was favorable for plant growth however; plant can also be successfully cultivated in open field. All the plants in glass house survived

without any mortality. While in open field, about 4 to 5 % mortality was found with some damage to leaves at both the sites of cultivation. The reason could be the strong winds which enhances the severity of cold. It was also observed that the plants were well grown in the glass house than open field indicating that adverse effect on the growth of plants in open field was due to weather and not because of nutrient deficient and/or acidic soil. The reddening as a coral or reddish tinge at the tips of the leaves was observed in the plants of open field, these results are similar to the observation by Kent (1980). This may be due to alteration in the chlorophyll synthesis due to cloudy conditions. The glass house condition was found to be more suitable for plant growth as it provides protection from wind, frost and low temperature at night and maintains the humidity and temperature.

It was found that plants were healthy in open fields only during summer season. During winter season leaves became dry and reddish due to excess cold and snowfall. The plant in polyhouses and glass houses remain healthy throughout the year and consistent growth was observed.

At all stages, it was found that glass house condition was favorable for plants growth than the open field condition as the plants in glass house had higher number of suckers and leaves. The length and width of leaves was also more than the plants in an open field conditions. The number, length and width of leaves were found to be almost three times higher in glass house than in open field. Also the number of days required for the initiation of sucker formation was almost half in glass house with almost 3 % more number of suckers.

In winter season plants in glass house remain unchanged while in open field reddening and drying of leaves at the tip portion was observed and minimum six months old plants could survive. No measurable growth was observed in this season. With the onset of the summer all plants in open field show rejuvenation.

From all the results it can be concluded that all the morphological parameters were found to be improved using this technology despite of the location of collection, varietal and climatic differences.

2.5 CONCLUSION:

Distribution studies carried out indicated that the genus *Aloe* was distributed throughout the Uttaranchal region of Himalayas. No of plants species and varieties collected were mainly collected from Kumaon region which is 1082-5397 MSL altitude. Among all, only three locations (Tehri Garhwal, Joshimath and Auli) had shown the availability of the *Aloe* in Garhwal region. It's interesting that out of these three locations two were under the very high altitude areas and all of them have very different species of *Aloe*. Total 5 species has been identified and collected from whole Uttaranchal region. Most of the species were found in rare pockets in the wild the common being *A. barbadensis*.

Total plants of *A. barbadensis* maintained in glass houses by the developed agro-technology were 60,000 (in the year 2005). Agro technology developed was found to be efficient and appropriate for *Aloe* plants. All morphological parameters were found to be improved in glass houses but open field conditions were not much favorable, the reason being the extreme cold temperature and dry chilled air during the winter season.

Based on these results it can be concluded that the genus can be cultivated for its pharmaceutical and nutritional constituents in Uttaranchal region. It can be also used for socioeconomic development of the region. Mass commercial cultivation will generate the employment opportunities through cottage and processing industries and also as food supplement for the undernourished population of the region. It has already been known that high degree of slopes, continuous land slides and high altitudes are responsible for nutrient deficiency of soil. Hence in Field Station Auli (Joshimath, 9000'), certain modifications were done in GAP. It was decided to cultivate equal number of plantlets in open field and in glass house to find out the effect of nutrient deficit and acidic soil on plant growth.

The present study suggests that cultivation of *Aloe barbadensis* is also possible in protected conditions at the regions of very high altitude and extremely cold temperatures like snow covered for more than six months which is very diverse from its natural habitat of *Aloe*. These finding are in agreement with Kent's suggestion (1980) that *Aloe barbadensis* plant can thrive in any soil and therefore can be considered as a potential

crop at higher altitudes. The study is helpful to extend the range of *Aloe barbadensis* cultivation as its market demand is increasing day by day due to its wide range of uses. It has been proved that it can also be used as a minor crop for extra source of income.

2.6 APPLICATION:

Aloe plants can be found in these hilly regions of Uttaranchal even though it is not their natural and normal habitat. By using the developed agricultural practices people in this region can cultivate *Aloe* for commercial purposes and can opt for small cottage industry or processing industry as an employment opportunities. Also the nutrient deprived land of hills can be utilized for mass cultivation and resource generation.

Defence Agricultural Research Laboratory (DARL), Pithoragarh, Uttaranchal is already conducting free training sessions and seminars for the retired army personals, poor and needy people and students for commercial cultivation and management of Himalayan herbs of food and medicinal importance.

The present work had been undertaken to study the cultivation of *Aloe barbadensis* in Western Himalayan region of Uttaranchal, India. The region is at the altitude of 2680 m from MSL characterized by deep freezing temperature (from-10°C to 23°C) and low soil pH with an average snowfall up to 250 cm. The place remains snow covered from November to March and temperature ranges between -10^0 to 25^0C with an average rainfall up to 1400 mm. Total snowfall in the season can go up to 250 cm. and the soil in this region is nutrient deficient due to its topography, intensive rain and high degree of slopes.

The ultimate aim of this research is to benefit the troops posted in this region as its gel has already been proved to be a good remedy for frostbite and gives protection from UV rays. In addition *Aloe barbadensis* has good nutritional value therefore it can also be used as a food supplement at higher altitudes where there is unavailability of nutritious foods. Considering these importance, promotion for cultivation of *Aloe barbadensis* in this state will not only meet the increasing demand of market for *Aloe* but will also help in improving the socioeconomic status of the local farmers.

Chapter 3

Nutraceutical Assessment

Extraction of active ingredients from the Aloe plants
means taking out the intelligence and throwing away the wisdom.

3.1 INTRODUCTION:

There is an emphasis on the improvement of nutritive value of foods in the developing countries and especially the hilly regions where soil is deprived of many essential nutrients. Effects of the environment and soil on uptake of nutrients by plants have direct consequences on nutritional quality of food and thereby on human health. Different geographical regions show variations in the nutrients composition of the plants i.e. the relative proportions of carbohydrates, proteins, and fats and hence the foods. In developing countries nutritional status affect through dependence on food availability (Westerterp Plantega, 1999). Due to population explosion India is among one of the regions in the world where number of undernourished people has increased by 18 million between 1990-92 and 1998-2000, although the proportion of undernourished dropped by one percent (Andrew Aitken). This proportion is more in rural and hilly regions.

Uttaranchal, the hilly state of in India is comprised of 13 districts divided into two regions- Kumaon and Garhwal (Chapter No. 1 and 2). About 93% area is covered by hills and only 7% is covered by plains. High mountains formed of sedimentary rocks broken by valleys and deep gorges characterize the terrain. The hill areas are sparsely populated, communication is difficult, and many areas are inaccessible. Natural calamities like droughts are a regular recurrence. The hill region lags behind in agro-industrial development and the level of poverty is also high.

As per the survey, the people living in this region, especially in rural areas are undernourished. Most of them can be categorized into severely malnourished people because of the varied agro climatic conditions that results in different cropping system. As a result people don't get complete nutrition from the food. The lifestyle also affects the nutritional condition of these people. The people living in urban areas have a better nutritional status due to literacy, dietary habits, availability of nutritious food and food supplements, small family and fare economical status.

There is need to screen this Himalayan region of rich plant biodiversity for its potential to provide the nutrients of plant origin which are endemic to the region. Hence the present studies were undertaken to screen the genus *Aloe* which found to be the potential source of nutrients (Chapter No. 2).

The genus *Aloe* is native to the Mediterranean region but now widely distributed in southern parts of North America, Europe and Asia. These are cactus plants and contains about 98-99 % water and its gel has a pH of 4.5. The remaining (1%) solid material contains about 246 different compounds that contribute to its medicinal properties. The most important species is *Aloe barbadensis* which is also known *as Aloe vera* (Allen *et al.*, 1974; Joshi, 1998). It is one of the highly exploited species of the world for its nutraceutical (Akpanabiatu *et al*., 1998; Smith, 1988), cosmeceutical and pharmaceutical applications (Mills, 1981; Nielsen 1984). It contains important ingredients like phenolics (anthraquinones) (Okamura *et al.,* 1996), vitamins, minerals (Shelton 1991), enzymes (Sabeh 1996), sugars i.e. carbohydrates, sterols, amino acids and salicylic acid (Sabeh 1996). The constituents of pharmaceutical importance are aloin, aloe-emodin, barbaloin and acemannan as they are of high pharmaceutical and clinical importance. These compounds are throughout distributed in leaf, gel and rind of plant.

Aloe is to be uniquely valued for its content of active biochemicals. These are substances which interact with living cells in very small amounts, producing significant changes to cell metabolism and cell behavior. Even though *Aloe* contains large amount of essential nutrients it itself is not a food, but pharmacologically active substances of the same general type as that distributed among unprocessed whole foods. None of our foods contain the same range of active cell-stimulating constituents as *Aloe* in the same proportions, but the principles involved in using *Aloe* are much the same as when one uses some foods as medicines. That is one of the most important elements of food therapy. The other aspects of food therapy is the way that the various whole unprocessed foods contribute pharmacologically active substances which constantly stimulate or otherwise modify the behavior and metabolism of our cells.

The consumption of fresh fruit and vegetables is very unevenly distributed between individuals of rural and urban areas. *Aloe* must be classified as an adjunct to the Nutritional Therapy, simply because *Aloe* is not a food. But it contains more potent stimulatory substances than any food, in its own unique combination**.** Used in this way it greatly enhances the efforts of the Practitioner to support the patient's immune system, to promote healing, to cleanse and to relieve inflammatory conditions (Forbes and Erdman, 1983).

Just because the pharmacologically active substances in *Aloe*, and also those in foods, interact with cell surface receptors, and because drugs also do the same thing, there is no need whatsoever to regard the *Aloe* substances as being drug-like in their action.

Aloe products have long been used in health foods for medical and preservative purposes (Grindley and Reynolds, 1986). The potential use of *Aloe* products often involves some type of processing, e.g. heating, dehydration etc. Processing may cause irreversible modifications to the polysaccharides, affecting their original structure, which may promote important changes in the proposed physiological and pharmacological properties of these polymers. At present, very little is known about the effects of processing on either the composition or the structure of the different types of polysaccharides found in *Aloe barbadensis* parenchyma.

Without the knowledge of apt quantities of nutraceuticals in *Aloe*; most of the manufacturers of *Aloe* products are using its crude gel in their preparations. It would be worthwhile embarking on an intensive scientific experimentation and investigation on this apparently valuable medicinal agent and to promote its large-scale utilization (Kojo and Quian, 2004).

Keeping in mind the importance of the biochemical composition of constituents in *Aloe* and their applications in food and medicinal products, present study was carried out to determine the amount of these different nutraceuticals in *Aloe barbadesnis* ecotypes and other species of *Aloe* collected from different parts of Uttaranchal. Even though the natural habitat of *Aloe* is semiarid and tropical regions, the plant is also successfully growing in the hilly regions of Uttaranchal which is a Western Himalayan region having agro climatic variation within the small geographical area (Chapter No.2). The medicinal products for eczema, leucoderma and toothache were already been developed at DARL, Pithoragarh in which *aloe* is one of the major constituents (Chapter No. 7).

The present work was done with a view to add information that could lead to its proper utilization as a food, food supplements and various related product formulations; and nutritional upliftment of the people living in Uttaranchal region.

3.2 MATERIALS:

Source of plant material: -

For nutritional evaluation of the *Aloe* plants leaf material of identified ecotypes and species was collected from different locations along the Uttaranchal region (Kumaon and Garhwal) of India. Collected material was dried and stored initially in air tight polyethylene bags or containers. Six ecotypes of *Aloe barbadensis* (Mill.) and four other species (*Aloe perryi, Aloe saponaria, Aloe humilis* and *Aloe zebrina*) were collected from 8 different locations. (Chapter No. 2, Table No. 2/7)

Glasswares and Plastic wares: -

Micropipettes of different precision measurements (1000, 200, 100, 20, 10 & 2 µl) were procured from Gilson medical Electronics, France. Micro tips, appendorff tubes (0.05, 1.5 & 2 ml) and microtip-boxes were procured from Axygen scientific Pvt. Ltd. (India). While centrifuge tubes (50ml) were purchased from Tarson products Pvt. Ltd, (Kolkata, India).

Chemicals/Reagents: -

Standards:

⇒ For minerals **-** E Merck AAS Spectrosol (100 mg/lit).
⇒ Aloin (Sigma, USA)
⇒ Glucose (SRL, India)
⇒ Cellulose (SRL, India)
⇒ Catechol (SRL, India)
⇒ Oxalic acid (SRL, India)
⇒ Tannic acid (SRL, India)
⇒ Ascorbic acid (SRL, India)

Solvents: (All from Merck, Germany)

- Methanol

- Acetic acid
- Chloroform
- Acetone
- Ethanol
- Petroleum ether

Chemicals: (From Sigma, Qualigens and SRL)

- ✓ Sulfuric acid
- ✓ Nitric Acid
- ✓ Hydrochloric acid
- ✓ Perchloric acid
- ✓ Phenol
- ✓ Sodium bicarbonate
- ✓ Sodium carbonate
- ✓ Folin Danis reagent
- ✓ Oxalic acid
- ✓ Sodium hydroxide
- ✓ Anthrone
- ✓ Folin Ciocalteu reagent
- ✓ Irish Moss extract

Equipments:

1. Atomic Absorption Spectrophotometer: (Model AAS 4129) (Electronics Corporation of India Ltd. Hyderrabad):

The basic principle of this technique is based upon Beer – Lamberts's law. It is used for the assessment of concentration of an analyte (elements) in a sample. The electrons of the atoms in the atomizer are promoted to higher orbitals for a short peroid of time by absorbing a set quantity of energy (i.e. light of a given wavelength). This amount of energy (or wavelength) is specific to a particular electron transition in a particular element, and in general, each wavelength corresponds to only one element. Thus it gives

its elemental selectivity. The quantity of energy (the power) put into the flame is known, and the quantity remaining at the other side (at the detector) can be measured.Thus by using the Beer – Lambert's law how many of these transitions took place can be calculated and a signal that is proportional to the concentration of the element is being measured.

In present study this instrument was used for the measurement of microelements like Co, Ni etc.

2. Flame Photometer (AIMIL India Limited, New Delhi, India):

The flame photometer was devised by Barnes, Richardson, Berry, and Hood in 1945. This technique uses a flame that evaporates the solvent and also sublimates and atomizes the metal and then excites a valence electron to an upper energy state. Light is emitted at characteristic wavelengths for each metal as the electron returns to the ground state that makes qualitative determination possible. Flame photometers use optical filters to monitor for the selected emission wavelength produced by the analyte species. Comparison of emission intensities of unknowns to either that of standard solutions (plotting calibration curve), or to those of an internal standard (standard addition method), allows quantitative analysis of the analyte metal in the sample solution.. The instrument is equipped with an amplifier which permits the analysis of solutions with greatly varying concentrations.

In present study this instrument was used for the measurement of microelements like Na, K, Ca, P etc.

3. UV-Vis Spectrophotometer- UV- 5704 SS (Electronics Corporation of India Ltd. Hyderabad):

The principle of this instrument is based on Beer – Lambert's law. A spectrophotometer consists of two instruments, namely a spectrometer for producing light of any selected color (wavelength) and a photometer for measuring the intensity of light. The instruments are arranged so that liquid in a cuvette can be placed between the spectrometer beam and the photometer. The amount of light passing through the tube is measured by the photometer. The photometer delivers a voltage signal to a display device, normally a galvanometer. The signal changes as the amount of light absorbed by

the liquid changes. If development of color is linked to the concentration of a substance in a solution, then that concentration can be measured by determining the extent of absorption of light at the appropriate wavelength. For example hemoglobin appears red because the hemoglobin absorbs blue and green light rays much more effectively than red. The degree of absorbance of blue or green light is proportional to the concentration of hemoglobin. This instrument was used for all the spectorphotometric estimations.

4. Kjeltec^{TM} Analyser 1026 system (Digestion, Distillation and Titration Unit) (FOSS Tecater, Sweden):

By using this equipment protein analysis based on the principle of Kjeldahl method was performed. It's a fully automated instrument provided with attached digestion and distillation unit. It works on principle of typical Kjeldahl distillation method. A test tube with digested sample, diluted with 75 ml distilled or deionised water is connected to the splash head. Alkali is pumped into the test tube when the alkali handle on the front panel is pulled down. The distillation is started by opening the steam valve, pushing the steam control handle on the front panel, marked STEAM, in the OPEN (down) position. The liberated ammonia goes, together with steam, through the splash head and is cooled in the condenser and collected in the receiver flask. By opening the steam valve on the front panel, power is connected to the heater in the steam generator, the cooling water inlet valve opens, and the distillation starts. The steam generator must contain a certain volume of water to operate. The correct water level in the steam generator is automatically controlled by level pins. When the steam valve is closed, boiling continues for about 10 seconds in the steam generator. The excess steam goes to drain via the waste tubing. At the same time the cooling water is switched off automatically. Thus no overpressure can develop. The generator is either connected to the test tube or to drain. In addition there is a safety valve on the steam generator if for any reason the steam outlet is blocked. In the present study this equipment was used for estimation of total proteins.

5. Tray dryer (Exacta Furnaces, Delhi, India):

It was used for the drying of the cut plant materials. Good circulating air, sensors for the moisture and temperature were the features. It was provided with multiple chambers and treys depending upon the requirement.

6. Lyophiliser (AIMIL India Limited, New Delhi):

Freeze-drying or lyophilization is a process that removes water from a substance. This dehydration process is performed under vacuum while the substance is in a frozen state. Lyophilization technology is used to freeze-dry products such as biologicals, bacterial cultures, analytical chemistry moieties, and therapeutic molecules (e.g., antibodies, vaccines, drugs, and heat-sensitive proteins). Lyophilizing such products, particularly liquid formulations, vastly increases their shelf-life and stability. The lyophilization process comprises three essential steps: pretreatment, freezing and drying.

Lyophiliser was used for the preservation of gel, aloin and other samples.

7. HPLC (Millenium ^{32} 600 E by Waters India Ltd.):

HPLC seperates mixture of compounds on the basis of polarity. Polarity refers to the greater the difference in electron affinity i.e. electronegativity between atoms in a covalent bond, the more polar the bond. Partial negative charges are found on the most electronegative atoms, the others are partially positive. Negative electrostatic potential corresponds to, partial negative charges. Positive electrostatic potential corresponds to, partial positive charges.

It is used to analyze, identify, purify and quantify compounds in the samples. It has a mobile phase, a stationary phase and a detector. The mobile phase is continuously pumped at a fixed flow-rate through the system and mixed by the pump. The injector is used to introduce a plug of a sample into the mobile phase without having to stop the mobile phase flow and without introducing air into the system. The mixture of components is carried in a narrow band to the top of the column. Some compounds in the sample mixture will have greater preference for stationary phase than the mobile phase and will be retained in the column longer. HPLC utilizes different types of stationary

phase (typically, hydrophobic saturated carbon chains), a pump that moves the mobile phase(s) and analyte through the column, and a detector that provides a characteristics retention time for the analyte. The detector may also provide other characteristic information (i.e. UV/Vis spectroscopic data for analyte if so equipped). Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase. The instrument was used for the quantitative determination of aloin in *Aloe barbadensis* ecotypes and species

8. Rotavapor® R-200 (Buchi Corporation, USA.):

It was used for the distillations and evaporation of solvents from extracts. It was provided with the flasks of the different volumes but having the same neck dimensions for fiting in the rotary evaporator. It was also provided with vertical condenser, temperature sensor adjustment and vacuum controller.

9. Soxhlet Apparatus (E – 816 by Buchi Corporation, USA):

The Soxhlet extraction and the specific extraction chamber were invented by Franz Soxhlet in 1879. The Extraction Unit E-816 Soxhlet follows the original principle: The sample is placed in an extraction chamber and solvent is heated to reflux. The Soxhlet extraction chamber is emptied when the set level is reached, with the solvent flowing to the heated beaker. During each cycle, a portion of the non-volatile compound (fat) dissolves in the solvent. At the end of the extraction, the desired compound is concentrated in the beaker. Automated systems have an optical level sensor instead of a siphon, which allows executing more cycles per hour and thus makes the extraction more efficient and faster. It was used for the analysis of crude fat and extraction of different anti microbial compounds by methanol and hexane solvent.

10. pH meter AIMIL (Delhi, India):

pH is the negative logarithm of hydrogen ion concentration. The measurement of pH in pH meter is based on ion exchange in between hydrated layers formed on glass surface. Change in ion exchange results in emf or voltage difference causing current

flow. The current intensity gives the value of pH. For adjusting the pH of the medium, digital pH meter was used.

11. Electric grinder (Local brand):

To grind the chopped and dried material into fine material suitable for extraction, an electric grinder was used. It was also provided with the vessels for different quantity of the material.

12. Vacuum Filter (Tillu, India):

After extraction of the material vacuum filter was used to remove the suspended debris. It was provided with flasks and funnels of different volumes but same neck dimensions. Depending upon the requirement different types of filters were used like muslin cloth, regular filter paper and Whatmann filter paper of various numbers. An electric vacuum pump was used for applying vacuum.

13. Hot Air Oven (Exacta Furnaces, Delhi, India):

Hot air oven with double walls where the inner chamber was made of mild steel or stainless steel and the outer body is made of GI sheets, duly powder coated was used. The gap between the inner and outer wall is filled with glasswool insulation. It is used for the sterilization of the glasswares.

14. Muffle Furnace (Exacta Furnaces, Delhi, India):

These lab furnaces (0-1500°C) specially designed to meet the requirements of high temperature applications was used for the through burning of the sample. In this furnace, the casing was manufactured using MS reinforced with iron angles. Along with this, the chamber was made of ceramic Zirconium Board having horizontally placed elements. Heating was carried out through silicon carbide rods and controlled through transformer. The furnaces have large surface radiation ratio to enhance thermal efficiency and reduce power consumption. There is a control panel fitted with the furnace and was having digital temperature indicator cum controller. It was operable at 220 volts, single phase AC supply. The muffle furnace with 100 to 1000^0C was used in the present study.

Silica crucibles especially used for the crude fibre and total minerals estimations were purchased form Lab India.

15. Milli Q water system (Millipore, USA):

The instrument is used for obtaining deionized water. There are series of ion exchanging columns. When ordinary water is passed through these columns, it is made free of salts and minerals that are naturally present in tap water. It is important to use deionized water for preparation of reagents as the presence of minerals and salts may alter the final composition and thereby alter the results.

16. Water bath (Julabo, Germany):

It is used for maintaining the constant temperature, in which, temperature setting, temperature indicator, and cooling effect are also available.

17. Weighing balance (Labtronics, India):

Digital pan balances covered with transparent chamber were used for weighing. For weighing fresh leaves rough balance with capacity 500 to 10000gm was used. Also for preparation of standards of very low concentration a balance of capacity 10 to 1000 mg was used.

18. Centrifuge (REMI, India):

It was used for the general purpose during the regular experiments. It was provided with the different rotors and tubes of various volumes. It was also provided with the cooling facility and thermal controller. The lowest temperatures 40^0C was maintained depending upon the requirement of the experiments.

19. Hot plate and Heating mantle (Exacta Furnaces, Delhi, India):

Industrial hot plate for general purpose having maximum temperature 370ºC was used. The temperature can be controlled with digital temperature controller cum indicator. Heating mantle suitable for the laboratory purpose was used. It was made up of gold yarn. The net of these heating mantles is designed with tight knitting with no joint

from top to bottom. This ensures strength for high temperature applications up to 350°C. The body of the heating mantles is made of one piece from non rusting aluminium in different capacities. Both these were used for the heating and evaporation of the samples.

3.3 METHODS:

A). Preparation of sample: -

The *Aloe* plants for this study were collected from different agro climatic regions of Uttaranchal and used as control. Fresh leaves of these plants were cut and dried in oven at 40^0C for about two days. These dried leaves then ground to fine powder and were used for the estimation of all nutraceuticals.

For drying of leaves specific method was followed. First fresh leaves were collected and washed properly under running tap water. The water was wiped with clean cloth. They were then chopped into small pieces with the help of a knife and kept in an open air condition for about two hours. Later they were kept in an oven at 40^0C for whole night. It was then taken out, spread properly and temperature was raised to 60^0C. After five to six hours temperature was increased to 100^0C and maintained till all the traces of water has been removed. Then it was ground to fine powder.

For quantitative determination of vitamin C, chlorophyll and proline, fresh leaves from the plants grown in glass houses with controlled conditions were used as it was observed that the plants grown here are healthy and did not get affected by harsh climatic conditions.

B). Moisture content: -

To determine the moisture content of the samples evaporation method given by AOAC was followed. For this fresh leaves of these plants were collected, cut into small parts and weighed properly into Petri-dishes. They were then kept in oven at 40^0C for two hours. After that temperature was raised to 60^0C for four hours. It was then raised to 100^0C to remove traces of water. The dried leaves were then weighed again and the loss in weight was recorded as moisture content.

C). Biochemical parameters: -

Standards were prepared for these biochemical parameters depending upon the compound to be estimated and as per given in reference methods (Table No. 3/16).

Estimation of total carbohydrates was carried out by phenol sulphuric acid method. Cellulose was estimated by acid hydrolysis method. Estimation of phenolics was carried out by Folin-Ciocalteu reagent method and tannins with Folin-Denis method. Estimation of crude fiber was carried out by the method specified by Maynard. Total protein was estimated by Kjeldahl analysis (Sadashivam and Manickam,2005). Estimation of total fat and total minerals was done by AOAC (1970) method. All the values are represented as gm/100 gm of dry weight. Vitamin C was determined by 2, 4 dichlorophenol-indophenol, Chlorophyll by chilled acetone and proline by acid ninhydrine method. Values for these parameters are represented in mg per 100 gm of fresh weight (Table No. 3/17).

Table No. 3/16: Preparation of standards

Sr. No.	Biochemical	Standard	O.D. (nm)
1.	Total Carbohydrates	Glucose (10mg/ml), Aliquots 2- 10 g/ml.	490
2.	Starch	---- do -------	630
3.	Cellulose	Cellulose (100 mg/ml), Aliquots 20- 100 mg/ml.	630
4.	Phenolics	Catechol (10mg/ml), Aliquots 2- 10 mg/ml.	650
5.	Tannins	Tannic acid (100 ug/ml), Aliquots 20- 100 ug/ml	700
6.	Proline	Proline (100 ug/ml) Aliquots 20- 100 ug/ml	520

Table No. 3/17: Methods for quantitative determination of the biochemical constituents

Sr. No.	Biochemical	Sample	Method/ Reagent	Reference
1.	Total Carbohydrates	0.1	Phenol Sulphuric acid	Sadashivam and Manickam, 2nd Ed. 2005.
2.	Starch	0.1	Anthrone	---- do -------
3.	Cellulose	0.1	Anthrone	---- do -------
4.	Total Proteins	1	Kjeldahl	---- do -------
5.	Total Minerals	5	AOAC	AOAC ,W. Horwitz, Washington, 10th Ed. (1970)
6.	Crude Fat	20	---- do -------	---- do -------
7.	Crude Fibre		Acid and alkali hydrolysis	---- do -------
8.	Phenolics	0.5	Folin Ciocalteu reagent	Sadashivam and Manickam 2nd Ed. 2005.
9.	Tannins	0.5	Folin Danis method	---- do -------
10.	Vit C	*5 gm of fresh leaf	2,6 dichloro phenol indophenol	---- do -------
11.	Proline	*0.5 gm of fresh leaf	Acid ninhydrine	---- do -------
12.	Chlorophyll a	*1 gm of fresh leaf	Chilled 80% acetone extraction	Witham et. al., 1971.
13.	Chlorophyll b	*1 gm of fresh leaf	---- do -------	---- do -------
14.	Total Chlorophyll	*1 gm of fresh leaf	---- do -------	---- do -------
15.	Moisture	*1 gm of fresh leaf	Gradual Evaporation	AOAC ,W. Horwitz, Washington, 10th Ed. (1970)

D). Minerals: -

Preparation of standard: -

Reference standards were obtained from E Merck AAS Spectrosol (100 mg/lit). Different aliquots 20, 40, 60, 80 and 100 mg/ ml were prepared.

Preparation of sample: -

For mineral estimation, 0.5 gm of dried powder was digested with two successive aliquots of concentrated HNO_3 (5 ml each). After digestion, organic matter was again slowly digested with 15 ml of triple acid mixture (10 part HNO_3 + 4 part $HClO_4$ + 2 part H_2SO_4). Digestion was carried out till it reduces to 1 ml. The residue after digestion was dissolved in small amount of distilled water, filtered and finally diluted to 100 ml with distilled water. This final solution was then used for the estimation of minerals. Similarly blank was also prepared.

Estimation of macroelements such as Na, K, Li and Ca was carried out by AIMIL Photoflame photometer. Estimation of microelements such as Fe, Co, Mn, Cu, and Zn was done by Atomic Absorption Spectrophotometer Model 4129 (Electronic Corporation of India Ltd., Hyderabad, AP, India).

The instruments were calibrated by using standard solutions (0.20 to 1.00 mg/lit) of above-mentioned metals. The method by Issac (1980) was used for estimation of these compounds. Estimation of P was carried out by colorimetric method as described by Allen (1974). Silica estimation was carried out by the method of Peach (1956) and Mishra (1968) (Sadashivam and Manickam, 2005).

Statistical analysis: -

These nutraceutical values were subjected to one-way analysis of variance (ANOVA) using 'Agrobase 99,' Agronomix Software, Inc., Manitoba, Canada (Mulitze, 1998). Significant differences among the ecotypes were tested by Duncun's Multiple Range Test ($P<0.01$) by MSTAT statistical software.

E). Aloin estimation: -

Aloe barbadensis is known for two main active principles one is the yellow sap or latex which is a major source of aloin which is extracted from outer rind portion. Another source of aloin is the central mucilaginous portion called pulp containing gel as an active principle.

Extraction of aloin: -

The extraction of aloin for the sap is done generally by two processes. These are

1) Mechanical process
2) Chemical process

1). Mechanical process: -

Mechanically the sap is extracted by two ways. In first method a transverse cut at the bottom of the leaf on lower adaxial side is made and the exudate is collected for two consecutive days such as opium from poppy. In second method the harvested leaves are collected and rested vertically in a container having stainless steel gauge at the bottom. The sap generally drops down and collected at the bottom of the container. The collected sap in both the above methods is dried directly in sun light or by heat or lyophilized. This is called as aloin.

For the present study aloin was extracted form the fresh leaves by a transverse cut at the bottom of the leaf on lower adaxial side, rested vertically in a container having stainless steel gauge at the bottom. This was dried by one of the methods mentioned above and used for the study.

2). Chemical process: -

The pulp is scraped out from the leaves with the help of sharp knife and the peeled portion is dried as mentioned above and then ground to fine powder. The grinded material is extracted thrice by methanol. The combined extract was concentrated in rotary vacuum evaporator. The extract was then made alcohol free by adding small amount of

water followed by subsequent evaporation in two or three steps. The concentrated extract is then lyophilized which is nothing but aloin.

Preparation of standard: -

Aloin standard was obtained from Sigma (USA). 1mg/ml of working solution was prepared in methanol just before the experiments.

Preparation of samples: -

Sample 1. 0.05 gm lyophilized leaf latex from freshly cut leaves were dissolved in 9.95 ml MeOH.

Sample 2. 0.1 ml fresh latex dissolved in 9.9 ml MeOH.

Sample 3. 0.1 gm dried leaf powder dissolved in 9.9 ml MeOH.

HPLC conditions: -

- Column – C-18 (µ Porasil- Water's (Millford, MA, USA), 3.9 mm ID X 300 mm L; 10µm irregular particles, stainless steel.)
- Mobile phase:- Methanol: Water containing 0.1% CH_3COOH (50:50)
- Injection volume 10 µl.
- Flow rate – 1 ml/ min.
- Run time: 15 min.
- Detection wavelength: - 355 nm.

Calibration of standard: -

Aloin standard was dissolved in methanol and using different aliquots (10 to 50 µg/ml) standard calibration curve was obtained (Fig. 3/9)

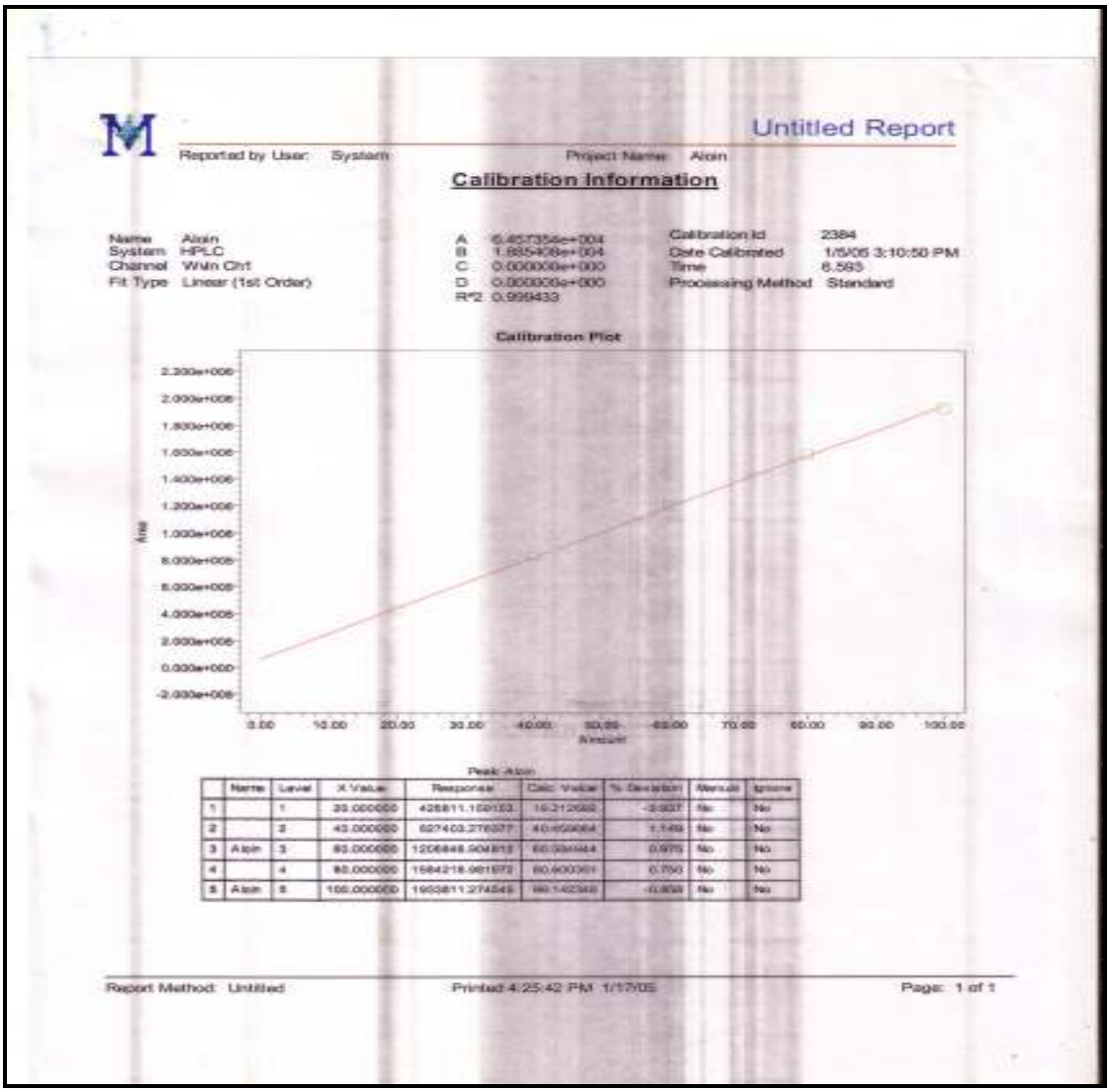


Figure 3/9: Calibration of Aloin standard

F.) General solvent extracts preparation from whole leaf dried material: -

Extraction of whole leaf is to be done from material obtained after drying and grinding process explained as above. The extraction is done by cold extraction method using 70% ethanol and water separately. Such extracts are widely used for different ailments. The cold extraction was done by soaking finely grounded dried powder in distilled water followed by 70% ethanol. The process was repeated thrice by soaking the

material for 24 hours in each solvent. The extract was filtered through muslin cloth over Buchner funnel using single, double, triple and quadruple layers of muslin cloth. Other methods of extraction are not recommended because the filtration membranes get choked due to gel and mucilage even under vacuum filtration.

The same method was followed in the present work.

Concentration of extracts: -

Both ethanol and water extracts are concentrated in rotary vacuum film evaporator. The extract is made alcohol free by adding small amount of water followed by subsequent evaporation in two or three steps. When the extract attains slurry like consistency it was taken out and left for cooling.

Drying of extract: -

The concentrated extract obtained as above are recommended to be deep freezed for 24 hours and then subjected to lyophilization for 70 to 96 hours depending on the quantity of extract at a temperature of -70 degree centigrade and 0.01 mb pressure.

G). Extraction from fresh leaves: -

The harvested fresh leaves after washing and wiping are crushed in grinder or juicer. The juice thus extracted is filtered through a clean muslin cloth as above or centrifuged at 12000 rpm. The clear juice so obtained is either preserved by adding 0.5% citric acid as preservative and stored in air tight pasteurized containers for future use as juice or it is concentrated under vacuum, evaporated and lyophilized as above and then stored.

Storage of extracts: -

Standard aloin as well as lyophilized extracts of aloin obtained as above was recommended to be stored in air tight containers of plastic or glass preferably of amber colour and stored in refrigerator.

H). Processing and preservation of pulp: -

The processing and preservation studies were carried out to find out the best possible method for the purpose.

The parenchymal portion of leaf is a mucilaginous material called as pulp. When it is removed from leaf it is called “gel filet”. This filet is grinded in the form of juice and fiber is removed by filtering through muslin cloth.

Preservation of pulp or gel filet: -

It was carried out by following three methods.

1. Chemical preservation: -

The pulp was scraped from rind portion of leaves and cut into small pieces. These pieces were blanched in lukewarm water. There after these were preserved in 25% ethanol or in mustard oil. The pulp remains intact for more than 2 years at room temperature.

Also it can be preserved by using 0.4 % ascorbic acid or 0.2 % sodium benzoate as a preservative.

The liquid was mixed with jellying agent such as Irish Moss extract.

2. Preservation of gel by precipitation: -

The liquid gel after the step of filtration as above was gradually reacted with acetone and stirred with the help of glass rod. The gel was precipitated out. It was left overnight and next day centrifuged. The precipitated gel was preserved by adding 0.3% citric acid and stored in sealed bottles in cool and dry place. Alternatively the precipitate was dried over hot water bath or lyophilized and stored as above.

3. Preservation by lyophilizing filet: -

The pulp pieces were deep freezed and then lyophilized. The lyophilized material was then stored in air tight containers at cool and dry place.

3.4 RESULTS:

% Moisture and dry matter content of the control and cultivated *aloe* plants was estimated. The plants collected from the wild were used and control and cultivated were used as test plants. From Table No. 3/18 it can be depicted that among all the cultivated ecotypes of *Aloe barbadensis*, Ecotype 2 showed maximum % moisture content and minimum dry matter content in all the plant parts studied for the experiments like whole leaf (98.55 ± 0.96 and 1.45 ± 0.96), peel (93.56 ± 0.31 and 6.42 ± 0.31) and pulp (99.1 ± 0.32 and 0.9 ± 0.32). Remaining cultivated ecotypes showed the significant increase in moisture and decrease in dry matter content. Ecotype 5 showed the least significant increase in moisture of whole leaf (94.58 ± 8.44 to 96.89 ± 0.19) and pulp (96.64 ± 2.01 to 98.83 ± 0.11) while Ecotype 6 showed least in peel (86.89 ± 0.81 to 90.82 ± 0.16) as compared to their respective controls.

Among the *Aloe* species *Aloe saponaria* was found to have maximum amount of % moisture and minimum dry matter content in whole leaf (96.32 ± 0.09 and 3.68 ± 0.09) and peel (90.82 ± 0.16 and 9.18 ± 0.16). It was also observed that in nature also its moisture content was more as compared to other controls among all the species. While *Aloe perryi* showed maximum % moisture and minimum dry matter content in pulp (98.57 ± 0.47 and 1.43 ± 0.47). Cultivated *Aloe perryi* showed the most significant increase in moisture and decrease in dry matter as compared to its control among all the species. Detail values of the % moisture and dry matter content are given in Table No.3/19.

Table No. 3/18: Moisture and dry matter content of the *Aloe barbadensis* ecotypes from Uttaranchal (n=5) (Mean ± S.D.)

	Control		Cultivated	
Plant Part	**% moisture**	**% dry matter**	**% moisture**	**% dry matter**
Ecotype 1				
Whole leaf	89.63 ± 1.23	10.37 ± 1.23	96.79 ± 0.19	3.21 ± 0.19
Peel	84.61 ± 0.91	15.39 ± 0.91	92.48 ± 2.14	7.52 ± 2.14
Pulp	91.21 ± 0.81	8.79 ± 0.81	98.7 ± 0.34	1.3 ± 0.34
Ecotype 2				
Whole leaf	91.47 ± 0.71	8.53 ± 0.71	**98.55 ± 0.96**	**1.45 ± 0.96**
Peel	86.12 ± 1.44	13.88 ± 1.44	**93.56 ± 0.31**	**6.42 ± 0.31**
Pulp	92.89 ± 2.77	7.11 ± 2.77	**99.1 ± 0.32**	**0.9 ± 0.32**
Ecotype 3				
Whole leaf	91.14 ± 0.73	8.86 ± 0.73	97.17 ± 0.08	2.83 ± 0.08
Peel	85.64 ± 4.77	14.36 ± 4.77	91.17 ± 2.46	8.83 ± 2.46
Pulp	93.41 ± 1.71	6.59 ± 1.71	99.06 ± 0.02	0.94 ± 0.02
Ecotype 4				
Whole leaf	94.75 ± 5.06	5.25 ± 5.06	97.21 ± 0.14	2.79 ± 0.14
Peel	89.99 ± 0.75	10.01 ± 0.75	93.05 ± 0.74	6.95 ± 0.74
Pulp	95.95 ± 2.22	4.05 ± 2.22	98.98 ± 0.02	1.02 ± 0.02
Ecotype 5				
Whole leaf	94.58 ± 8.44	5.42 ± 8.44	96.89 ± 0.19	3.11 ± 0.19
Peel	86.47 ± 0.41	13.53 ± 0.41	90.72 ± 0.14	9.28 ± 0.14
Pulp	96.64 ± 2.01	3.36 ± 2.01	98.83 ± 0.11	1.17 ± 0.11
Ecotype 6				
Whole leaf	93.57 ± 4.08	6.43 ± 4.08	96.32 ± 0.09	3.68 ± 0.09
Peel	86.89 ± 0.81	13.11 ± 0.81	90.82 ± 0.16	9.18 ± 0.16
Pulp	95.64 ± 0.33	4.36 ± 0.33	98.41 ± 0.18	1.6 ± 0.18

Table No. 3/19: Moisture and dry matter content of the *Aloe* species from Uttaranchal (n=5)

	Control		Cultivated	
Plant Part	**% moisture**	**% dry matter**	**% moisture**	**% dry matter**
Aloe perryi				
Whole leaf	89.91 ± 0.43	10.09 ± 0.43	95.1 ± 0.35	4.91 ± 0.35
Peel	84.32 ± 1.71	15.68 ± 1.71	90.73 ± 1.32	9.27 ± 1.32
Pulp	91.22 ± 0. 34	8.78 ± 0.34	**98.57 ± 0.47**	**1.43 ± 0.47**
Aloe saponaria				
Whole leaf	93.57 ± 4.08	6.43 ± 4.08	**96.32 ± 0.09**	**3.68 ± 0.09**
Peel	86.89 ± 0.81	13.11 ± 0.81	**90.82 ± 0.16**	**9.18 ± 0.16**
Pulp	95.64 ± 0.33	4.36 ± 0.33	98.41 ± 0.81	1.6 ± 0.18
Aloe humilis				
Whole leaf	86.34 ± 1.41	13.66 ± 1.41	91.23 ± 3.08	8.77 ± 3.08
Peel	82.94 ± 1.07	17.06 ± 1.07	85.41 ± 0.59	14.59 ± 0.59
Pulp	88.91 ± 0.63	8.79 ± 0.63	93.74 ± 0.41	6.26 ± 0.41
Aloe zebrine				
Whole leaf	89.63 ± 1.23	10.37 ± 1.23	91.56 ± 1.07	8.44 ± 1.07
Peel	84.61 ± 0.91	15.39 ± 0.91	87.17 ± 0.42	12.83 ± 0.42
Pulp	91.21 ± 0.81	8.79 ± 0.81	94.01 ± 0.39	5.99 ± 0.39

Mean ± S.D.

From Table No. 3/20 it was observed that starch, total protein, and proline in Ecotype 1 to 4 showed almost similar values with significant differences as compared to their respective controls and more values than Ecotype 5 and 6. Proline was found to be decreased as compared to control in all the ecotypes and least value (0.99 ± 0.07) was

found in Ecotype 5. Proteins were found to be almost in equal amount i.e. approximately 7 % among Ecotypes 1, 2, 3 and 4. It was found minimum (2.6 %) in Ecotypes 5 and 6. Starch was found to be maximum (7.35 ± 0.92) in Ecotype 5. Phenolics, tannins, crude fat and total minerals were having the least significant difference**.** Phenolics values ranged from 0.144 ± 0.01 to 0.243 ± 0.02 % and tannins from 0.646 ± 0.01 to 0.449 ± 0.01 % among the ecotypes. Crude fat values were 0.74 ± 0.18- 1.78 ± 0.15 % and values for total minerals were ranged from 9.62 ± 0.88- 11.79 ± 0.05 %. Highest amount of crude fat was found in Ecotype 3. Other compounds showed significant difference among all the ecotypes. The amount of total carbohydrates varied considerably from 25.17 ± 4.58 % (maximum) in Ecotype 1 to 14.3 ± 1.78 % (minimum) in Ecotype 6. Cellulose values ranged from 6.61 ± 1.29 Ecotype 4 - 4.97 ± 0.59 in and 4.97 % in Ecotype 6, while Vitamin C was found to be maximum in Ecotype 5 i.e. 116.81 mg % as compared to Ecotype 1, 2,3, 4 and 6. Crude fiber and starch values varied considerably among the plants in between 9.55 % - 12.32 % and 5.83 - 7.35 % respectively. Chlorophyll contents were ranging from 1.587 % - 2.471 % among the ecotypes. All the biochemical constituents were found in least amount in Ecotypes 6.

Table No. 3/20: Amount of various nutrients in *Aloe barbadensis* ecotypes

	Ecotype 1		Ecotype 2		Ecotype 3	
	Control	**Cultivated**	**Control**	**Cultivated**	**Control**	**Cultivated**
Total Carbohydrates	17.24 ± 3.54	**25.17 ± 4.58 a**	11.61 ± 0.61	16.05 ± 0.58 c	12.92 ± 0.12	18.42 ± 1.55 bc
Starch	4.17 ± 1.94	5.83 ± 0.86 a	3.09 ± 0.98	5.85 ± 0.20 a	4.07 ± 0.93	6.44 ± 0.83 a
Cellulose	3.98 ± 0.54	5.38 ± 0.26 b	4.27 ± 0.29	6.03 ± 0.26 ab	3.77 ± 0.09	5.19 ± 0.08 b
Total Proteins	2.08 ± 1.89	**7.53 ± 1.24** a	2.93 ± 1.64	6.48 ± 1.05 a	3.66 ± 0.99	7.47± 1.35 a
Total Minerals	5.98 ± 0.64	9.62 ± 0.88 c	6.07 ± 0.67	10.82 ± 0.04 abc	8.37 ± 0.20	**11.79 ± 0.05 abc**
Crude Fat	0.83 ± 0.41	1.74 ± 0.15 a	0.33 ± 0.55	0.74 ± 0.18 b	0.96 ± 0.07	**1.78 ± 0.15 a**
Crude Fibre	8.47 ± 1.21	11.4 ± 0.28 b	6.49 ± 0.64	9.55 ± 0.17 c	4.61 ± 0.66	8.33 ± 0.16 d
Phenolics	0.212 ± 0.78	**0.243 ± 0.02 ab**	2.08 ± 0.04	0.144 ± 0.01 c	2.04 ± 0.94	0.176 ± 0.01 bc
Tannins	0.498 ± 3.55	0.575 ± 0.06 abc	6.73 ±1.88	0.449 ± 0.01 c	0.496 ± 017	0.515 ± 0.05 bc
Vit C *	29.64 ± 3.94	38.84 ± 5.13 c	38.73 ± 0.67	41.47 ± 5.93 c	58.39 ± 0.99	**59.46 ± 0.60 b**
Proline*	3.95 ± 0.62	**3.09 ± 0.20 a**	3.04 ± 0.37	2.75 ± 0.14 a	3.96 ± 0.67	**3.09 ± 0.24 a**
Chlorophyll a*	0.982 ± 1.86	0.825 ± 0.01	0.255 ± 0.33	0.246 ± 0.01	0.394 ± 0.23	0.406 ± 0.02
Chlorophyll b*	1.579 ± 1.86	1.646 ± 0.01	1.013 ± 0.91	1.255 ± 0.00	0.674 ± 0.91	1.63 ± 0.01
Total Chlorophyll*	2.561 ± 1.38	2.471 ± 0.10 a	1.268 ± 1.15	1.501 ± 0.01 c	1.068 ± 0.07	2.036 ± 0.02 b

Table No. 3/20: Continued----------

	Ecotype 4		Ecotype 5		Ecotype 6	
	Control	**Cultivated**	**Control**	**Cultivated**	**Control**	**Cultivated**
Total Carbohydrates	13.43 ± 0.75	18.78 ± 0.42 bc	11.94 ± 0.53	14.49 ± 0.91 ab	8.96 ± 0.66	14.3 ± 1.78 c
Starch	5.09 ± 1.49	7.08 ± 0.63 b	5.62 ± 0.05	7.35 ± 0.92 a	4.09 ± 0.34 b	6.24 ± 0.32 b
Cellulose	4.30 ± 0.86	**6.61 ± 1.29 ab**	5.25 ± 0.42	5.49 ± 0.11 a	2.98 ± 0.08	4.97 ± 0.59 b
Total Proteins	3.37 ± 1.51	6.59 ± 0.97 a	1.00 ± 1.07	2.69 ± 0.97 b	1.04 ± 0.04	2.67 ± 1.03 b
Total Minerals	8.00 ± 0.94	11.58 ± 0.74 ab	6.93 ± 0.07	10.13 ± 0.43 bc	6.99 ± 0.96	10.57 ± 0.37 abc
Crude Fat	0.84 ± 0.41	1.6 ± 0.15 a	0.58 ± 0.94	0.95 ± 0.07 b	0.79 ± 1.07	1.06 ± 0.15 b
Crude Fibre	10.23 ± 1.97	**12.32 ± 0.25 a**	8.32 ± 1.23	11.03 ± 0.82 b	6.96 ± 0.07	8.26 ± 0.24 d
Phenolics	0.185 ± 0.53	0.162 ± 0.03 bc	0.327 ± 0.55	0.219 ± 0.07 a	2.638 ± 0.06	0.205 ± 0.00 bc
Tannins	0.402 ± 0.54	0.592 ± 0.02 abc	0.566 ± 0.09	**0.646 ± 0.01 ab**	0.600 ± 0.28	0.643 ± 0.04 a
Vit C *	44.53 ± 0.32	43.89 ± 2.93 c	123.69 ± 0.37	116.81 ± .60 a	41.38 ± 1.96	45.17 ± 2.07 c
Proline*	3.38 ± 0.37	2.82 ± 0.12	1.63 ± 0.64 a	0.99 ± 0.07 b	1.99 ± 0.33	1.26 ± 0.09 b
Chlorophyll a*	0.382 ± 0.44	**0.572 ± 0.00**	0.267 ± 0.73	0.444 ± 0.01	0.647 ± 0.09	0.464 ± 0.02
Chlorophyll b*	1.277 ± 0.27	**2.535 ± 0.10**	1.012 ± 0.82	1.932 ± 0.03	0.65 ± 0.77	1.123 ± 0.01
Total Chlorophyll*	1.659 ± 0.94	**1.963 ± 0.07** b	1.279 ± 0.08	1.488 ± 0.07 c	1.297 ± 1.01	1.587 ± 0.01 c

Mean in gm / 100 gm dry wt. basis ± S.D., * Values are given in mg /100 gm fresh wt. basis ± S.D. (n=5)

Values followed by small letters showed significantly different (P<0.01) by Duncun's Multiple Range Test

In Table No. 3/21 quantitative determination of the biochemicals in different species of *Aloe* showed that *Aloe perryi* contains highest amount of total minerals (12.83 ± 0.11), crude fat (1.99 ± 0.21), vitamin C (49.05 ± 0.09) and Chlorophyll a (1.041 ± 0.07), b (2.438 ± 0.01) and total chlorophyll (3.48 ± 0.01). *Aloe perryi* also showed maximum amount of phenolics (0.285 ± 0.00) and tannins (0.815 ± 0.04) among all the species. It was observed that all the species cultivated in glass houses showed less amount of phenolics and tannins as compared to their controls collected from wild sources. Total carbohydrates (14.74 ± 2.11) and crude fibres (11.4 ± 0.28) were found in more amounts in *Aloe saponaria.* Proline was found maximum and equal in *Aloe saponaria* and *Aloe zebrina* i.e. 3.09. In case of proline also it was found that all species showed less amount as compared to their controls collected from wild sources. *Aloe zebrina* showed maximum amount of starch (9.44 ± 1.04) and total proteins (5.47± 2.35). Cellulose was found to be high in *Aloe humilis* (6.03 ± 0.26).

According to Duncun multiple range test applied to cultivated ecotypes, total protein, starch and proline were the least significantly varied constituents among the while maximum significant variation was found in crude fat, crude fibre and total carbohydrates. Significant vraitions were found in each of the biochemical constituent among all the cultivated.

Individual mineral content of ecotypes of *Aloe barbadensis* are given in Table No. 3/23 which showed that Sodium (Na) (438.18 ± 0.09), Potassium (K) (1471 ± 0.08), Phosphorous (P) (204.28 ± 0.09), Copper (Cu) (4.42 ± 0.05) and Lithium (Li) (7.38 ± 0.06) were found to be in high concentration in Ecotype 4 as compared to its control. Elements found in highest amount in the cultivated ecotypes were Calcium (Ca) (1039.64 ± 0.08) and Silicon (Si) (1.59 ± 0.08) in Ecotype 2, Iron (Fe) (65.07 ± 0.02) in Ecotype 3, Zinc (Zn) (5.4 ± 0.02) in Ecotype 5, Mangnese (Mn) (30.04 ± 0.04) in Ecotype 6 as compared to their respective controls. Cobalt (Co) was found to be absent or in concentration beyond the detectable limit.

Here also Duncun multiple range test was applied to find out the most significant differences among the cultivated ecotypes. Table No. 3/23 showed that all the minerals were varied significantly and Ecotypes 4 and 5 were shown the more significant values among the ecotypes.

Table No. 3/21: Amount of various nutrients in different *Aloe* species

	Aloe perryi		*Aloe saponaria*		*Aloe humulis*		*Aloe zebrina*	
	Control	**Cultivated**	**Control**	**Cultivated**	**Control**	**Cultivated**	**Control**	**Cultivated**
Total Carbohydrates	8.63 ± 0.08	13.75 ± 1.49	8.13 ± 4.07	**14.74 ± 2.11**	7.51 ± 0.34	11.85 ± 0.52	10.82 ± 0.12	14.23 ± 0.55
Starch	3.26 ± 1.00	5.74 ± 0.70	6.33 ± 1.64	8.07 ± 0.45	5.09 ± 0.22	6.85 ± 0.28	6.07 ± 0.33	**9.44 ± 1.04**
Cellulose	3.67 ± 1.97	5.83 ± 0.08	3.98 ± 0.54	5.38 ± 0.26	4.27 ± 0.29	**6.03 ± 0.26**	3.77 ± 0.09	5.19 ± 0.08
Total Proteins	2.68 ± 1.19	3.10 ± 0.11	2.08 ± 1.12	3.53 ± 2.24	2.93 ± 1.44	3.48 ± 1.71	3.66 ± 0.45	**5.47± 2.35**
Total Minerals	8.34 ± 0.33	**12.83 ± 0.11**	3.55 ± 0.51	5.721 ± 0.86	6.07 ± 0.67	10.82 ± 0.04	8.37 ± 0.20	11.79 ± 0.05
Crude Fat	0.87 ± 0.37	**1.99 ± 0.21**	0.83 ± 0.41	1.74 ± 0.15	0.33 ± 0.55	0.74 ± 0.18	0.96 ± 0.07	1.78 ± 0.15
Crude Fibre	6.02 ± 0.10	8.27 ± 0.08	8.47 ± 1.21	**11.4 ± 0.28**	6.49 ± 0.64	9.55 ± 0.17	4.61 ± 0.66	8.33 ± 0.16
Phenolics	0.337 ± 0.09	**0.285 ± 0.00**	0.212 ± 0.78	0.243 ± 0.02	2.08 ± 0.04	0.144 ± 0.01	2.04 ± 0.94	0.176 ± 0.01
Tannins	1.007 ± 0.18	**0.815 ± 0.04**	0.498 ± 3.55	0.575 ± 0.06	6.73 ±1.88	0.449 ± 0.01	0.496 ± 017	0.515 ± 0.05
Vit C *	42.56 ± 0.089	**49.05 ± 0.09**	23.64 ± 3.53	26.31 ± 4.81	19.66 ± 1.24	21.63 ± 4.70	30.07 ± 0.99	33.64 ± 0.60
Proline*	2.08 ± 0.65	1.5 ± 0.08	3.95 ± 0.62	**3.09 ± 0.40**	3.04 ± 0.37	2.75 ± 0.14	3.96 ± 0.67	**3.09 ± 0.39**
Chlorophyll a*	1.311 ± 0.34	**1.041 ± 0.07**	0.982 ± 1.86	0.825 ± 0.01	0.255 ± 0.33	0.246 ± 0.01	0.394 ± 0.23	0.406 ± 0.02
Chlorophyll b*	2.275 ± 0.19	**2.438 ± 0.01**	1.579 ± 1.86	1.646 ± 0.01	1.013 ± 0.91	1.255 ± 0.00	0.674 ± 0.91	1.63 ± 0.01
Total Chlorophyll*	3.586 ± 0.07	**3.48 ± 0.01**	1.964 ± 1.04	2.0417 ± 0.10	1.268 ± 1.15	1.596 ± 0.01	0.891 ± 0.07	1.321 ± 0.02

Mean in gm / 100 gm dry wt. basis ± S.D., * Values are given in mg /100 gm fresh wt. basis ± S.D. (n=5)

Table No. 3/22: Analysis of variance for nutrients in different species of *Aloe*

Trait (Parameter)	df (degree of freedom)	MS (Mean square)	P value	Rank
Total Carbohydrates	3	5.017	0.0243	2>4>1>3
Starch	3	8.861	0.0001	4>2>3>1
Cellulose	3	0.599	0.0137	3>1>2>4
Total Proteins	3	3.187	0.0011	4>2>3>1
Total Minerals	3	30.253	0.0000	1>4>3>2
Crude Fat	3	0.888	0.0012	1>4>2>3
Crude Fibre	3	7.647	0.0002	2>3>1>4
Phenolics	3	0.009	0.0026	1>2>4>3
Tannins	3	0.0075	0.0002	1>2>4>3
Vit C *	3	415.657	0.0000	1>4>2>3
Proline*	3	1.905	0.0003	2>4>3>1
Chlorophyll a*	3	0.399	0.0000	1>2>4>3
Chlorophyll b*	3	0.785	0.0001	1>2>4>3
Total Chlorophyll*	3	2.772	0.0000	1>2>4>3

(1- *Aloe perryi*; 2- *Aloe saponaria*; 3- *Aloe humilis*; 4- *Aloe zebrina*)

Table No. 3/23: Amount of minerals in *Aloe barbadensis* ecotypes

Elements	Plants	Ecotypes					
		1	**2**	**3**	**4**	**5**	**6**
Na	**Control**	124.94 ± 0.55	257.37 ± 1.22	275.44 ± 0.07	389.67 ± 0.87	301.94 ± 0.07	280.99 ± 2.00
	Cultivated	192.24 ± 0.57 f	326.14 ± 0.24 e	340.12 ± 0.10 c	**438.18 ± 0.09 a**	368.44 ± 0.51 b	329.21 ± 0.21 d
K	**Control**	389.61 ± 1.89	123.54 ± 0.11	589.36 ± 0.04	1401.28 ± 0.09	98.99 ± 0.06	496.29 ± 0.55
	Cultivated	485.59 ± 0.11 d	189.78 ± 0.04 e	659.79 ± 0.04 b	**1471 ± 0.08 a**	164.85 ± 0.04 f	516.51 ± 0.04 c
Ca	**Control**	623.19 ± 0.07	965.24 ± 0.05	889.12 ± 0.11	786.68 ± 0.11	895.67 ± 0.07	929.37 ± 0.07
	Cultivated	693.96 ± 0.09 f	**1039.64 ± 0.08 a**	947.75 ± 0.08 d	847.36 ± 0.04 e	994.06 ± 0.06 ca	1007.52 ± 0.04 b
P	**Control**	86.52 ± 0.05	93.14 ± 0.08	99.63 ± 0.77	147.59 ± 0.09	63.88 ± 0.08	45.19 ± 0.03
	Cultivated	115.09 ± 0.06 d	130.02 ± 0.05 c	146.77 ± 0.07 b	**204.28 ± 0.09 a**	102.39 ± 0.05 e	85.12 ± 0.04 f
Cu	**Control**	1.62 ± 0.06	1.87 ± 0.04	2.16 ± 0.01	3.88 ± 0.04	1.89 ± 0.09	2.73 ± 0.01
	Cultivated	2.7 ± 0.04 c	2.6 ± 0.03 d	2.7 ± 0.02 c	**4.42 ± 0.05 a**	2.68 ± 0.01 c	3.03 ± 0.03 b
Zn	**Control**	3.64 ± 0.04	2.74 ± 0.02	2.49 ± 0.07	1.61 ± 0.04	4.66 ± 0.07	3.99 ± 0.07
	Cultivated	4.02 ± 0.07 c	3.14 ± 0.06 d	3.37 ± 0.06 e	2.02 ± 0.05 f	**5.4 ± 0.02 a**	4.76 ± 0.03 b

Fe	**Control**	26.34 ± 0.07	21.54 ± 0.04	53.68 ± 0.02	29.99 ± 0.02	29.89 ± 0.07	33.18 ± 0.07
	Cultivated	32.4 ± 0.04 e	28.54 ± 0.03 f	**65.07 ± 0.02 a**	40.79 ± 0.04 b	36.64 ± 0.04 c	40.02 ± 0.02 d
Mn	**Control**	8.14 ± 0.08	11.56 ± 0.09	7.98 ± 0.03	9.66 ± 0.40	10.56 ± 0.09	24.66 ± 0.06
	Cultivated	13.93 ± 0.06 d	17.03 ± 0.03 b	13.31 ± 0.03 e	13.32 ± 0.04 e	16.11 ± 0.03 c	**30.04 ± 0.04 a**
Li	**Control**	3.99 ± 0.06	4.87 ± 0.07	5.90 ± 0.08	6.66 ± 0.08	4.05 ± 0.01	4.79 ± 0.08
	Cultivated	5.05 ± 0.06 d	5.14 ± 0.05 d	6.56 ± 0.05 b	**7.38 ± 0.06 a**	4.88 ± 0.06 e	5.93 ± 0.06 c
Si	**Control**	0.85 ± 0.06	1.06 ± 0.01	0.61 ± 0.00	0.94 ± 0.04	0.76 ± 0.10	0.09 ± 0.04
	Cultivated	1.18 ± 0.02 ab	**1.59 ± 0.08 a**	1.11 ± 0.04 b	1.49 ± 0.22 ab	1.27 ± 0.37 ab	0.57 ± 0.04 c
Co	**Control**	**NIL**	**NIL**	**NIL**	**NIL**	**NIL**	**NIL**
	Cultivated						

Mean in mg/100 gm dry wt. basis ± S.D. (n=5)

Values followed by small letters showed significantly different (P<0.01) by Duncun's Multiple Range Test

In case of the mineral content (Table No. 3/24) of the *Aloe* species it was found that all the mineral were in highest amount in *Aloe perryi* with the large differences among the species with its control except Si, which was found to be maximum in *Aloe saponaria*. Each mineral element was found in lowest concentration in *Aloe humilis*. Here also Co was found to be absent or in concentration beyond the detectable limit.

Analysis of vaiance for the mineral content of other species (Table No. 3/25) collected from Uttaranchal showed that Aloe perryi contained maximum concentration of all the minerals among rest of the species. All the minerals found in the same ascndingsequence of *Aloe perryi* > *Aloe saponaria* > *Aloe humilis* > *Aloe zebrina.*

Table No. 3/24: Amount of minerals in different *Aloe* species

		Aloe perryi	***Aloe saponaria***	***Aloe humilis***	***Aloe zebrina***
Na	**Control**	193.54 ± 0.31	87.23 ± 0.61	64.94 ± 0.55	75.24± 0.54
	Cultivated	**243.69 ± 0.16**	123. 54 ± 0.55	71.49 ± 0.02	83.64± 0.45
K	**Control**	1687.29 ± 0.15	561.02± 0.56	389.61 ± 1.89	420.88 ± 091
	Cultivated	**1853.96 ± 0.08**	630.45± 0.08	461.34 ± 0.48	530.41 ± 0.81
Ca	**Control**	789.37 ± 0.04	523.46± 0.04	369.19 ± 0.07	410.91± 0.08
	Cultivated	**846.36 ± 0.06**	641.87 ± 0.06	412.74 ± 0.18	473.54 ± 0.09
P	**Control**	254.91 ± 0.07	103.91 ± 0.07	86.52 ± 0.05	93.61± 0.04
	Cultivated	**302.39 ± 0.06**	133.39 ± 0.06	102.98 ± 2.9	113.51 ± 0.09
Cu	**Control**	3.64 ± 0.09	0.85 ± 0.06	0.62 ± 0.06	0.70 ± 0.01
	Cultivated	**4.46 ± 0.14**	1.18 ± 0.02	1.06 ± 0.19	1.13 ± 0.08
Zn	**Control**	3.97 ± 0.31	0.86 ± 1.31	0.66 ± 0.55	0.71 ± 0.08
	Cultivated	**5.04 ± 0.05**	1.69 ± 0.13	0.99 ± 0.02	1.46 ± 0.09

Fe	**Control**	14.52 ± 0.04	8.64 ± 0.17	5.83 ± 0.89	7.06 ± 0.05
	Cultivated	**16.76 ± 0.04**	10.63 ± 1.08	6.34 ± 0.36	8.33 ± 0.9
Mn	**Control**	11.08± 1.00	9.37 ± 0.04	6.23 ± 0.07	8.05 ± 0.40
	Cultivated	**13.52 ± 0.54**	10.16 ± 0.06	8.74 ± 0.18	9.13 ± 0.20
Li	**Control**	5.31 ± 0.90	2.91 ± 0.07	0.86 ± 0.05	1.63 ± 0.08
	Cultivated	**8.30 ± 0.03**	3.39 ± 0.06	1.02 ± 0.09	2.31 ± 1.00
Si	**Control**	0.54 ± 0.84	0.64 ± 0.09	0.26 ± 0.06	0.54 ± 0.01
	Cultivated	0.92 ± 0.09	**0.94 ± 0.14**	0.49 ± 0.19	0.61 ± 0.01
Co	**Control**	**NIL**	**NIL**	**NIL**	**NIL**
	Cultivated				

Mean in mg /100 gm dry wt. basis ± S.D. (n=5)

Table No. 3/25: Analysis of variance for minerals in different species of *Aloe*

Trait (Parameter)	df (degree of freedom)	MS (Mean square)	P value	Rank
Na	3	18440.229	0.0000	1>2>4>3
K	3	1307149.067494	0.0000	1>2>4>3
Ca	3	113342.889976	0.0000	1>2>4>3
P	3	30665.822	0.0003	1>2>4>3
Cu	3	8.083	0.0000	1>2>4>3
Zn	3	10.253	0.0000	1>2>4>3
Fe	3	59.713	0.0000	1>2>4>3
Mn	3	13.978	0.0000	1>2>4>3
Li	3	30.236	0.0000	1>2>4>3
Si	3	0.127	0.0000	1>2>4>3
Co	3	NIL		

(1- *Aloe perryi*; 2- *Aloe saponaria*; 3- *Aloe humilis*; 4- *Aloe zebrina*)

The major pharmaceutical compound Aloin (Fig. 3/10) was found in high concentration in cultivated Ecotype 1 (Fig. 3/11) among all the ecotypes in all the three samples viz whole leaf (51.53 ± 0.59, control- 29.48 ± 1.01), fresh latex (346.94 ± 0.73, control- 287.41 ± 0.84) and lyophilized latex (1465.38 ± 0.77, control- 786.32 ± 1.58). Among the species all values of aloin were more in whole leaf (156.96 ± 0.89, control- 89.62 ± 0.88), fresh latex (576.92 ± 0.47, control ± 387.41 ± 0.94) and lyophilized latex (1629.14 ± 1.32, control- 1093.67 ± 2.08) of *Aloe perryi* (Fig. 3/12). It was observed that aloin was found in much higher concentration in *Aloe perryi* among all the ecotypes and species. Also values in lyophilized latex were more than whole leaf and latex.

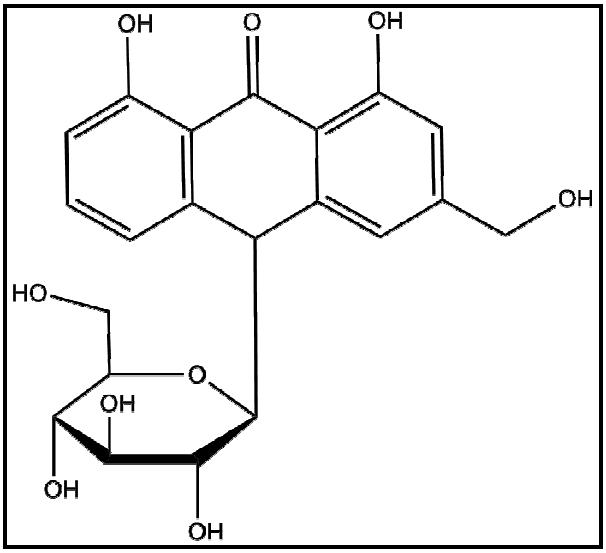


Figure 3/10: Structure of Aloin

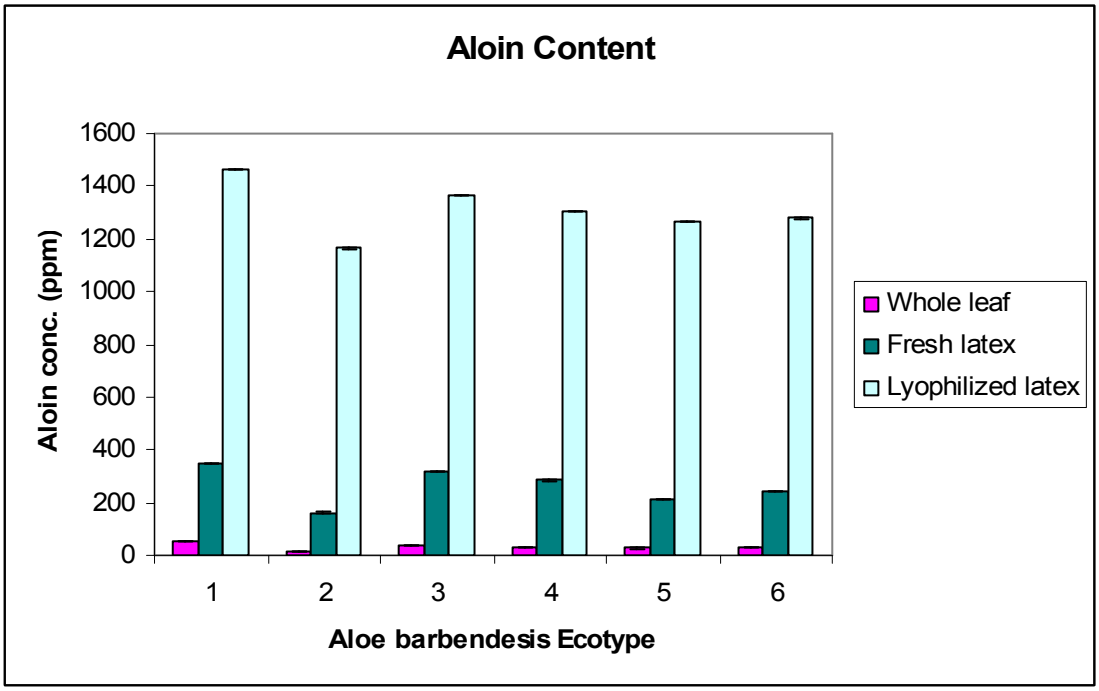


Figure 3/11: Aloin content of *Aloe barbadensis* ecotypes

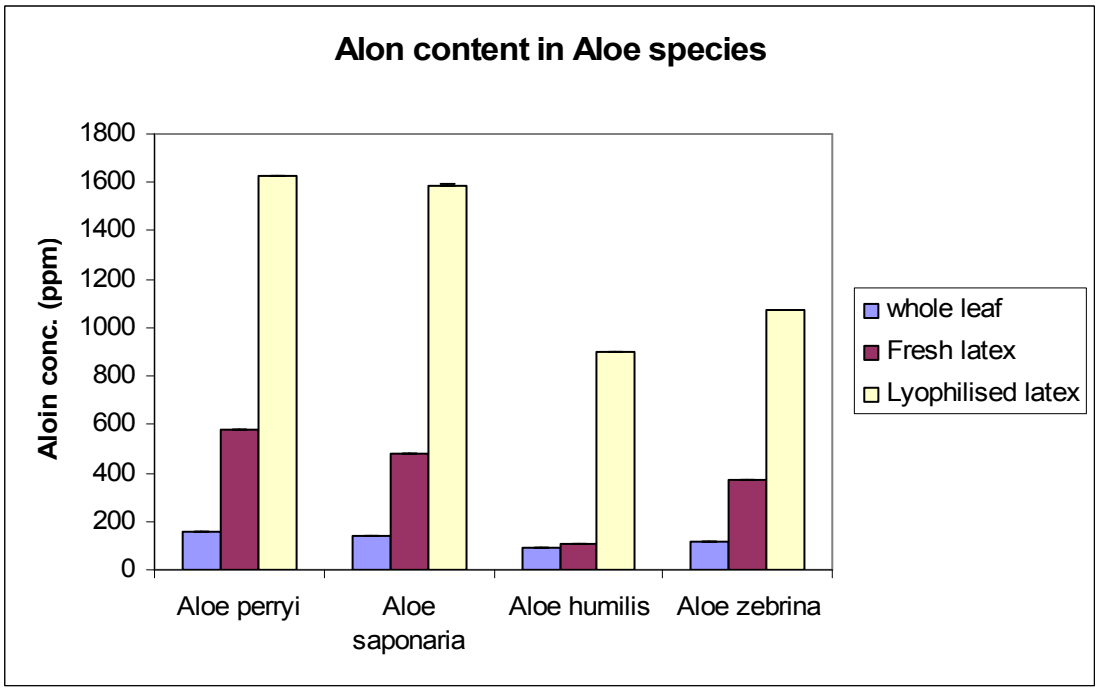


Figure 3/12: Aloin content of different *Aloe* species

To find out the efficacy of the developed agrotechnology, Ecotype 1 collected from Pithoragarh (5397' MSL) was cultivated at higher altitude at Auli (9000' MSL). Table No. 3/26 showed the reduced values of % moisture and dry matter content in whole leaf, peel and pulp in cultivated plants as compared to its control.

Table No. 3/26: Moisture and dry matter content of *Aloe barbadensis* Ecotype 1 cultivated at Auli (9000' MSL) (n=5)

	Control		Cultivated	
Plant Part	**% moisture**	**% dry matter**	**% moisture**	**% dry matter**
Ecotype 1 cultivated at Auli (9000' MSL)				
Whole leaf	89.63 ± 1.23	10.37 ± 1.23	86.34 ± 1.41	13.66 ± 1.41
Peel	84.61 ± 0.91	15.39 ± 0.91	82.94 ± 1.07	17.06 ± 1.07
Pulp	91.21 ± 0.81	8.79 ± 0.81	88.91 ± 0.63	8.79 ± 0.63

Mean ± S.D.

Table No. 3/27 showed that in cultivated *Aloe*, biochemical constituents like total carbohydrates, total proteins, crude fat, phenolics and all the chlorophyll contents i.e. chlorophyll a, b and total chlorophyll were found in less amount. While cellulose, total minerals, tannins, vitamin C and proline were found in high concentrations. Starch was found to be increased (4.26 ± 1.05 control- 4.17 ± 1.94) but significant difference was minimum. Here also Ecotype 1 collected from wild region of Pithoragarh used as a control.

Table No. 3/27: Amount of various nutrients in *Aloe barbadensis* Ecotype 1 cultivated at Auli (9000' MSL)

Sr. No.	Biochemicals	Control	Cultivated
1.	Total Carbohydrates	17.24 ± 3.54	14.96 ± 0.14
2.	Starch	4.17 ± 1.94	4.26 ± 1.05
3.	Cellulose	3.98 ± 0.54	6.27 ± 0.57
4.	Total Proteins	2.08 ± 1.89	1.98 ± 0.04
5.	Total Minerals	5.98 ± 0.64	12.78 ± 1.05
6.	Crude Fat	0.83 ± 0.41	0.56 ± 0.02
7.	Crude Fibre	8.47 ± 1.21	8.32 ± 0.10
8.	Phenolics	0.212 ± 0.78	0.141 ± 0.02
9.	Tannins	0.498 ± 3.55	0.592 ± 0.07
10.	Vit C *	29.64 ± 3.94	55.63 ± 0.38
11.	Proline*	3.95 ± 0.62	5.69 ± 0.28
12.	Chlorophyll a*	0.982 ± 1.86	0.12 ± 0.03
13.	Chlorophyll b*	1.579 ± 1.86	0.926 ± 0.02
14.	Total Chlorophyll*	2.561 ± 1.38	1.045 ± 0.01

Mean in gm /100 gm dry wt. basis ± S.D. (n=5)

*** Values are given in mg /100 gm fresh wt. basis (n=5)**

Table No. 3/28 showed that concentration of minerals like K, Ca, P, Cu, Zn, Fe, Li and Si increased and concentration of Na was decreased drastically as compared to its control during cultivation at higher altitude. Mn also showed reduced concentration in cultivated Ecotype 1. Cobalt (Co) was found to be absent or in concentration beyond the detectable limit.

Table No. 3/28: Amount of minerals in *Aloe barbadensis* Ecotype 1 cultivated at Auli (9000' MSL)

Na		K		Ca		P	
Control	**Cultivated**	**Control**	**Cultivated**	**Control**	**Cultivated**	**Control**	**Cultivated**
124.94 ± 0.55	61.49 ± 0.02	389.61 ± 1.89	782.34 ± 0.48	623.19 ± 0.07	879.74 ± 0.18	86.52 ± 0.05	102.98 ± 2.9
Cu		**Zn**		**Fe**		**Mn**	
Control	**Cultivated**	**Control**	**Cultivated**	**Control**	**Cultivated**	**Control**	**Cultivated**
1.62 ± 0.06	2.62 ± 0.19	3.64 ± 0.04	4.44 ± 0.41	26.34 ± 0.07	34.50 ± 0.04	8.14 ± 0.08	6.86 ± 0.06
Li		**Si**		**Co**			
Control	**Cultivated**	**Control**	**Cultivated**	**Control**	**Cultivated**		
3.99 ± 0.06	10.55 ± 0.22	0.85 ± 0.06	0.97 ± 0.1	NIL			

Mean in mg/100 gm dry wt. basis ± S.D. (n=5)

Aloin content of Ecotype 1 cultivated at Auli (Table No. 3/29) was found in significantly high concentration in all the three samples viz whole leaf (130.82 ± 2.45), fresh latex (429.75 ± 1.54) and lyophilized latex (1464.94 ± 1.26) as compared to its control.

Table No. 3/29: Aloin content of *Aloe barbadensis* Ecotype 1 cultivated at Auli (9000' MSL)

Aloin Content		Whole Leaf (WL, ppm)	Fresh Latex (FLx, ppm)	Lyophilised Latex (LLx, ppm)
Ecotype 1 at Auli	**Control**	35.63 ± 2.97	385. 15 ± 2.33	981.00 ± 0.99
	Cultivated	130.82 ± 2.45	429.75 ± 1.54	1464.94 ± 1.26

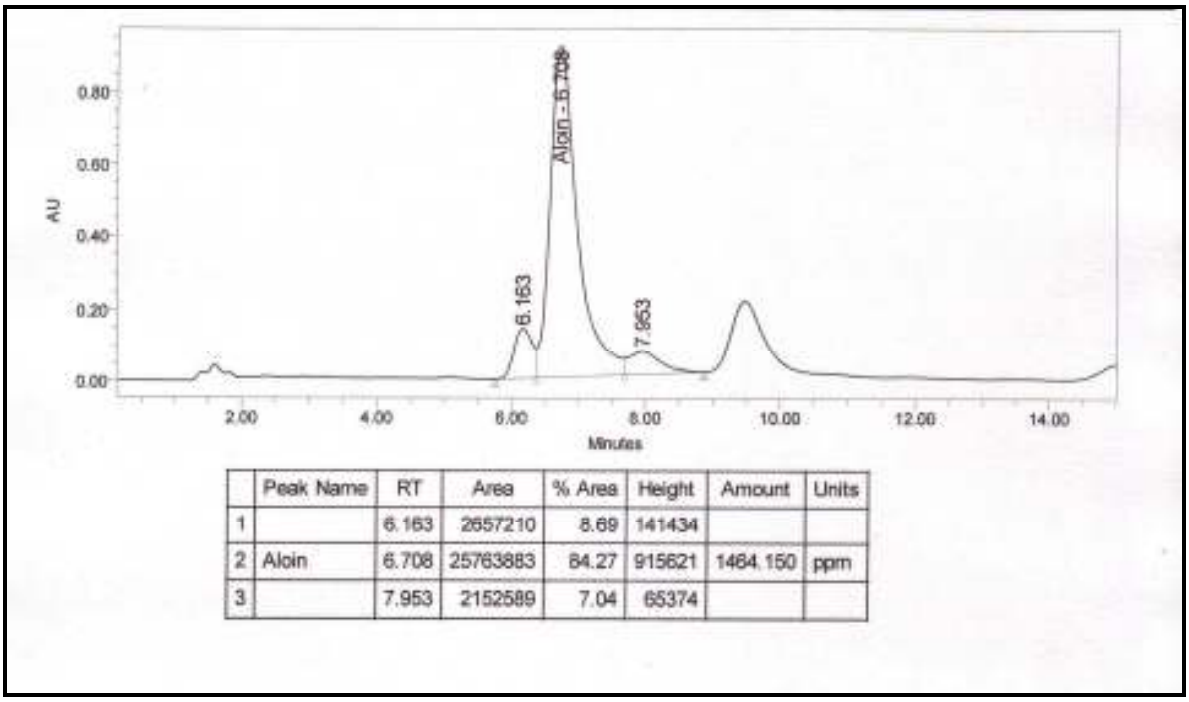
Mean ± S.D. (n=5)

The methodology developed for processing and preservation of *Aloe* whole leaf powder and gel was found to be efficient as the quality of the material was found to be maintained at least for two years. The regular assessment of the quality was done by visual observations like development of fungus in gel, its transparency and change in colour etc. Texture of dried powder was also observed for moisture. Shelf life of both types viz dried material and gel was found to be increased. The gel can be preserved at least up to six months and dried powder up to two years, which can stretch further.

It was also found that Aloin content in lyophilized latex of Ecotype 1 cultivated at Pithoragarh and Auli have shown the similar values (1464 ppm) (Fig. 3/13).

3.5 DISCUSSION:

In the present study variable moisture content in *Aloe* ecotypes and species collected from different altitude was observed. The reason may be that in Uttaranchal region the climate is cloudy and locations of collection were varying in altitudes, in addition to the species specificity of genus *Aloe*. Cultivation of these plants showed relative increase in % moisture content. This may be due to proper scheduled irrigation



	Peak Name	RT	Area	% Area	Height	Amount	Units
1		6.163	2657210	8.69	141434		
2	Aloin	6.708	25763883	84.27	915621	1464.150	ppm
3		7.953	2152589	7.04	65374		

Figure 3/13: Concentration of Aloin in lyophilized latex of cultivated Ecotype 1 (At Pithoragarh, 5397' and Auli 9000' MSL)

in open fields and maintenance of humidity in glass houses. Ecotype 2 showed high moisture content as it was collected from altitude of 5375' MSL which was found sloppy, hence it may have developed water retention tendency due to scarcity of water.

Aloe saponaria (1105') and *Aloe perryi* (1082') which showed higher amount of moisture in whole leaf, peel and in pulp respectively were collected from the plains. Water availability in these plains is sufficient due to less slopes hence high moisture content was observed.

From the literature it was proved that the variations in chemical constituents among the genus aloe are prevalent due to different species, agro-climate, soil type and soil nutrients (Boudreau and Beland, 2006; Dagne and Yenesew, 1994; Akinyele and Odiyi, 2007). The results of biochemical constituents and minerals obtained in the present study were found to be in accordance with the previous findings as they showed lot of variation in ecotypes and species with respect to altitude, actual site of collection, agro climatic variations, soil type, soil nutrition levels etc. It was found that the value of

proline was more in all the controls of *Aloe barbadensis* ecotypes. It is well known that the proline is secreted in excess amount in plants whenever they get stressed like cold, nutrients deficiency etc. Uttaranchal region was hilly and sloppy. The actual sites of collection were also rocky, water deficient areas. The major role was of climate as already discussed in Chapter No. 2. In Uttaranchal the season remains extremely cold in winter with snow fall at night, in summer also due to high altitude the intensity and heat of sun radiation is high. Hence proline may have been synthesized in greater concentration. In glass house conditions all the environmental parameters like temperature, availability of water, soil nutrients were very well maintained so no major changes were observed.

Similar results were obtained in the concentrations of tannins and phenolics. These compounds are mainly secreted as a secondary metabolites and it's a part of the defense mechanism against the adverse condition. As discussed earlier the adverse condition may have lead to the higher concentration of these compounds. Hence the cultivated plants may not have shown the significant differences. Same was the case with vitamin C. It acts as antioxidant and used by plants as a part of defense mechanism.

It was proved that limitation in light availability was found to affect primarily total dry mass production and carbon allocation to plant components, such as the number of leaves per plant (Paez *et al*., 2000). Ecotype 1 showed high amount of total polysaccharides. It was collected form higher altitude of 5397' MSL. This region is also deficient in soil nutrients due to high degree of slopes and temperature variation at day and night (-10 to 25^0C) hence this ecotype has restored food in the form of carbohydrates. High amount of starch and cellulose was found in Ecotype 5 collected form plains (1105'). But its total carbohydrate content was less. This means that Ecotype 1 due to higher altitude stored non starch polysaccharides which are hard to digest, degrade and can be utilized in much adverse conditions. This is the reason Ecotype 1 served best prebiotic source among all the ecotypes (Chapter No. 4). Ecotypes 1 also showed maximum amount of total proteins which are also the source of energy and heat in challenging environmental conditions. Duncun multiple range test was used for the most significant variations among the cultivated ecotypes. It also proved that the Ecotype 1 was the best source of most of the nutraceuticals among all the ecotypes. *Aloe perryi*

showed high amounts of vitamin C, phenolics, tannins and chlorophyll compounds even though it was collected from plains (1105'). No relation among the biochemical constituents and location was found among these species. Hence it can be concluded that the reason of variation among the biochemical are purely species dependant. Only proline was found high in *Aloe zebrina* which was collected from higher altitude (3786' MSL) than all the other species.

It is known that plants uptake the minerals from the soil and provided fertilizers. It is the major source of micro and macro elements for plants. Very less information is available on synthesis of minerals by plants. In the present study Ecotype 4 showed the maximum amount of minerals in most of the elements. It was collected from Haldwani (1257). This region is close to plains and the soil might have been rich in nutrients. Other ecotypes collected from higher altitude showed less amount because at higher altitude soil is deficient in minerals due to high degree of slopes, soil erosion, floods etc due to which nutrients gets leached and washed off (Indrayan *et al*., 2005). The results were found in accordance with this finding. The minerals were found in less concentration as the site (Pithoragarh, 5397') is very nutrient deficient soil and soil is the major source of mineral for plant uptake.

It was proved in the present study (by ANOVA also) that *Aloe perryi* showed maximum concentration of the minerals among all the species. This may be attributed to species differences and collection site i.e. plains of Kumaon. Other species were collected from Garhwal region. The soil in this region is very poor in nutrients as compared to Kumaon. Also very high attitude might have been responsible for nutrient deficient soil. Appropriate sunlight and temperature in plains may have facilitated the synthesis of chlorophyll compounds which were also found to be high among all the species.

From the literature it was found that the content and distribution of the anthraquinones are related to environmental conditions, such as season and illumination, and the content varies at different growth stages and even in different parts of the same leaf (Chauser-Volfson and Gutterman, 1996; Gutterman and Chauser-Volfson, 2000A, B; Shen *et al.*, 2001A; Chauser-Volfson *et al*., 2002). The aloin content is the highest among these anthraquinones (Beaumont et al., 1984; Reynolds, 1985A, B, 1990; Reynolds and Nicholls, 1986; Yamamoto *et al*., 1991). In the present study Ecotype 1 was found to

have maximum amount o the aloin among the ecotypes. It can be correlated with the high altitude, abiotic stress which facilitated the aloin secretion. Among other species aloin in *Aloe perryi* was found to be at highest concentration. It can be directly correlated to the species difference as well as high concentration of phenolics, tannins and anthraquinone moiety.

Cultivation at high altitude (Auli, 9000' MSL) of *Aloe barbadensis* Ecotype 1, showed most of the biochemicals and minerals in reduced concentrations as compared to its control. The reason was very high altitude affected these compositions. The soil in this region was found rocky, very acidic in nature and climate was very harsh. The temperature in winter reaches to -30^0C and in day it's around 10 to 12^0C. This location remains snow covered for more than 7 months. These conditions may have adversely affected the composition even though the cultivation was carried out in glass houses. Only proline concentration was increased as part of survival molecule in such conditions. Because it has been proved that the plants under stress conditions synthesizes more amount of proline.

Aloin content was also found to be increased drastically in Ecotype 1 cultivated at Auli as it is also known that the anthraquinones are synthesized in more quantities in adverse condition as a part of defense system.

In the present study lyophilized latex of all the plants showed high concentration of aloin as compared to whole leaf powder and fresh latex. Lyophilization of any liquid sample reduces moisture content and freezing under vacuum during this process prevents the oxidation of aloin. Aloin due to its constituents undergoes oxidation.

Processing method was found appropriate when it was observed that the technique reduces the moisture content of the materials. It also helps in the preservation particularly of *Aloe* gel where the moisture content is around 99%. Many techniques like heat and cold treatment, pectinolytic enzymes and antioxidants are recommended for processing and preservation of gel (Ramchandra and Rao, 2008). The organic solvents like ethanol, methanol, and acetone used in preservative technique actually acts as dehydrating agents. But it should be used in optimum amount if the materials are supposed to be used in any edible preparation. Compounds like Na- benzoate are permitted preservative and also acts as antimicrobial agents. However, care should be

taken during the frequent use and in dose dependant preparation as high concentration of such permitted preservative can be fatal.

3.6 CONCLUSION and APPLICATION:

As already been reported by Rawat and Mathpal (1981), the micronutrient deficiency is more prevalent in Uttaranchal's hilly region. This is due to the acidic and light textured soil, intensive rain and high degree of slopes because of which nutrients gets leached and washed off (Indrayan et al., 2005). But the data obtained by this study shows that the overall nutritional values of the *Aloe barbadensis ecotypes* is substantially significant in spite of nutritionally deficient soil of this region. It was observed that in *Aloe barbadensis* all the nutraceuticals are in considerable amount in all the ecotypes. Ecotype 1 however showed maximum nutraceutical contents. Thus *Aloe barbadensis* if cultivated as commercial crop in this region can be used as food supplement. Ecotype 1 of *A. barbadensis* can be potential variety for large scale cultivation. It can also be used as a minor crop in routine cropping system as there is a high demand for *Aloe* in industry. Distribution of these nutrients among the ecotypes can be used as selection criteria in determining the superior germplasm in terms of high content of the compound of interest. In addition, cultivation of the appropriate ecotypes, transporting the raw material to large and cottage scale industries, processing of the raw material in this region itself will provide means of employment to the local people which in turn will improve their socioeconomic status.

It can also be concluded that *Aloe barbadensis* Ecotype 1 can be best utilized for its high content of biochemicals and minerals. *Aloe perryi* can be used as a source of aloin and other pharmaceutically important anthraquinones.

Chapter 4

In vitro evaluation of prebiotic and synbiotic potential

The fundamental changes which Aloe is capable of making within the body will help the body to fend off each and every chronic disease

4.1 INTRODUCTION:

People in the modern world are very much cautious about their health, food and food contents which they are consuming. A trend has been developed among the modern consumers for not only the tasty and attractive but also safe and healthy foods. Diet and health gathers pace, many consumers feel well and stay healthy by eating nutritionally designed foods (Xu *et al.,* 2009). In the industrial world, change in eating habits and diet has increased the number of people with reduced defense system. In this context the study of bacterial association with the humans is a great challenge for the future research (Hammes and Hertel, 2002). There are many organs in the human body with their own microflora important for the normal well being. Some of these prominent organs are saliva, liver, vagina and the most important is gut or Gastro Intestinal (GI) tract. The microflora of the gut consists of at least 1013–14 microorganisms (Xu and Gordon, 2003) that includes not only 800–1000 species of bacteria, but fungi, viruses, archea and eukaryotic microorganisms such as helminthes and protozoa.

This useful microflora stimulates angiogenesis, controls inflammation and develops immune system along with the prevention of colonization of potential pathogenic microorganisms. Hence, it becomes possible that the cascade could be initiated by a change in the intestinal flora or an inappropriate host response to the intestinal flora, which could pave the ways for novel prophylactic and therapeutic approaches (Wilks, 2007). In recent years, the cost of medical applications has increased tremendously. The frequent use of antibiotics has led to the reduction in its potency and efficiency.. It has been proved that the use of antibiotics decreases the total numbers of useful microbes in the gut like *Bifidobacteria* and Bacteroides (Penders *et al.,* 2006) and changes faecal flora which may persist long after cessation of antibiotic treatment (Hoverstad *et al.,* 1986; Tannock, 2002).

The loss of gut microflora can only be rejuvenated by the consumption of healthy and safe tailored food. Diet serves this purpose without any side effects. Hence, attention has turned towards food materials that may offer improved health bonuses, either prophylactically or curatively. The current emphasis is into improved gut flora management and strategies targets to improve resistance to infections, irritable bowel

syndrome, chronic gut disorder (inflammatory bowel disease, colon cancer), lactose intolerance, coronary heart disease, recurrent vaginal thrush, skin problems, food allergy, autism, cognitive function and mineral bioavailability.

Within the last decade, there is a distinct change in the understanding of the role of foods in human health promoting explosion of consumer interest in the active role of foods in the well-being and life prolongation as well as in the prevention of initiation, promotion and development of cancer, cardiovascular diseases and osteoporosis. As a result, a new term — functional food — was proposed (Wlodzimierz *et al.,* 2005).

In 1991 the Japanese government started an approval system for "Foods for Specified Health Use" (FOSHU) (Taniguchi, 2004) and the concept Functional food is introduced. Functional foods deliver the positive message of eating well, rather than to reduce and avoid (Warrand, 2006). The health potential of foods is well perceived by consumers as a functional food (Fogliano and Vitaglione, 2005) or medicinal food. This is considered as the emergence of the food of medicinal importance and/or supplemented with the nutritional ingredients, called as nutraceuticals.

Nutraceuticals are medicinal foods that play a role in maintaining well being, enhancing health, modulating immunity and thereby preventing as well as treating specific diseases (Ramaa *et al.,* 2006). The nutraceuticals fall basically into three categories: first- alternative low-calorie sugars, second- polysaccharides and oligosaccharides and third- B vitamins (Jeroen and Eddy, 2002; Wildman, 2001). Their addition to food matrices develops innovative functional foods with the potential to produce physiological benefits or reduce the long-term risk of developing diseases (Elliott and Ong, 2002). The delivery of these molecules will therefore require food formulators and manufacturers to provide protective mechanisms that

- Maintain the active molecular form until the time of consumption and
- Deliver this form to the physiological target within the organism (Lingyun and Gabriel*,* 2006).

Hygiene hypothesis developed functional and medicinal food that involves live beneficial microbial additions into the diet to manipulate the composition of colonic

micro flora. This was the beginning of- Probiotics and Prebiotics (Aryana and McGrew, 2007; Coppa *et al*., 2006; Losada and Olleros, 2002; Rao, 2001).

Probiotics, Prebiotics and Synbiotic: -

Probiotics: -

The human gastrointestinal tract is a kinetic microecosystem that enables normal physiological functions of the host organism unless harmful and potentially pathogenic bacteria dominate it. Maintaining a proper equilibrium of the microflora may be ensured by systematic supplementation of the diet with probiotics, prebiotics or synbiotics. The most widely used definition of probiotic has been proposed by Fuller (1989, 1992), i.e. a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance. Yet, for human nutrition Salminen *et al*. (1998) proposed the following definition: 'a live microbial food ingredient that is beneficial to health'. *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces*—particularly *L. casei*, *L. rhamnosus*, *L. acidophilus*, *L. plantarum*, *B. longum*, *B. bifidum*, and *S. cerevisiae boulardii*— are the most commonly used probiotics and actively researched organisms (Bourlioux *et al*., 2003; Mishra and Prasad, 2005) at the basic level and in clinical trials.

Bacteria used as probiotic adjuncts are commonly delivered in a food system and, therefore, begin their journey to the lower intestinal tract via the mouth. Berrada *et al.* (1991) reported that the time from entrance to release from the stomach to be 90 min. However, further digestive processes have longer residence times; hence, there is a need for the bacteria to be resistant to the stressful conditions of the stomach and upper intestine, which contain bile. Cellular stress begins in the stomach, which has pH as low as 1.5 (Lankaputra and Shah, 1995). After the bacteria pass through the stomach, they enter the upper intestinal tract where bile is secreted into the gut. The concentration of bile in the human gastrointestinal system is variable and is difficult to predict at any given moment (Lankaputra and Shah, 1995). After traveling through this harsh environment, the organism colonizes the epithelium of the lower intestinal tract (Conway *et al*., 1987; Delzenne and Roberfroid, 1994). Thus, strains selected for use as probiotic bacteria should be able to tolerate acid for at least 90 min, tolerate bile acids,

attach to the epithelium, and grow in the lower intestinal tract before they can start providing any health benefits (Chou and Weimer, 1999). There is a very specific mechanism of action of probiotics on human health. It is given in Fig. 4/14.

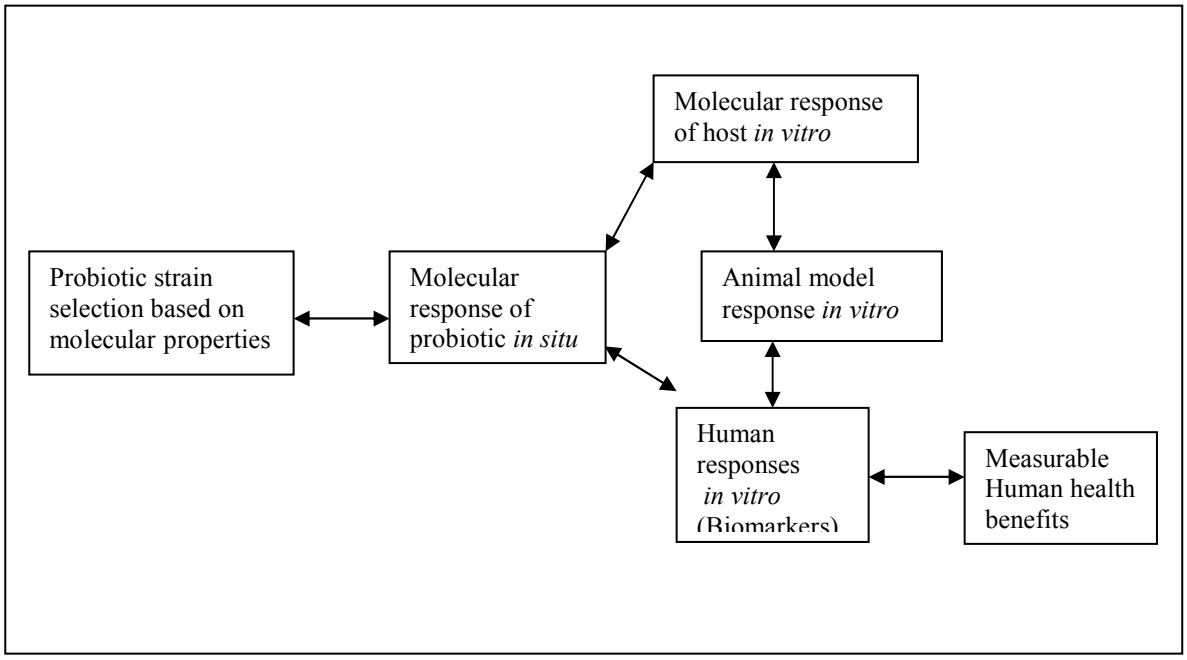


Fig. 4/14: Schematic representation of bi-directional, reciprocal molecular approaches to elucidate the molecular mechanisms underlying probiotic function in relation to human health.

Thus for organisms to be considered as probiotics, the following criteria need to be fulfilled

- It should be isolated from the same species as its intended host
- It should have ability to adhere and colonize the intestinal tract
- It should have a demonstrable beneficial effect on the host
- It should have acid and bile tolerance (Lan- Szu *et al.,* 1999)
- It should be non-pathogenic
- It should have antimicrobial activity against intestinal pathogens
- It should be able to survive transit through the gastrointestinal tract (Fuller, 1992)

- On storage, large number of viable bacteria must be able to survive for prolonged periods (Harish and Varghese, 2006).

It is estimated that over one million consumers in the UK regularly (each day) consume probiotics. The European market value is in excess of one billion euros per annum. Many different commercial products of probiotics exist in the market (Table No. 4/30) and new developments are continuing at a rapid pace (Gibson, 2004).

The probiotics satisfying the above criteria are responsible for health benefits like

- Control of diarrhoea (Reddy *et al.,* 1998),
- Alleviation of lactose intolerance (Fonden *et al.,* 2000),
- Inhibition of intestinal pathogens (Bhatia *et al.,* 1989).
- Reduction in cholesterol level (Agarbaek *et al.,* 1995),
- Enhanced immune response (Kimura *et al.,* 1997)
- Antimutagenic and anticarcinogenic activity (Fuller and Gibson, 1997; Collins and Gibson, 1999).
- Synthesis of vitamins (Gibson, 1998).

Table No. 4/30: Commonly used species of probiotics, their strain designations and commercial products

Sr. No.	Species	Strain	Commercial brand name(s)
1.	*Lactobacillus casei*	DN114001	Actimel ®
2.	*Lactobacillus casei*	Shirota	Yakult®
3.	*Lactobacillus plantarum*	299v	ProViva®
4.	*Lactobacillus rhamnosus*	GG	ActifitPlus®, Gefilus®, LGG®, Onaka He GG®, Vifit® etc.
5.	*Lactobacillus johnsonii*	La1	LC1®
6.	*Bifidobacterium lactis*	BB12	Various brand names

The most specific health benefits proven in human intervention studies are given in Table No. 4/31

Table No. 4/31: Beneficial effects of probiotic bacteria documented in human intervention studies

Sr. No.	Effects	Strain(s)	Comments	References
1.	Treatment of acute diarrhoea in children, especially caused by rotavirus	META	Positive correlation	Van Niel *et al.,* 2002; Szajewska and Mrukowicz, 2001
2.	Reduction of the risk of antibiotic-associated symptoms in children and in adults	META	Positive correlation	Wullt *et al.,* 2003; Cremonini *et al.,* 2002
3.	Reduction of the recurrence of *Clostridium difficile* enterocolitis	299v	Preliminary, more research needed	Plummer *et al.,* 2004; Wullt *et al.,* 2003
4.	Reduction of the risk of acute diarrhoea in children	LGG; BB12	Documented for certain strains	Szajewska *et al.,* 2001; Chouraqui *et al.,* 2004
5.	Relief of milk allergy/atopic dermatitis in infants	LGG; BB12 combination of *L. rhamnosus* 19070-2 and *L. reuteri* DSM 22460	Documented for certain strains, more research needed	Majamaa and Isolauri, 1997; Isolauri, 2000; Viljanen *et al.,* 2005; Rosenfeldt *et al.,* 2004; Rosenfeldt *et al.,* 2003
6.	Relief of allergic rhinitis	*L. paracasei* 33	Preliminary, more research needed	Wang *et al.,* 2004 B
7.	Reduction in the risk of atopic diseases in infants	LGG	4 years single study — repetition needed	Kalliomaki *et al.,* 2003
8.	Reduction in the risk of respiratory infections	LGG	Preliminary, more research needed	Gluck and Gebbers, 2003; Hatakka *et al.,* 2001.

Table No. 4/31: continued

Sr. No.	Effects	Strain(s)	Comments	References
9.	Amelioration of the immune response	Various	Documented for certain strains, more research needed	de Vrese *et al.,* 2004; Yasui *et al.,* 2004; Marcos *et al.,* 2004; Schultz *et al.,* 2003
10.	Reduction in the risk of dental caries	LGG	Preliminary, more research needed	Nase *et al.,* 2001
11.	Suppression of *Helicobacter pylori*	Various	Preliminary, more research needed	Cruchet *et al.,* 2003; Linsalata *et al.*; 2004; Sakamoto *et al.,* 2001; Wang *et al.;* 2004 A
12.	Reducing the recurrence of pouchitis	VSL#3; LGG	Documented for certain strains, more research needed	Gosselink *et al.,* 2003; Gionchetti *et al.,* 2004
13.	Relief of IBS symptoms	299v	Preliminary, more research needed	Niedzielin *et al.,* 2001
14.	Relief of rheumatoid arthritis symptoms	LGG	Preliminary, more research needed	Hatakka *et al.,* 2003

The strains used are indicated, as are specific comments on the trials. META, indicates meta-analyses and LGG, 299v and BB12 refer to *L. rhamnosus* GG, *L. plantarum* 299v and *B. lactis* BB12, respectively.

Prebiotics: -

Gibson and Roberfroid (1995) coined the term prebiotic and defined it as a 'nondigestible food ingredients that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health'. This definition was upgraded by Gibson *et al.,* (2004) and prebiotics is now defined as "selectively fermented ingredients that allow specific changes, both in the composition and /or activity in the gastrointestinal microbiota that confers benefit upon host well being and health". Prebiotics comprise some oligosaccharides, especially fructo-oligosaccharides, which preferentially promote *lactobacillus* and *bifidobacteria* growth in the large intestine. To date, the greatest scientific interest was focused on the nutritional and health benefits of oligofructose and inulin (Hartemink, 1997; Roberfroid 2000). More than any other nutrient/food ingredient, a prebiotic is essential to human and mammal's nutrition. In the context of dietary guidelines, it is recommended for daily intake. Prebiotic treated as food ingredient enable manufacturers to meet and exceed the expectations of today's health conscious consumer (Yanbo, 2009). Consumption of appropriately selected prebiotics may enhance the beneficial effect.

A range of oligosaccharides have been tested using various *in vitro* methods, animal models and human clinical trials as a potential sources of the prebiotics. The functional oligosaccharides are intermediate in nature between simple sugars and polysaccharides and are claimed to behave as dietary fibres and prebiotics (Kunz *et al.,* 2000). The most widely used functional oligosaccharides in world are given in Table No. 4/32. They are commercially available in the market with the different brand names depending upon their ingredients and sources (Table No. 4/33).

Table No. 4/32: Functional oligosaccharides along with their monomers as a widely used prebiotics

Sr. No.	Functional Oligosaccharides	Monosaccharide
1.	IMOS (isomaltooligosaccharides), Glucose-oligo-saccharides, Gentio-oligosaccharides, Malto-oligosaccharides	Glucose
2.	SBOS (soybean oligosaccharides), Raffinose, Stachyose	Fructose, galactose, glucose
3.	FOS (fructooligosaccharides)	Sucrose, fructose
4.	XOS (xylo-oligosaccharides)	Xylose
5.	MOS (maltooligosaccharides)	Mannitose, glucose
6.	Palatinose, Clycosylsucrose	Glucose, fructose
7.	Lactosucrose, Lactulose	Galactose, fructose
8.	Galato-oligosaccharides	Galactose

Table No. 4/33: Type, commercial name and manufacturer of the carbohydrates as a potential prebiotics

Sr. No.	Type of substrate	Commercial product	Manufacturer
1.	Fructooligosaccharide (FOS)	Raftilose P95	Orafti, Belgium
2.	Inulin	Frutafit IQ, Frutafit TEX!	Sensus, Netherlands
3.	Arabinogalactan	FibreAids® AG Fibregum B Fibregum P Fibregum TAN	Larex, USA CNI, France
5.	b-glucan	Barley b-glucan hydrolysate	Cevena, Canada
6.	Soybean oligosaccharide (SOS)	Soybean oligo Saccharides powder	Calpis, Japan
7.	b-glucan concentrate	Viscofibre^{TM} barley bglucan	Cevena, Canada
8.	D(+)-Raffinose pentahydrate (B94 only)		Fluka, Switzerland
9.	Glucose (positive control)		Univar, USA

Prebiotic compounds from plants can be obtained by following of three processes:

A. Direct extraction of natural carbohydrates (monosaccharides and oligosaccharides) from plants e.g. oligofructose from chicory inulin
B. Controlled hydrolysis of natural plant polysaccharides in mono and oligosaccharides (Xu *et al*., 2009) e.g. glycanases from plant cell wall polysaccharides (Van Laere *et al*., 2000; Oosterveld *et al*., 2002).
C. Enzymatic synthesis, using hydrolases and/or glycosyl transferases (Grizard and Barthomeuf, 1999; Vidanarachchi *et al.,* 2009) such as sucrose (fructooligosaccharides) or lactose (trans-galactosylated oligosaccharides or galactooligosaccharides) (Playne and Crittenden, 1996).

Among these prebiotics, inulin and oligosaccharides are the most studied prebiotics and have been recognized as dietary fibers in most countries (Moshfegh *et al*., 1999). Thus the dietary fibres found in vegetables, fruits, cereal grains and legumes have a potential as a prebiotic. But not all dietary carbohydrates are prebiotics. Dietary fibres display different degrees of solubility e.g. pectins, hemicellulose, guar gum and inulin (Woods and Gorbach, 2001) which leads to the formation of gels in the gastrointestinal tract. This aids their fermentability by the gut microflora by virtue of an increased surface area available for enzymatic attack.

The effective prebiotic should fulfill the following criteria-

- Prebiotics indirectly causes selective modification of the host's intestinal (especially colonic) micro flora as selectively feeds on one or a limited number of microorganisms. Thus the changes induced in indigenous microflora composition like *lactobacilli* and *bifidobacteria* (Teitelbaum and Walker, 2002) by prebiotics is responsible for its effects.
- They must be able to withstand digestive processes before they reach the colon and preferably persist throughout the large intestine (Gibson *et al.,* 2004).
- They must get fermented by potentially beneficial selective bacteria in the colon (Gmeiner *et al.,* 2000).

- They must stimulate the growth and/or activity of selective intestinal bacteria (Fooks *et al.,* 1999)
- They should also be suitable for the growth and activities of the probiotics,
- They should remain stabile to food processing treatments (Yanbo, 2009)

In general, prebiotics are considered to be safe for human consumption as many of them are constituents of the normal diet, albeit in lower concentrations. Almost every food oligosaccharide and polysaccharide including dietary fibre has been claimed to have prebiotic activity, but not all dietary carbohydrates are prebiotics.

The interest has been developed for prebiotics due to several factors like- exciting scientific and clinical findings using well documented probiotic organisms, concern over limitation and side effects of pharmaceutical agents and consumer demand for natural products. It has been predicted that the sales of prebiotic and probiotics will increase thrice by 2010 (Gregor, 2006). It has already made a place in many food preparations which are accepted throughout the world. Some of the applications of prebiotics in food are given in Table No. 4/34.

Table No. 4/34: Food applications of prebiotics

Sr. No.	Applications	Functional properties
1.	Yoghurts and desserts	Sugar replacement, texture and mouthfeel, fiber, and prebiotics
2.	Beverages and drinks	Sugar replacement, mouthfeel, foam stabilization, and prebiotics
3.	Breads and fillings	Fat or sugar replacement, texture, fiber, and prebiotics
4.	Meat products	Fat replacement, texture, stability and fiber
5.	Dietetic products	Fat or sugar replacement, fiber, and prebiotics
6.	Cake and biscuits	Sugar replacement, moisture retention, fiber, and prebiotics
7.	Chocolate	Sugar replacement, heat resistance and fiber
8.	Sugar confectionary	Sugar replacement, fiber, and prebiotics
9.	Soups and sauces	Sugar replacement, and prebiotics
1.0	Baby food	Texture, body and mouthfeel, fiber, stability, and prebiotics

Synbiotic: -

The synbiotics, is a combination of probiotics and prebiotics. Some authors (Fooks *et al.,* 1999; Roberfroid, 2000) suggest that they might improve the survival of the bacteria crossing the upper part of the gastrointestinal tract, thereby enhancing their effects in the large bowel. Perhaps their individual advantages might be additive or even synergistic. It has not been intensively studied to date. Generally, there is lack of studies on development of synbiotics from properly selected probiotics and prebiotics, followed by examination of their *in vivo* effectiveness (Bielecka *et al.,* 2002).

Perspectives of *Aloe*: -

None of our foods contain the same range of active cell-stimulating constituents as *Aloe* in the same proportions, but the principles involved in using *Aloe* are much the same as some medicines. It has already been analyzed that *Aloe barbadensis* contains a large amount of carbohydrates (Chapter No. 3). The functional oligosaccharides may be a good candidate for use in food and pharmacological applications. *In vivo* biological assays demonstrate that functional oligosaccharides have antioxidant, antimutagenic, antibacterial and other pharmacological activities. This can be used as nutraceuticals and in preparation of nutrition rich food supplements for enhancing the nutritional status of the people in Uttaranchal region.

The present study was carried out to analyze and assess *Aloe barbadensis* ecotypes collected from the Uttaranchal region of India, as a potential source of prebiotic and to evaluate the synbiotic activity towards the selected probiotics – *Lactobacillus* spp. and *Bifidobacterium bifidum*. It has already been proved by Pogribna *et al*. (2008) that the non starch polysaccharides in whole leaf extract of *Aloe vera* alters the growth and short chain fatty acid synthesis of intestinal microorganisms – *Bacteroides fragilis, Bifidobacterium infantis* and *Eubacterium limosum.*. To the best of our knowledge the efficacy of *Aloe barbadensis* gel on *Lactobacillus* spp. and *Bifidobacterium bifidum* by the used method is being reported for the first time.

4.2 MATERIALS:

- Nutrient broth with 2% NaCl and *Aloe* gel as

NaCl	5.0 g
Peptone	10.0 g
Distilled water	1.0 L

  Adjust pH to 7.0-7.5

  Aloe gel as C source (prebiotics plant sources, Table No. 4/35)

- Nutrient agar (Add 0.8% agar in above broth)
- Bacterial cultures as probiotics

  Lactobacillus plantarum (2084)

  *Lactobacillus lactis (*2589*)*

  Lactobacillus acidophilus(2285)

  *Lactobacillus delbrueckii (*2292)

  Bifidobacterium bifidum

- Saline solution (0.8% NaCl)
- Whatmann filter paper
- Slants of nutrient agar
- Maintenance media

Glucose	5.0 g
Lactose	5.0 g
Liver extract	10.0 g
Sodium acetate	6.0 g
Yeast extract	5.0 g
*Salt A solution	5.0 ml
*Salt B solution	5.0 ml
Distilled water	1.0 L
Agar	20.0 g
Adjust pH to	7.6

  Salt A solution

KH_2PO_4	10.0 g

K_2HPO_4	10.0 g
Distilled water	100 ml
Salt B solution	
$MgSO_4.7H_2O$	4.0 g
NaCl	0.2 g
$MnSO_4.4H_2O$	0.2 g
$FeSO_4.7H_2O$	0.2 g
Distilled water	100 ml

Table No. 4/35: Source of the *Aloe barbadensis* ecotypes collected form Uttaranchal region

Sr. No.	Plants Collected	Location	Latitude	Longitude	Altitude (MSL)
1.	Ecotype 1	Pithoragarh	29^0 35' 00"	80 13' 00"	5397’
2.	Ecotype 2	Lamgarha	29^0 50' 45"	78 42' 39"	5375’
3.	Ecotype 3	Champawat	29^0 20' 00"	80 06' 00"	5414’
4.	Ecotype 4	Betalghat (Haldwani)	29^0 35’ 33"	79 53’03"	1257’
5.	Ecotype 5	Dehradun (Raiwala)	30^0 01' 00"	78 13' 00"	1105’
6.	Ecotype 6	Haldwani	29^0 13' 00"	79 31' 00"	1082’

Glasswares and Plastic wares:

Micropipette of different precision measurements (1000, 200, 100, 20, 10 & 2µl) was procured from Gilson medical Electronics, France. Micro tips and microtip-boxes were procured from Axygen scientific Pvt. Ltd. (India).

Equipments:

1. UV- Vis Double beam Spectrophotometer (Schimatzu, Japan):-

The number of colonies of the microorganism was determined by using UV- Vis Double beam Spectrophotometer. Generally optical density is maintained as 1 which corresponds to the 1 X 10^{-9} colony forming unit per milliliter (cfu/ml).

2. Shaker with incubator (Lab India):

Shaker with the temperature controller in the range 25 to 60º C was used. The inoculum was kept at 37ºC for 24 hours to obtain the sufficient growth of the microorganisms.

3. Laminar Air Flow (AIMIL, India):

Horizontal laminar air flow bench was used for the sterile handling during the experiments. The UV rays, laminar flow of air through the HEPA (High Efficiency Particulate Air) filters maintains the sterile environment in laminar air flow bench. Laminar Air Flow is based on the flow of air current to create uniform velocity, along the parallel lines, which helps in transforming microbial culture in aseptic conditions. When fresh air is passed in the laminar air flow it replaces the contaminated air inside and keeps it contamination free.

4. Autoclave (Local Brand):

Autoclaving is done for sterilization of culture media. The main principle is to maintain high temperature and high pressure inside equipment. The maintained pressure is 15 lbs and temperature is 121 ºC for 15 minutes. In that condition of temperature and pressure the microorganisms and their spores are destroyed.

5. Hot Air Oven (Lab India):

Hot air oven with the temperature control in the range of 100 to 300 ºC was used for the sterilization of the glasswares. The instrument was provided with the additional facility for air circulation.

4.3 METHOD:

Bacterial strains and growth conditions: -

For the experiment lactobacillus strains were collected from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India. Those strains were *Lactobacillus plantarum* (2084), *Lactobacillus lactis*

*(*2589*), Lactobacillus acidophilus (*2285*), Lactobacillus delbrueckii (*2292). *Bifidobacterium bifidum* was isolated from the commercially available sample. 24 hr old culture strains were used for the experiments. The growth of suspension was checked by using UV-Vis double beam spectrophotometer at 600nm to have concentration of bacteria as $1X10^{-9}$ cfu/ml.

Basal media: -

The basal media selected was Nutrient broth. Basal medium was modified and carbohydrate source was replaced solely by *Aloe* gel.

Maintenance of stock: -

Stock cultures of *Lactobacillus* were maintained on maintenance media recommended by National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL) and *Bifidobacterium* was maintained on Nutrient agar media. The stock cultures were maintained at 4^0C in refrigerator.

Inoculum: -

The inoculum was prepared in nutrient broth and kept at 37^0C on rotary shaker at 300 rpm. After 24 hours absorbance of turbidity was measured at 600 nm using UV-Vis double beam spectrophotometer. Once it was observed that the broth has concentration of bacteria as $1X10^{-9}$ cfu/ml, 1% of this broth was used as an inoculum.

Isolation of *Bifidobacterium bifidum*: -

Commercially available tablets of bacteria were purchased from the local market. One of the tablets was dissolved in normal saline solution (0.8% NaCl). One drop from this solution was spread on the nutrient agar plate and kept overnight for incubation at 37^0C. The separated colonies were picked up and added into the broth for further growth overnight at 37^0C on rotary shaker at 300 rpm. The Petri plates of bacterial colonies were kept at 4^0C in a refrigerator for further work. For every experiment colonies were sub-cultured on nutrient agar plate, incubated overnight at 37^0C and then used for experiments.

Preparation of gel solution: -

The plant materials of *Aloe barbadensis* ecotypes were collected from the well maintained polyhouses of the Defence Agricultural Research Laboratory (DARL), Pithoragarh. Fresh leaf material was used. The outer rind of the leaf was removed with knife and transparent gel was scooped out. The gel was then diluted with distilled water in the ratio 1:10 and mixed homogenously in juicer. It was then filtered with muslin cloth and then with filter papers. Thus fibres were removed and the extracted gel was used for the experiments.

Optimization of gel concentration: -

Different concentrations of the gel (Table No. 4/36) prepared by above mentioned method were used for the optimization of gel concentration. The gel was added into the basal media in given concentrations and volume was made up. When sufficient turbidity was observed in the given concentration of gel i.e. 5% for *Lactobacillus* and 4.5% in *Bifidobacterium,* the dilutions were carried out to maintain the bacterial count with the help of optical density and dilution factors. Initial viable counts for cultures were standardized by the use of O.D. at 600 nm after 24 hours so that they were approximately the same for all cultures i.e.$1X10^9$ cfu/ml. To follow the standard procedure for evaluation of prebiotic potential 5% concentration of gel was used for all the microbes for the experiments as an optimum concentration.

Prebiotics assay: -

The assay was based on that of Roy and Ward (1990), using nutrient broth as a basal medium supplemented with gel solutions. It monitors culture absorbance instead of pH. Six different types of *Aloe barbadensis* ecotypes collected from various regions of Uttaranchal, India (Table No. 4/35) were evaluated for each of the probiotic mentioned above.

Growth of the probiotic strains was monitored in a basal medium supplemented with the test prebiotic at different concentrations of *Aloe* gel as the sole carbon source. Initially different concentrations of gel from 0.5 to 7.5 % were tested and it was found that 5% gel showed required growth ($1X10^9$ CFU/ml) for the experiments. To prepare the

medium, the test gels were first dissolved in basal broth, filtered and then autoclaved for 15 min at 110^0C and 15 lbs pressure. Following the media preparation, 1% inoculum of an overnight culture of each probiotic having 1X 10^9 CFU/ml was added to the medium. This was then kept for 24 hr incubation at 37^0C. Growth of each strain was monitored by measuring the optical density of the cultures at 600 nm at regular intervals of 2, 4, 8, 12, 16, 20 and 24 hrs using UV visible spectrophotometer (Schimatsu, Japan) keeping broth without gel sample as a control.

Table No. 4/36: Optimization of concentration of *Aloe barbadensis* gel

Sr. No.	Gel concentration	*Lactobacillus* spp. growth	*Bifidobacterium* spp. growth
1.	0.5	No turbidity	No turbidity
2.	1.0	No turbidity	No turbidity
3.	1.5	No turbidity	Minute turbidity
4.	2.0	No turbidity	Minute turbidity
5.	2.5	No turbidity	Very less turbidity
6.	3.0	Minute turbidity	Less turbidity
7.	3.5	Very less turbidity	Good turbidity
8.	4.0	Less turbidity	**Very good turbidity**
9.	4.5	Good turbidity	**Very good turbidity**
10.	5.0	**Very good turbidity**	Low turbidity
11.	5.5	**Very good turbidity**	Very low turbidity
12.	6.0	Low turbidity	Minute turbidity
13.	6.5	Very low turbidity	No turbidity
14.	7.0	Minute turbidity	No turbidity

Statistical analysis: -

For each experiment, the data was analyzed using the Excel statistical package. The results presented i.e. Optical Density (O.D.) readings and standard deviations were calculated from triplicate samples and the experiments were repeated twice.

4.3 RESULTS:

The basal media selected for the growth of the probiotics was nutrient broth. It was used with the only modification of carbon source which was completely replaced by the *Aloe* gel as it contains large amount of total carbohydrates which serves as the source of carbon for the growth. The bacteria which were grown aerobically on the rotary shaker showed better results. 1% of broth having colony concentration $1X10^9$ CFU/ml was used as an inoculum.

The optimization experiments of the *Aloe* gel concentration showed that the species of *Lactobacillus* showed the required growth in the medium provided with the 5 and 5.5% gel as the sole source of the carbon. While *Bifidobacterium* showed similar growth in gel concentrations of 4 and 4.5%. Required colony concentration of the bacteria for the experiments was $1X\ 10^9$ CFU/ml. Very low (0.5 to 3.5%) and very high (6 to 7%) gel concentrations in case of *Lactobacillus* species did not show required growth for the experiments. *Bifidobacterium* didn't show the required growth in gel concentration of 0.5 t0 3.5% and more than 5%. This may be due to the very low content of prebiotics at low concentration of gel and high content of phenolics and anthraquinones at high concentration of gel.

From the growth patterns obtained for all the experimental probiotics using *Aloe barbadensis* ecotypes gel as a prebiotic source (Fig. 4/15 to 4/19), it can be depicted that all the probiotics showed the usual growth pattern. Lag phase of growth curve was observed from 0 to 3 hours in all the experimental probiotics. While log phase was observed from 3 to around 8 hrs. in *L. acidophilus and L. lactis*. In remaining probiotics it was extended up to 16 hrs. The probiotics *B. bifidum* showed the longest log phase while the prolonged stationary phase was observed in *L. plantarum*. The decline phase started after 20 hrs in all the probiotics. The shortest stationary phase was found in *L. acidophilus.* The decline phase started after 28 hrs. in *L. plantarum.*

Highest O.D. was found in *B. bifidum* at 16 hrs of stationary phase. The order of O.D was *L. acidophilus* > *L. lactis* > *L. plantarum* > *L. delbrueckii* for the remaining probiotics.

In terms of the source of prebiotics Ecotype 1 collected from Pithoragarh (5397' MSL) was the best source as all the five probiotics showed maximum growth. This is one of the highest altitudes of occurrence of *Aloe barbadensis* ecotypes. It was also found that the total carbohydrate and crude fibre content of this ecotype was highest among all the ecotypes. The polysaccharides accumulated served the purpose of probiotic growth. After that Ecotype 4 (1257') and Ecotype 5 (1105') were found to be the potential sources. These two locations are at around equal altitude and hence they showed the similar growth pattern. These two ecotypes differ in total carbohydrate content but the crude fibers were found to be in about equal amount. Ecotypes 2, 3, 6 were found to be at lower rank of prebiotic sources and so did their total carbohydrates and crude fibre content. Ecotypes 2 (Lamgarha, 5375') and 3 (Champawat, 5414') were found in the high degree of slopes and the environment is very harsh as compared to other locations even though the altitudinal difference is not very considerable. Ecotype 6 was found in the plains of Haldwani (1082') and the environmental factor would have been the limiting for accumulation of prebiotics.

Growth of *Lactobacillus acidophilus* was found to be maximum in Ecotype 1 and then in 5 (Fig. 4/15). Growth pattern was found to be almost similar in Ecotype 2, 3 and in 4, 5. Exactly similar pattern was observed in *Bifidobacterium bifidum* (Fig. 4/16). Hence it was found that *L. acidophilus* and *B. bifidum* showed the synbiotic activity for Ecotype 1.

For *L. lactis* maximum growth was found in Ecotype 1, then in Ecotype 5 followed by Ecotype 4 (Fig. 4/17). Remaining ecotypes showed the same growth. Thus *L. lactis* showed synbiotic potential for Ecotype 5. *L. plantarum* was growing to its maximum in Ecotype 4 and 1, hence showed synbiotic activity (Fig. 4/18). . *L. delbrueckii* has also shown the highest growth in Ecotype 4 and 1 (Fig. 4/19). The pattern was similar to that of *L. plantarum.*

Ecotype 1 followed by Ecotype 4 and 5 proved to be the most efficient sources for growth of all the experimental probiotics on the basis of showed synbiotic activity for

almost all test probiotics. Hence they can be recommended for the further research and applications. All the results obtained during the present study were in accordance with their polysaccharide contents as documented in Chapter No. 3.

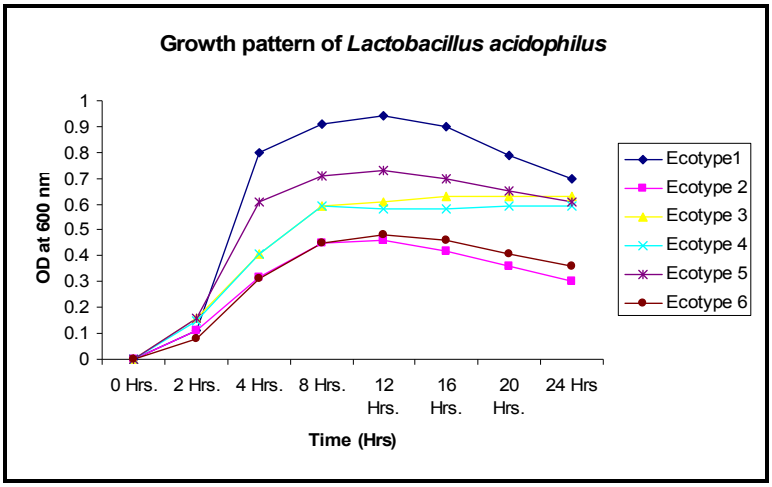


Figure 4/15: Growth pattern of *Lactobacillus acidophilus* in basal media supplemented with gel of different ecotypes of *Aloe barbadensis*.

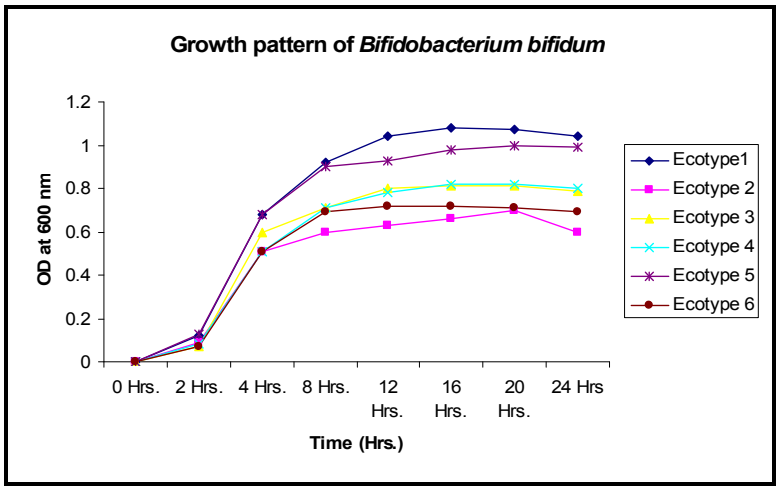


Figure 4/16: Growth pattern of *Bifidobacterium bifidum* in basal media supplemented with gel of different ecotypes of *Aloe barbadensis*.

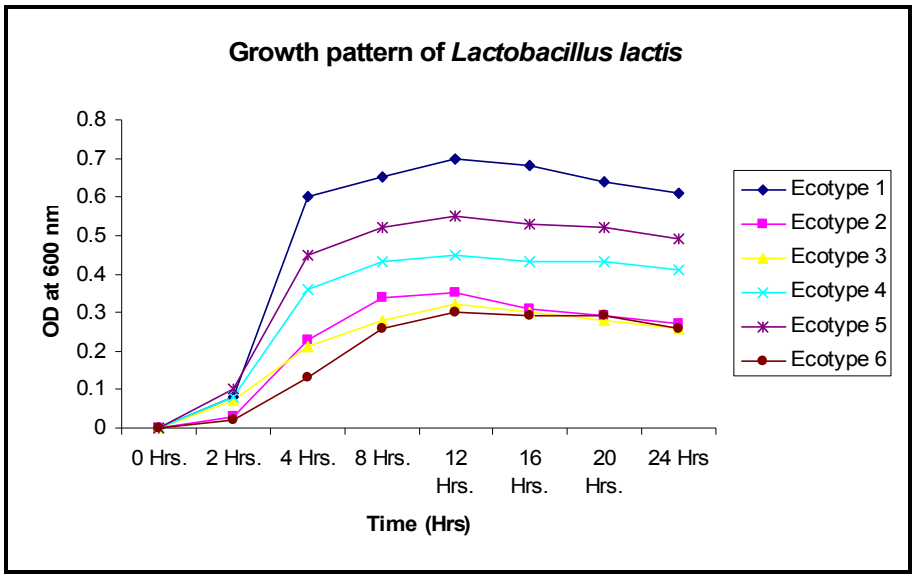


Figure 4/17: Growth pattern of *Lactobacillus lactis* in basal media supplemented with gel of different ecotypes of *Aloe barbadensis*.

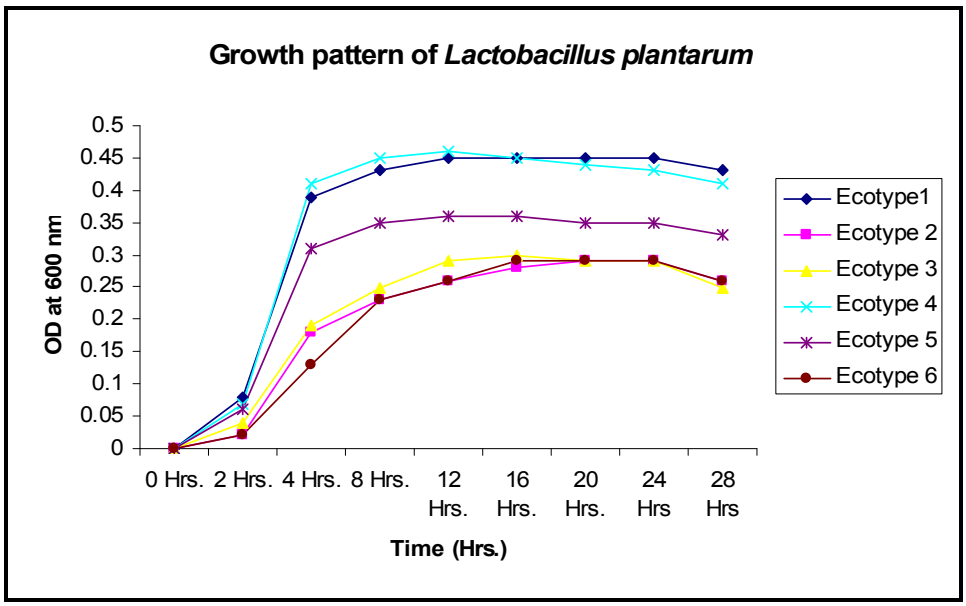


Figure 4/18: Growth pattern of *Lactobacillus plantarum* in basal media supplemented with gel of different ecotypes of *Aloe barbadensis*.

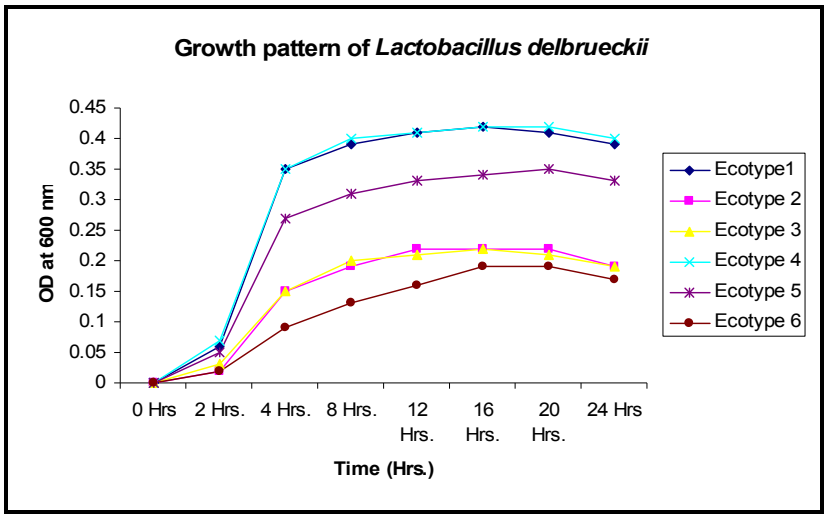


Figure 4/19: Growth pattern of *Lactobacillus delbrueckii* in basal media supplemented with gel of different ecotypes of *Aloe barbadensis*.

4.4 DISCUSSION:

It is known that *Lactobacillus* and *Bifidobacteria* are sensitive to exposure to air and hence may prove difficult to cultivate on an industrial scale, or sensitive to feed processing techniques, which could make them unsuitable candidates for probiosis. The ability to grow in the presence of oxygen, which is shared by commonly used probiotic bacteria such as *Enterococcus faecium* (Jin *et al*., 2000) may allow these organisms introduced with the feed to replicate prior to their passage to the large intestine. Survival in the presence of oxygen (Beerens *et al*., 2000) could also result in survival in faeces and transmission to other animals in contact. The aerotolerance of these cultures may reflect the impact of cultivation for long periods in the laboratory (Maxwella *et al*., 2004).

In the present study even though *Lactobacillus* strains were provided with the recommended enrichment media but to make the process of evaluation and use of these probiotics cost effective and easily acceptable in terms of processing, nutrient basal media was used for the experiments. For *Bifidobacterium* no such recommendations were

provided by the manufacturers hence it was isolated, maintained and grown on the same nutrient broth media composition.

Results of the optimization of the gel concentrations showed that very high and very low amount didn't give the required growth of the probiotics. This is because in very low amount of gel, probiotics get deprived of the essential growth nutrients particularly the carbohydrates and crude fibres. Hence turbidity as a growth indicator was less or absent. It has been proved that the compounds like phenolics and anthraquinones are responsible for inhibition of growth of microbes (Chapter No. 5). The *Aloe barbadensis* ecotypes were collected from the very high altitudinal regions, adverse climatic conditions and nutrient deficient soils. It is well known that the plants on such types of conditions have tendency to accumulate the phenolics and anthraquinones as part of the defense mechanism. *Aloe barbadensis* ecotypes contains very high amount of both of these components in the gel (Chapter No.3). Hence high amount of gel may have provided these compounds due to which the probiotics didn't survived.

In case of higher values of O.D., higher was the turbidity. It was an indicator of the formation of the more bacterial colonies and hence the required growth.

From the results it was observed that even though the test probiotics showed similar growth pattern there was variation in the optical density for the different sources of the prebiotics. It was because of the contents of the different prebiotics. It is well known that predominant growth substrates for gut bacteria are of dietary origin and consist of foodstuffs that have not been absorbed in the upper gastrointestinal tract e.g. resistant starch, dietary fibre, sugars, oligosaccharides, proteins, peptides and amino acids etc. There is also a quantitatively lower contribution from endogenous sources such a mucins (Fooks *et al*. 1999, Ouwehand *et al*. 2005). Also non-starch polysaccharides (NSP) can not be digested by human being and most of monogastric animals, but can be utilized as nutrients by beneficial bacteria (e.g. *Lactobacillus* and *Bifidobacterium*) (Ze-yuan Deng *et al*., 2007). These polysaccharides, being possible prebiotics, could enhance the proliferation of beneficial bacteria and produce short chain organic acids, which help to inhibit the growth of harmful bacteria by reducing the intestine pH and keeping the intestine healthy (Savory, 1992; Shi *et al.*, 1999; Marie *et al*., 1999).

In the present study it was found that the amount of total carbohydrates and crude fibre was very high in Ecotype 1 (Chapter No. 3) which was proved to have most synbiotic potential. Even though Ecotype 1 has maximum amount of carbohydrates Ecotype s 4 was found to be the best for *L. plantarum and L. delbrueckii*. Hence particular ecotypes can be used for the growth of specific probiotic. It was also proved that the plants accumulate the necessary components in the adverse environments which serve as the sources of carbon for the growth of probiotics.

It was also found that the plants were collected from the different agro climatic conditions like soil type, water availability and climate. Species, climate, and growing conditions tremendously affect the physical and chemical composition of *Aloes*. (Briggs, 1995; Klein and Penneys, 1988; Shelton, 1991). The soil is highly acidic and deficient in nutrition (Rawat and Mathpal, 1981). In the present study with respect to the O.D. and the duration of the stationary phase lot of variation was observed which was due to the variation in the compositions. This variation has resulted into the availability of the prebiotics from the different *Aloe barbadensis* ecotypes.

If probiotics and a prebiotic occur together in one product, it is referred as a synbiotic or eubiotic. The above-mentioned combination aims at increased survivability of the administered probiotic and facilitates its inoculation in the large intestine (Scantlebury Manning and Gibson, 2004). In the present study it was recorded that all the probiotic microorganisms showed the maximum growth. The organisms showed the almost same growth in Ecotype 2 and 5. This means these probiotics showed the substrate specificity. This substrate specificity in the provided prebiotics sources is the synbiotic activity i.e. specific probiotic grows to its maximum in specific prebiotic. Even though *Lactobacillus* and *Bifidobacterium* do have the same micro environment, depending upon their metabolic activities and functions they have different requirement for the substrates i.e. prebiotic composition. The ecotypes were also collected from the varied locations and altitudes. So they showed variation in utilization of ecotype prebiotics. Thus the synbiotic or eubiotic potential of the *Aloe barbadensis* ecotypes along with their prebiotic activity was proved.

4.5 CONCLUSION:

Growth of probiotics on *Aloe* gel media indicates the use of this plant as food or food supplement. Crude gel can directly be used as source of functional oligosaccharides and potential source of manufacturing of plant based novel prebiotics by modern biotechnological tools and techniques. Large amount of carbohydrates in these plants can be utilized for tailoring the targeted oligosaccharides with microbial enzymes and other biotechnological processes like fermentation. In India, where vegetarian culture has great influence over huge population and plants are available in ample quantity, this research is helpful for providing prebiotics of plant origin. Along with the high carbohydrate content *Aloe* plants have a large amount of other important nutritional ingredients like dietary fibres, vitamins, minerals and proteins etc. Its pH which was about 4.5 can withstand the colonic pH and favors the acidic micro environment for growth of gut flora (Yolanda, 2007). Hence use of biotechnological tools and techniques to manufacture novel and functional prebiotic, processing and/or treatment of crude gel to manufacture oligosaccharides of interest, generates the possibilities of better food and health in this region along with the development of employment and socioeconomic development. The ecological consideration has significance for the safety of prebiotics (Hammer and Hertel, 2002). With regard to their safety evaluation one has to consider the inherent properties of the prebiotic compound as well as their effect on the microbial flora altogether. In context to the prebiotic, it is the induction or repression of virulence factors in food pathogens by defined food ingredients as pointed out by Gibson and Collins (1999).

4.6 APPLICATION:

The use of diet to fortify certain gut flora components is a popular current aspect of functional food sciences and prebiotics have a significant role. Improved techniques for analysis of the gut microflora i.e. probiotics, new food manufacturing biotechnologies, and increased understanding of the metabolism of prebiotic by

probiotics are facilitating development of prebiotics for specific functional properties and health outcomes.

In the food industry, *A. barbadensis* has been utilized as a resource of functional food, especially for the preparation of health food drinks and other beverages, including tea. The amount of *A. barbadensis* gel that finds its application in the pharmaceutical industry is not negligible as far as the manufacturing of topical ointments, gel preparations, tablets and capsules are concerned. The *A. barbadensis* ecotypes collected form Uttaranchal region will provide the rich source of prebiotics and favor the improvement of human health by enhancing the population of the beneficial gut microflora.

Probiotics are used as health or functional foods whereby they are ingested for their purported positive advantages in the digestive tract and/or systemic areas like the liver, brain, vagina or bloodstream (Gibson, 2004). Hence it has found its application in herbal medicines for curing the clinical and pathological conditions.

The simplicity and cost effectiveness of the present study makes these *A. barbadensis* ecotypes as potential sources of prebiotics and synbiotics for cultivation of oxygen sensitive probiotics like *Lactobacillus* and *Bifidobacterium* to cultivate aerobically on an industrial scale, to feed processing techniques, which could make them suitable candidates for probiosis for long periods in the laboratory.

Safety of pro and prebiotics: -

As the use of the probiotics and prebiotics has increased drastically through out the globe there are certain safety, efficacy and regularity issues one must consider while using.

Long history of safe consumption of some probiotics could be considered the best proof of their safety. Although some *lactobacilli* and *bifidobacteria* have been associated with rare cases of bacteraemia, usually in patients with severe underlying diseases, the safety of members of these genera is generally recognized due to their history of safe use and lack of toxicity (Gueimonde *et al.,* 2004). On the other hand, the low incidence of infections attributable to these microorganisms, there is no increase in the incidence of

bacteraemia due to *lactobacilli* in Finland despite the increased consumption of probiotic lactobacilli (Salminen *et al.,* 2002). With regard to other bacteria, such as *enterococci*, *Saccharomyces boulardii*, *Clostridium butyricum* or some members of the genus *Bacillus* which have been used as probiotics, the situation is more complicated even when they have been used for some time, needs assessment. Hence the most common probiotics and their health benefits need to be assessed, on a strain by strain basis, before any health related product claims could be approved (Reid, 2006). Knowledge of the mechanisms of action complemented with target functions and biomarkers are accepted as relevant to the state of health and well-being or reduction of risk of disease. The hypothesis can be tested by *in vitro* or *in vivo* studies using animal models. However, the most important studies are clinical studies in human subjects (Miguel and Seppo, 2006).

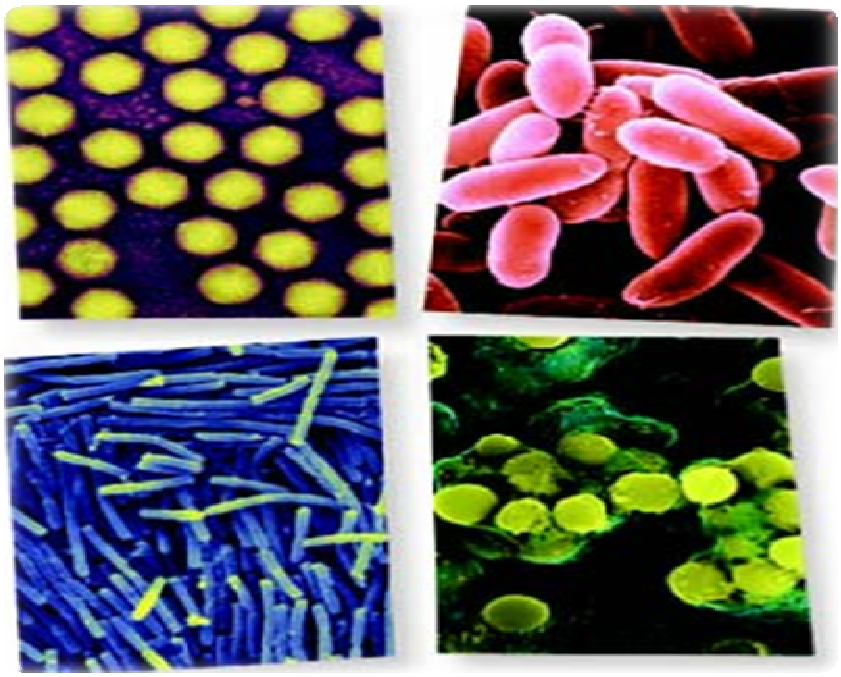
Recently, many patents concerning prebiotics unease and safety issues of prebiotics are raised (Grajek *et al.,* 2005). Prebiotics can be incorporated into many foodstuffs as agents to improve or maintain a balanced intestinal microflora to enhance health and well-being. Today the classification of inulin- type fructans and galactose and their sources as food or food ingredients is not debated based on their history of safe use. The ecological consideration has significance for the safety of prebiotics (Hammes and Hertel, 2002). Alterations in the intestinal microflora could result in adverse effects, depending on which bacterial populations are stimulated. Correspondingly, the enhancement of butyrate formation by the intestinal flora may be of concern (Hammes and Hertel, 2002). Selective stimulation of growth and/or activity of a limited number of the health-promoting bacteria in gut microbiota is one of the most important criteria (Fooks *et al.,* 1999) for pro/prebiotic selection.

Furthermore, some microorganism species in our gut tract such as the genus *Enterococcus* are considered as opportunistic pathogens and are involved in human infections (Hancock and Gilmore, 2000). This background made raised a question whether it is wise to use enterococci as probiotics (Franz *et al*., 1999).

It is a desirable aim of this research to protect the population from adverse reactions and to select the most effective prebiotics.

Chapter 5

Assessment for antimicrobial activity of the extracts



The scientific spirit is of more value than its products, and irrationally held truths may be more harmful than reasoned errors

5.1 INTRODUCTION:

In modern and developing industrial world, health is at a great risk with respect to many clinical and pathological conditions due to change in life styles and dietary habits. Food is among the major origins for these conditions as it acts as a medium for growth and transfer of pathogenic microorganisms. These microorganisms are cause of spoilage, deterioration of food and hence its consumption leads to severe food borne diseases and disorders. Microbial activity is a primary mode of deterioration of many foods and is often responsible for the loss of quality and safety. Concern over pathogenic and spoilage microorganisms in foods is increasing due to the increase in outbreaks of food-borne diseases (Rahman and Kang, 2009). The Gram-positive bacterium *Staphylococcus aureus* is mainly responsible for post-operative wound infections, toxic shock syndrome, endocarditis, osteomyelitis and food poisoning (Mylotte *et al.*, 1987). *Listeria monocytogenes* is responsible for the severe food-borne illness, listeriosis, which has been recognized as one of the emerging zoonotic diseases during the last two decades (Farber, 2000). The Gram-negative bacterium *Escherichia coli* present in human intestines causes urinary tract infection, coleocystitis or septicaemia (Singh *et al*., 2000). Therefore, provision of safe food, control of these pathogens in food and food related products, preventive and curative aids for food borne conditions are becoming matter of concern.

In most of the traditional systems of medicines worldwide, herbal medicines are used for such types of infectious diseases (Brantner and Grein, 1994). Particularly in Asian and other developing countries 80% of the primary requirement of the health care is fulfilled by these traditional systems (Matu and Van Staden, 2003). About 4 billion people today rely on plants as sources of drugs even in developed countries (Nighat Fatima *et al*., 2009). Presently, at least 25% of standard drugs prescribed by a physician originate from folk medicines (Farnsworth, 1988).

The side effects of modern allopathic drugs on human health and multi-drug resistance of pathogenic microbes is a world-wide concern (Alemdar and Agaoglu, 2009), attributed to the extensive use of antibiotics, selection pressure on bacterial strains, and lack of new drugs, vaccines, and diagnostic aids. These shortcomings lead to a

continuous need for new antimicrobial drugs, like extracts or biologically active compounds particularly from natural resources (Essawi and Srour, 2000). Majority of medicinal plant species are rich in biomolecule contents which can cope with health hazard. Recently, antibacterial activity of many plant species has been reported (Pandey and Mishra, 2009).

Among these medicinal plants, *Aloe barbadensis* has been used therapeutically for centuries and is of particular interest due to lengthy historic reputation as a curative agent and its widespread use in complementary therapies. *Aloe* is a well-known natural dietary supplement and chemopreventive agent (Pandey and Mishra, 2009). *Aloe* gel has been used for a long time for topical treatment of skin irritations, but *Aloe* can also be used as a beverage and *Aloe* products are considered to provide relief from constipation. *Aloe* extract is also taken orally as a dietary supplement.

Aloe barbadensis gel is the leaf pulp or mucilage, a thin clear jelly-like substance obtained from the parenchymal tissue that makes the inner portion of the leaves. The gel consists of about 99.3% water and remaining 0.7% is made up of solids with glucose and mannose constituting a large part. The active components of *A. barbadensis* include aloesin (2-acetonyl-8-*β*-D-glucopyranosyl-7-hydroxy-5-methylchromone), anthra-quinones (aloin and aloe emodin), aloemannan, acemannan (gel polysaccharides), aloeride, verectin, giberellin like substance, aloeresin I, 5-methylchromone, flavonoids, glycoprotein fraction, G1G1M1DI2, anthraglycosides, reducing sugars, cardiotonic glycosides, saponins, nafthoquinones, sterols, triterpenoids, amino acids and vitamins. These compound are the source of polysaccharides and possess antibacterial, antiviral, antifungal, antioxidant, angiogenic, immune system modulator, antidiabetic, antihypertensive, cathartics, analgesic, antiinflammatory, wound healing, antihepatitis, antigastric ulcer, and antineoplastic activities.

Very few studies have been carried out on leaf extract of *Aloe* and most of them have been conducted on gel. The available resources in Uttaranchal region i.e. *Aloe barbadensis* ecotypes were used to evaluate the anti microbial property in the present study. For the antimicrobial activities, water, methanol and hexane extracts of *A. barbadensis* leaf were carried out. Other species collected from the region were not used for the purpose because they are not recommended for the edible purposes. Although a

lot of work has been carried out on the medicinal applications of *A. barbadensis* gel, there is still little information on the uses of the whole leaf. This work therefore provides information on the antibacterial activity of leaf extracts against the common food pathogens.

5.2 MATERIALS:

- Test microorganisms
  - *Escherichia coli*
  - *Salmonella typhii*
  - *Bacillus subtilis*
  - *Stayphylococcus aureus*
- Nutrients agar media/ liter

NaCl	5.0 g
Peptone	10.0 g
Distilled water	1.0 L
Adjust pH to	7.0-7.5
Agar agar	0.8 %

- Whatmann filter paper No. 1
- Muslin cloth
- Stainless steel borer (0.8 mm)
- Petri plates
- Klin wrap
- Slants of nutrient agar

Glass wares and plastic wares: -

As mentioned in Chapter No. 3 and 4

Equipments: -

As mentioned in Chapter No. 3 and 4

5.3 METHODS:

Samples: -

Fresh leaves of *Aloe barbadensis* Ecotype s 1 to 6 were collected from glass house and drying was done as mentioned in Chapter No. 3.

Test organisms: -

Pure bacterial cultures of test organisms were obtained from Department of Microbiology, Fergusson College, Pune, India.

Inoculum: -

The inoculum was prepared in nutrient broth and kept at 37^0C on rotary shaker at 300 rpm. After 24 hours absorbance of turbidity was measured at 600 nm using UV-Vis double beam spectrophotometer. Once it was observed that the broth has the concentration of bacteria as $1X10^9$ cfu/ml. 0.1 ml of this broth was used as an inoculum.

Preparation of extract: -

For water extract the 5 gm of dried leaf powder was soaked in 250 ml of mili Q water and kept overnight. After that it was filtered through muslin cloth and then through Whatmann Filter paper no.1

For preparation of methanol and hexane extracts 5 gm of dried leaf powder was kept in a thimble made up of filter paper. This thimble was kept in Sauxhlet apparatus and 100 ml of respective solvents were added in the round bottom flask kept on the heating mantle. The extraction was carried out at 60^0C for six hours.

All the three viz water, methanol and hexane extracts were evaporated at 45°C temperature under reduced pressure to syrup like residue. The solvent was completely removed and dried extracts were re-dissolved in respective solvents (Mili Q water, methanol and hexane) to determine MIC.

Determination of Minimum Inhibitory Concentration (MIC): -

Minimum inhibitory concentration (MIC) was determined by the dilution method as recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (2000). Different concentrations of aqueous, methanol and hexane leaf extracts of *A. barbadensis* ecotypes (ranging from 0.1 mg/ml to 41 mg/ml) were tested separately for each bacterium and inhibition zone of microbial growth in the plates containing tested solutions was judged by comparison with blank control plates. Minimum inhibitory concentration is defined as the lowest concentration of test samples that result in a complete inhibition of visible growth. Experiments were carried out three times.

Antibacterial activity: -

The bacterial susceptibility tests were carried out using the diluted extracts by agar diffusion method (Janssen *et al*., 1987). Petri plates were prepared by pouring 20 ml of nutrient agar medium for all the bacteria. The bacterial inoculum was spread on the top of the solidified media and allowed to dry for 10 min. Leaf extracts (MIC of each bacterium) were delivered into the well and plates were incubated at 37 °C for 24 h. The presence of zone of inhibition was regarded as the presence of antimicrobial action and antimicrobial activity was expressed in terms of average diameter of the zone of inhibition measured in millimeter (Fig. 5/20). Each test was carried out in triplicate.

Statistical analysis: -

For each experiment, the data was analyzed using the Excel statistical package. The results presented are mean of five readings with standard deviations.

5.4 RESULTS:

During the experiments it was found that MIC for water extract ranged in between 25 to 41 mg/ml. It was found higher in case of *B. subtilis* for Ecotype 2 and lowest in case of *S. aureus* for Ecotype 1. In each microbe MIC of Ecotype 1 was found minimum. Average higher values were found in Ecotype 4. MIC for methanol ranged in between 8 to 27 mg/ml and for hexane it was 10 to 28 mg/ml. MIC of water extract of all the *Aloe*

barbadensis ecotypes was observed to be very high as compared to the methanol and hexane extracts (Table No. 5/37, 5/39, 5/41).

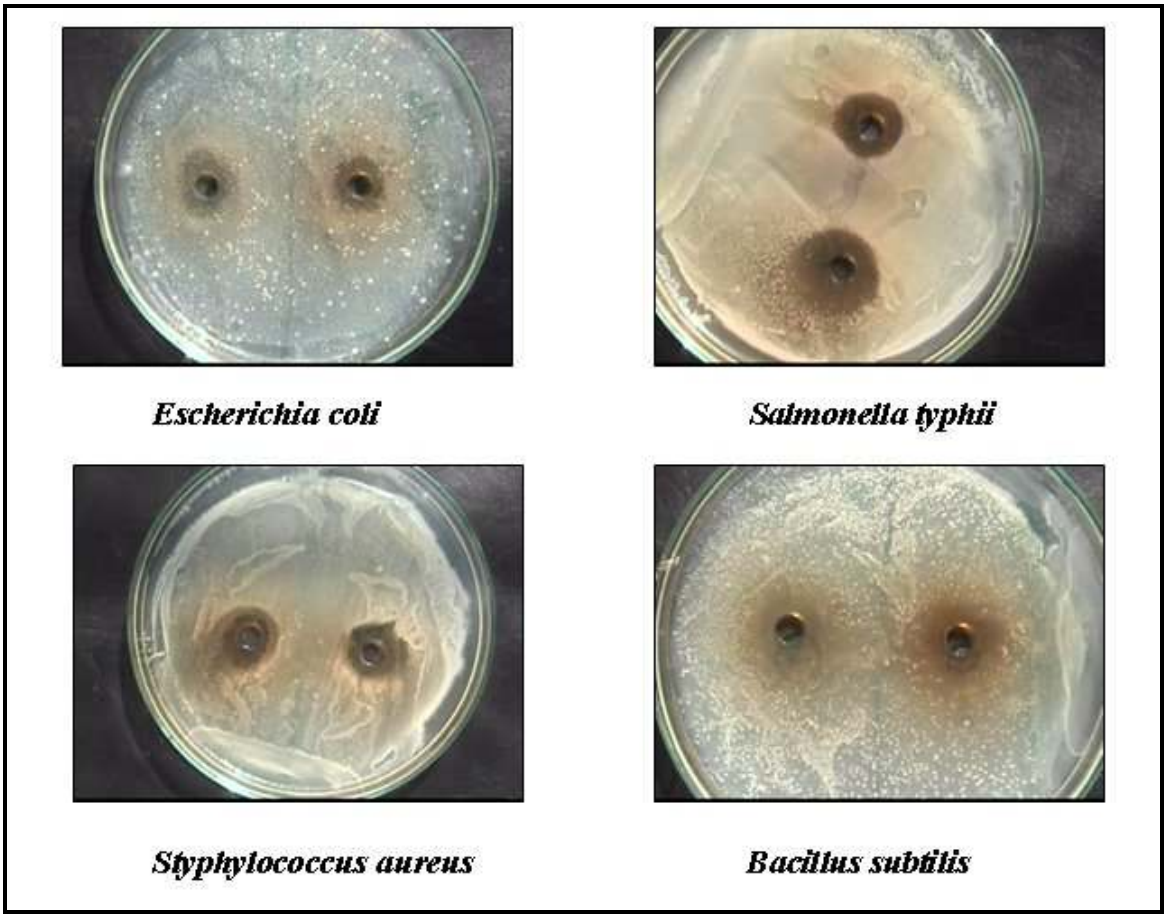


Figure 5/20: Zone of inhibition (mm) of methanol extract of *Aloe barbadensis* Ecotype 1

From Table No. 5/38 it can be noted that all the high values of zone of inhibition were found for the water extracts of the Ecotype 1. Remaining ecotypes didn't show much variation. The zone of inhibition excluding the bore diameter 0.8 mm ranged in between 7.09 ± 0.07 to 21.81 ± 0.04 mm for Ecotype 2 and Ecotype 1 respectively. Performance of Ecotype 4 was found to be consistent for all the microbes. *E. coli* (21.81 ± 0.04 mm) showed highest zone of inhibition for water extract of Ecotype 1. The

difference was very minimum as compared to *S. aureus* (20.08 ± 0.04) for same extract. Least value was observed in *S. typhii* (7.09 ± 0.07) in extract of Ecotype 2.

Table No. 5/37: MIC (mg/ml) of water extract of ecotypes on bacteria

	E. coli	*S. typhii*	*B. subtilis*	*S. aureus*
Ecotype 1	28	27	33	25
Ecotype 2	35	38	41	34
Ecotype 3	31	35	29	33
Ecotype 4	33	33	33	37
Ecotype 5	34	38	37	29
Ecotype 6	37	31	40	36

Table No. 5/38: Zone of inhibition of water extract excluding bore diameter 0.8 mm

	E. coli	*S. typhii*	*B. subtilis*	*S. aureus*
Ecotype 1	**21.81 ± 0.04**	**16.27 ± 0.07**	**18.53 ± 0.08**	**20.08 ± 0.04**
Ecotype 2	13.67 ± 0.06	7.09 ± 0.07	11.41 ± 0.07	11.94 ± 0.07
Ecotype 3	19.34 ± 0.02	13.18 ± 0.01	16.84 ± 0.07	17.42 ± 0.08
Ecotype 4	17.22 ± 0.03	11.94 ± 0.03	15.00 ± 0.05	16.30 ± 0.07
Ecotype 5	16.83 ± 0.03	10.96 ± 0.08	14.56 ± 0.09	15.86 ± 0.04
Ecotype 6	14.07 ± 0.04	8.09 ± 0.00	12.44 ± 0.04	13.06 ± 0.10

Concentration used – 35 mg/ml. Mean ± SD of five replicates.

The MIC values for the methanol extracts of *A. barbadensis* ecotypes ranged in between 8 (Ecotype 1, *S. typhii*) to 27 (Ecotype 3, *E. coli*). The consistant performance was observed for Ecotype 5 and Ecotype 6 (Table No. 5/39) in all the bacteria

For zones of inhibition the values ranged in between 41.07 ± 0.02 to 23.03 ± 0.06 mm. Maximum inhibition was found in E. coli in extract of Ecotype 1 and least diameter was observed in *S. aureus* in extract of Ecotype 2. *S. typhii* showed the maximum

inhibition diameter of 33.52 ± 0.08 mm in extract of Ecotype 3. Rest of the bacteria showed more values in Ecotype 1. The values indicate that methanolic extracts showed the more zone of inhibition for all the microbes and ecotypes as compared to water.

Table No. 5/39: MIC (mg/ml) of methanol extract of ecotypes on bacteria

	E. coli	***S. typhii***	***B. subtilis***	***S. aureus***
Ecotype 1	12	8	11	10
Ecotype 2	16	13	17	15
Ecotype 3	27	15	15	11
Ecotype 4	21	18	21	19
Ecotype 5	23	19	22	20
Ecotype 6	19	17	17	16

Table No. 5/40: Zone of inhibition of methanol extract excluding bore diameter 0.8 mm

	E. coli	***S. typhii***	***B. subtilis***	***S. aureus***
Ecotype 1	**41.07 ± 0.02**	33.17 ± 0.04	**32.02 ± 0.06**	**33.11 ± 0.07**
Ecotype 2	30.68 ± 0.05	24.87 ± 0.07	31.99 ± 0.01	23.03 ± 0.06
Ecotype 3	38.41 ± 0.05	**33.52 ± 0.08**	29.51 ± 0.07	31.08 ± 0.05
Ecotype 4	35.27 ± 0.02	29.96 ± 0.06	26.09 ± 0.14	28.61 ± 0.07
Ecotype 5	34.09 ± 0.18	27.09 ± 0.41	25.08 ± 0.08	26.99 ± 0.04
Ecotype 6	32.86 ± 0.09	26.07 ± 0.09	23.99 ± 0.07	24.62 ± 0.07

Concentration used – 15 mg/ml. Mean ± SD of five replicates.

Table No. 5/41 is showing the minimum concentration for inhibition of experimental microorganisms with hexane extracts of *A. barbadensis* ecotypes. The MIC was found low (10 mg/ml) in case of *E. coli* in hexane extract of Ecotype 1 and high (31

mg/ml) in case of *S. typhii* in extract of Ecotype 6. This time Ecotype 4 showed the consistent performance for all the pathogens.

Zone of inhibition (Table No. 5/42) was found to be maximum in all these experimental microorganisms in the extract of Ecotype 1. Maximum inhibition was observed in *E. coli* (32.07 ± 0.08 mm) and *S. aureus* (32.4 ± 0.04 mm) with least difference. While minimum inhibition (20.01 ± 0.04 mm) was observed in *B. subtilis* in extract of Ecotype 2. Consistent zone of inhibitions were observed in Ecotype 3.

Finally it was observed that methanol extracts of all the ecotypes showed less values for MIC for all the bacteria followed by hexane and water extract.

Table No. 5/41: MIC (mg/ml) of hexane extract of ecotypes on bacteria

	E. coli	***S. typhii***	***B. subtilis***	***S. aureus***
Ecotype 1	10	18	11	13
Ecotype 2	18	27	19	27
Ecotype 3	21	24	22	19
Ecotype 4	23	21	25	26
Ecotype 5	25	29	21	24
Ecotype 6	27	31	23	28

Table No. 5/42: Zone of inhibition of hexane extract excluding bore diameter 0.8 mm

	E. coli	***S. typhii***	***B. subtilis***	***S. aureus***
Ecotype 1	**32.07 ± 0.08**	**27.84 ± 0.09**	**30 ± 0.17**	**32.4 ± 0.04**
Ecotype 2	24 ± 0.08	20.46 ± 0.06	20.01 ± 0.04	22.89 ± 0.03
Ecotype 3	31.99 ± 0.03	26.82 ± 0.05	28.99 ± 0.19	30.75 ± 0.06
Ecotype 4	29.69 ± 0.06	24.99 ± 0.03	26.37 ± 0.05	28.04 ± 0.05
Ecotype 5	28.04 ± 0.11	23.74 ± 0.03	24.67 ± 0.05	26.17 ± 0.11
Ecotype 6	26.34 ± 0.03	22.07 ± 0.04	22.7 ± 0.18	25.63 ± 0.03

Concentration used – 25 mg/ml. Mean ± SD of five replicates.

5.5 DISCUSSION:

From the results it was observed that the MIC was found to be low for all the *Aloe barbadensis* ecotypes. The water, methanol and hexane extracts of the ecotypes proved to be efficient for inhibiting the growth of these experimental microorganisms. It was clear from the previous chapters (Chapter No. 3 and 5) that these ecotypes contain various amounts of different phytochemicals which may be responsible for the antimicrobial activity of these plant extracts. In the present study antimicrobial activity can be due to alkaloids, phenols, polyphenols, saponins, tannins, triterpenes, anthraquinones and steroids found in crude extract and fractions. These phytochemical groups are known to posses antimicrobial compounds (Watt and Breyer-Brandwijk, 1962; Bruneton, 1999; Cowan, 1999). Their presence in the plant extract and fractions could therefore justify the observed activity.

It was also found that the variation in the phytochemical composition depends upon the altitude, agro-climatic condition and soil types (Chapter No. 2, 3 and 4); the variation obtained in the present study can be correlated to these factors.

Water even though considered as the universal solvent has less capacity to extract the compound from the plants. This is due to its less polarity and solubility of these compounds in it. Hence in the present study, it was found the MIC was found maximum (41 mg/ml) in case of water extracts and diameter of zone of inhibition was found minumum in this high amount of MIC. Water extract of Ecotype 1 performed well as amount of anti microbial compounds extracted in water would have been high. Hence MIC was found minimum.

A review of the literature on the antimicrobial activity of different plant extracts shows that methanol extracts have a high level of activity. Parekh and Chanda (2007) reported that the crude methanol extract of *Woodfordia fruticosa* contains certain constituents such as tannins with significant antibacterial properties, which enables the extract to overcome the barrier in Gram-negative cell wall. Parekh *et al*., (2005) reported that methanol extracts were more active than aqueous extracts. Methanol extracts of *Syzygium cumini* contains many chemicals such as alkaloids, amino acids, flavonoids, glycosides, phytosterols, saponins, steroids, tannins and triterpenoids (Kumar *et al*.,

2009). The results in the present study were found exactly in accordance with this literature. The methanol extract was found most efficient because the MIC was found very low and the zone of inhibitions were found more in diameter as compared to the water and hexane extracts. Only Ecotype 3 (5414' MSL) showed maximum zone of inhibition in case of *Salmonella typhii.* This may be because of the tendency of high altitude plants to synthesis more amount of secondary metabolites as a part of defense mechanism. The type and quantity of respective metabolites may vary because the climatic conditions of this location (Champawat) are different than the rest of the others (Chapter No. 2). Hence, the compound specific for the inhibition of *Salmonella typhii* may have been synthesized more and got extracted in methanol.

Hexane was used very rarely for the anti microbial activities. But in the present study it was found that it showed better anti microbial activity against all the microorganisms after methanol which was determined by zone of inhibition. MIC was also found to be less as it also may have extracted anti microbial compounds responsible for the activity against these experimental microorganisms. Hexane extract of Ecotype 1 performed well for all the microbes. However, hexane is less used for this purpose as it is toxic.

From the results it was found that Ecotypes 1 has performed well in all the respect of anti microbial activity. It was collected form higher altitude of 5397' MSL. This region is also deficient in soil nutrients due to high degree of slopes and temperature variation, hence this ecotype has restored food in the form of carbohydrates. It was also found that the amount of vitamin C, phenolics and tannins were very high in Ecotype 1 (Chapter No. 3) which are synthesized to cope up with above situations. All these phytochemicals may have contributed for Ecotype 1 to have more amount of anti microbial compounds and consequently maximum anti bacterial activity.

It was also found in the present study that the Ecotype 4 (1257'), 5 (1105') and 6 (1082') were the least performer. These plants were collected from the plains and favorable conditions may have altered the secondary metabolite profile resulting into less amount of antimicrobial compounds and hence the activity.

Thus, the plants collected from the different agro climatic conditions like soil type, water availability, climate, species, climate, and growing conditions tremendously

influence the physical and chemical composition of *Aloes*. (Briggs, 1995; Klein and Penneys, 1988; Shelton, 1991). The present study showed all the results in accordance of the above findings.

Up till now it was proved that the *Aloe barbadensis* juice shows inhibitory activity only against the Gram positive bacteria. But *Staphylococcus aureus* was not found to be inhibited. It was also found that it didn't show any activity against the Gram negative bacteria like *E. coli* except *Klebsiella pneumonae* (Alemdar and Agaoglu, 2009). In the present study all ecotypes showed inhibition for both Gram positive and Gram negative bacteria. That means whole leaf extracts contains the essential compounds responsible for the antimicrobial activities. The type and amount of these compounds and their activities are solvent dependant. These activities might depend on the compounds being extracted by each solvent, the polarity of the solvents, and their intrinsic bioactivity.

The results of the anti microbial assay have proved *A. barbadensis* to hold excellent potential as an antibacterial agent. *A. barbadensis* has acemannan (acylated mannose) which makes a mucilaginous layer around the urinogenital, gastrointestinal, and respiratory tract when consumed orally. The layers trap the microbial flora and make them unable to invade the system. *A. barbadensis* has anthraquinones as an active compound, which is structural analogue of tetracycline. The anthraquinones act like tetracycline and inhibit bacterial protein synthesis by blocking the ribosomal A (where the aminoacylated t-RNA enters) site (Eun- Young *et al*., 2009). Therefore, the bacteria can not grow in the media containing *A. barbadensis* extract. Acemannan and anthraquinones both work together *in-vivo,* while *in-vitro*, only anthraquinones are effective. Anthraquinones are soluble in organic solvents like alcohol, acetone, etc. but poorly or insoluble in water, therefore we observed significant antibacterial effect with ethanolic extract. Some bacteria exhibit least sensitivity (as the zone of inhibition is much less in comparison to others) in *in vitro* conditions, but if the gel or whole leaf or juice is taken orally both acemannan and anthraquinones will work simultaneously and it may be more effective.

5.6 CONCLUSION:

Safe food can be produced by adhering to good hygienic practices (GHP), good manufacturing practices (GMP), good agricultural practices (GAP), etc. and implementation of food safety risk management systems such as hazard analysis critical control points (HACCP). Microbiological testing is one of the potential tools that can be used to evaluate whether a food safety risk management system is providing the level of control it was designed to deliver. It is one of the number of tools that, when used correctly, can provide industry and regulatory authorities with tangible evidence of control (Van Schothorst *et al*., 2009).

According to World Health Organisation (WHO), herbal medicine is said to be toxic if the LD_{50} is lower than 5 g/kg body weight. From this assertion it can be said that the methanolic extract is not toxic, since after oral administration at the doses of 16 g/kg body weight, no death were recorded (Kuete *et al*., 2006). The methanolic extract of the *A. barbadensis* leaf extrcat could therefore be considered as presenting no danger at therapeutic doses and thus represents potential source of antimicrobial drug.

5.7 APPLICATION:

The water, methanol and hexane extracts of *Aloe barbadensis* ecotypes collected from the Uttaranchal region showed antimicrobial activity against pathogens which are often responsible for skin conditions. The higher activity of the extracts containing complexes of components of the drug to fight against these bacteria, relative to that of the fractions comprising compounds of different polarity, establishes its use as a functional food. It can also be used as preservative against food pathogens and part of medicines for curing infection in humans and animals.

These results could be the reason for the popular use of *A. barbadensis* leaf and gel to relieve different types of gastrointestinal irritations, skin irritations, upper respiratory tract, intestinal tract, and urinogenital infections.

Chapter 6

Genetic diversity analysis by ISSR

Scientific knowledge is in perpetual evolution;
it finds itself changed from one day to next

6.1 INTRODUCTION:

Over the long term, the ability of a plant population to respond adaptively to environmental changes depends on the level of its genetic variability or diversity (Ayala and Kiger, 1984). During the process of evolution, genetic differentiation by natural selection to facilitate reproductive isolation involves the presupposition of the origin of geographic races, subspecies, and species (Stebbins, 1999). A species without enough amount of genetic diversity is thought to be unable to cope with changing environments or evolving competitors and parasites (Schaal *et al*., 1991). Morphological characters remains the fluctuating criteria owing to the fact that the similar cultivar can be expressed in different way according to the ecological conditions like soil, climate etc. and the present considerable morphological differences viz length, width, spotting patterns of leaves and differences in the flowering patterns. Under these conditions the exact identification of the cultivars becomes difficult and complicated. Even the complex economically important traits are highly influenced by the environment.

Therefore, investigation of population genetic diversity and the structure of populations within a species may not only illustrate the evolutionary process and mechanism, but also provide information useful for biological conservation (Schaal *et al*., 1991).

The most important tool developed for the genetic diversity analysis is molecular markers and the technique of PCR has revolutionized the study area.

6.1.1 Molecular markers and PCR analysis: -

Most molecular markers for genetic diversity studies fall into one of two basic categories of techniques that use either hybridization or are based on the PCR:

1. Hybridization based (non-PCR) techniques: -

It includes Restriction Fragment Length Polymorphism (RFLP) where DNA is first digested with restriction enzymes. The resultant fragments are separated by gel electrophoresis and transferred onto filters by southern blotting and then sequence specific probes are used to detect polymorphism.

2. Arbitrarily primed PCR and other PCR based multi-locus profiling techniques: -

The development of the PCR removed the necessity for probe hybridization steps. In this category we can group together all PCR based techniques, which use arbitrary or semi arbitrary primers for amplification of DNA. A common feature of these techniques is the lack of requirement for sequence information from the genome under investigation. Arbitrarily chosen primers are used in PCR conditions in Random Amplified Polymorphic DNA (RAPD) analysis. The primers will initiate synthesis even when the match with the template is imperfect. In this case each amplified product will be derived from a region of the genome that contains short segments which share sequence similarity to the single primer and which are on opposite strands and sufficiently close together for the amplification to work. The PCR products are separated on agarose gels in the presence of ethidium bromide and visualized under ultraviolet light.

In a second subgroup the primers used are semi arbitrary. In that they are based upon restriction enzyme sites or sequences that are interspersed in the genome, such as repetitive elements, transposable elements and micro satellites. The use of primers based on restriction sites is the basis of the technique of Amplified Fragment Length Polymorphism (AFLP). In AFLP, DNA is restricted with two restriction enzymes, adaptors are ligated, and then PCR is carried out with primers which comprise a common part corresponding to the adaptors and restriction site and a unique part corresponding to selective bases.

DNA based molecular markers are most widely used. The reason being they are ubiquitous, innumerable, discrete, non-deleterious, inherited by Mendelian laws, unaffected by the environment and free of epistatic interactions (Beckman and Soller, 1986, Tanksley *et al*., 1989) and have following advantages over morphological and isozyme markers:

- They detect the differences at the DNA level in both translatable and non-translatable regions. They are available in large numbers (Helantjaris, 1991, Stuber, 1992).
- As the polymorphisms are detected directly at the DNA level and not by the gene product which may be influenced by alterations in gene coding sequence

or by changes in gene level expressions, they are neutral in terms of phenotypic and environment characters (Beckman and Soller, 1986; Laurie *et. al.,* 1992).

- It can be carried out in any season and with small amount of sample. Being non tissue specific it can also be used at any stage of plant growth.
- They do not show either dominant recessive interaction or epistatis and are devoid of pleotropic effect (Gokhale, 2003).
- They can give the efficient results in very small quantity of DNA

PCR analysis for genetic diversity: -

In 1984, a team of scientists at Cetus Corporation developed a DNA amplification procedure based on an *in vitro* rather than an *in vivo* process. Known as the polymerase Chain Reaction (PCR) (Saiki *et al*., 1985; Mullis and Faloona, 1987; Saiki, 1988), this method can produce large amounts of a specific DNA fragment from a complex DNA template in a simple enzymatic reaction. PCR is characterized by the three Ss: selectivity, sensitivity, and speed. PCR is a recently developed technique which has had a significant impact in many areas of science. PCR is a rapid and simple method for specifically amplifying a target DNA sequence in an exponential manner. Virtually pure DNA fragments from complex genomes can be obtained in a matter of hours rather than the weeks or months. The method utilizes a DNA polymerase and two oligonucleotide primers to synthesize a specific DNA fragment from a single stranded template sequence. The amount of starting material needed for PCR can be as little as a single molecule rather than the usual millions of molecules required for standard cloning and molecular biological analysis. Although purified DNA is used in many applications, it is not required for PCR, and crude cell lysates also provide excellent templates. The DNA need not even be intact, in contrast to the requirements of other standard molecular biological procedures, as long as some molecules exist that contain sequences complementary to both primers. The speed and sensitivity of PCR have been widely recognized by scientists in both medicine and basic biology, and the method has been applied to problems that a few years ago were thought to be inaccessible to molecular analysis.

Briefly, the method consists of synthesizing a set of primers that have nucleotide sequences complementary to the DNA that flanks the target sequence. The primers are

then mixed with a solution of the target DNA, a thermostable DNA polymerase and all four deoxynucleotides (A, T, C and G). The solution is then heated to a temperature sufficient to separate the complementary strands of DNA (approximately 95° C.) and then cooled to a temperature sufficient to allow the primers to bind efficiently and specifically to the flanking sequences. The reaction mixture is then heated again (to approximately 72° C.) to allow the DNA synthesis to proceed. After a short period of time the temperature of the reaction mixture is once again raised to a temperature sufficient to separate the newly formed double-stranded DNA, thus completing the first cycle of PCR. The reaction mixture is then cooled and the cycle is repeated. Thus, PCR consists of repetitive cycles of DNA melting, annealing and synthesis. Twenty replication cycles can yield a million fold amplification of the target DNA sequence. The ability to amplify a single DNA molecule by PCR has applications in environmental and food microbiology (Wernars *et al.* 1991; Hill and Keasler 1991). PCR has also been used in phylogenetic studies of various organisms. For example, PCR has been used to authenticate and differentiate three medical species of Panax from one another and also from their common adulterants (Cheung *et al*., 1994; Shaw and Pui-Hat Butt, 1995).

Among the entire DNA based molecular makers in PCR analysis, ISSR are important because of their advantages over the others like availability and acceptability.

6.1.2 ISSR markers: -

The Inter-Simple Sequence Repeat (ISSR) markers are easiest of all molecular approaches. Oligonucleotide primers of ISSR-PCR are usually designed by random playing with the core sequence motifs of microsatellites by adjunction of either the 5’ or 3’ flanking anchor. These anchors ensure a higher resolution and better reproducibility of the bands in ISSR patterns (Zietkiewicz, 1994; Bornet and Branchard, 2001; Wiesner, 2001), which is desirable for automated computer-assisted interpretation of ISSR patterns.

ISSR amplifies intermicrosatellite sequences at multiple loci throughout the genome (Salimath *et al*., 1995, Li and Xia, 2005). An ISSR molecular marker technique permits the detection of polymorphism in microsatellites and inter-microsatellite loci without previous knowledge of DNA sequences (Zietkiewicz *et al*., 1994). Furthermore,

they are highly reproducible due to their primer length and to the high stringency achieved by the annealing temperature. This technique has been widely used to investigate genetic diversity and population genetic structure (Reddy and Nagaraju, 1999; Li and Xia, 2005; Chen *et al*., 2005) because of its advantages in overcoming limitations of allozyme and RAPD techniques (Wolfe *et al*., 1998; Ratnaparkhe *et al*., 1998; Esselman *et al*., 1999).

Because of the high mutability of simple sequence repeats (SSR), there are many versions in which microsatellites are used as primers. It is the proven tool for the interspecies and intraspecies genetic diversity studies. Some of the important breakthroughs because of ISSR are given in Table no. 6/43.

During the studies all Uttaranchal region were screened for the collection of genus *Aloe.* The ecotypes of *Aloe barbadensis* and other species were the only types available in this region (Chapter No.2). It was observed that the phenotypic characters of collected ecotypes of *Aloe barbadensis* were similar e.g. colour of the leaves, number of leaves per plant, number of suckers etc. (Chapter No. 2). The differences were obtained after the sufficient statistical analysis was carried out. The study was extended to the quantitative determination of biochemical constituents of the *Aloe barbadensis* ecotypes and the other species (Chapter No. 3) which resulted wide rage of constituents among the ecotypes and the species. Hence the present study was carried out

- To find out the basis of morphological and biochemical variations
- To investigate the genetic diversity among *Aloe* ecotypes and species found as wild source
- To facilitate conservation management

Till now PCR has been used for the fidelity testing (Sanghmitra and Satyabrata, 2008) and identification of *Aloe* species by using RAPD markers (US Patent No. 6001572). The ISSR markers have been widely used to investigate genetic diversity and population genetic structure (Reddy and Nagaraju, 1999; Li and Xia, 2005; Chen *et al*., 2005) because of its advantages in overcoming limitations of allozyme and RAPD techniques (Wolfe *et al*., 1998; Ratnaparkhe *et al*., 1998; Esselman *et al.,* 1999). Hence

the ISSR primers were selected for the present study. Their reproducibility, authenticity and availability are also key factors making them most acceptable these purposes.

Table No. 6/43: Reports on inter specific and intra specific genetic diversity studies using ISSR molecular markers

Sr. No.	Name of plant	Study	Reference
Inter specific Genetic Diversity			
1.	Lycoris (Amaryllidaceae)	Interspecific relationship	Shude *et al*., 2006
2.	Sulla genus	Genetic diversity	Houda *et al.,* 2007
3.	*Pistachio* tree	Genetic diversity	Fares *et al*., 2009
4.	*Aloe* species	Identification of species	US Patent 6001572
5.	Dendrobium species	Phylogenetic study and molecular identification	Hui- Zong *et al*., 2009
6.	Common bean	Comparison between wild and domestic population	Gonzalez *et al*., 2005
Intra specific Genetic Diversity			
7.	*Stevia Rebaudiana* Bertoni	Genetic relationship	Heikel *et al*., 2008
8.	*Thymus daenensis* subsp. Daenensis	Genetic Variability and Geographic Differentiation	Mehdi *et al*., 2009
9.	*Jatropa*	Molecular characterization	Senthil kumar *et al*., 2009
10.	*Gmelina arborea* Roxb.	Morphological and genetic diversity	Naik *et al*., 2009
11.	*Vitex rotundifolia*	Population genetic structure	Hu *et al.,* 2008

6.2 MATERIALS and METHODS:

Source of plant material: -

For Genomic DNA isolation, mature and fresh leaf material was collected from different locations along the Uttaranchal region (Kumaon and Garhwal) of India. Those were the only ecotypes and species available in this region. Collected material was stored initially in air tight polyethylene bags. For long term storage, collected material was first frozen in liquid nitrogen and the stored in deep refrigerator at -70ºC. Six ecotypes of *Aloe barbadensis* (Mill.) and four other species (*Aloe perryi, Aloe saponaria, Aloe humilis* and *Aloe zebrina*) were used which were collected from 8 different locations. (Chapter No. 2, Table No. 2/7,)

Glasswares and Plastic wares: -

Micropipette of different precision measurements (1000, 200, 100, 20, 10 & 2µl) was procured from Gilson medical Electronics, France. Micro tips, appendorff tubes (0.05, 1.5 & 2 ml) and microtip-boxes were procured from Axygen scientific Pvt. Ltd. (India). While centrifuge tubes (50ml) were purchased from Tarson products Pvt. Ltd, (Kolkata, India).

Reagents:

- **Extraction buffer: -**
    - Tris –HCl 0.22 M (pH 7.8)
    - NaCl 0.89 M
    - CTAB 0.8 %
    - EDTA 0.022 M
    - Mannitol 0.14 M
    - 2- mercaptoethanol 1 µl/ml
    - PVP 2 gm
- **Fixing solution: -**
    - Chilled absolute ethanol.
- **PCR mixture (for 25 µl): -**
    - dNTPs 5 µl (Bangalore Genei, India)

- 10X Buffer 2.5 µl (Bangalore Genei, India)
- Taq polymerase 1 µl (1 U) (Bangalore Genei, India)
- Primer 4 µl (0.24 umol) (ISSR Primer Set #9 (801…900) obtained from University of British Columbia, USA was used (Table No. 6/44)
- $MgCl_2$ 2 µl
- Template DNA 8 µl (40 ng)
- Mili Q water 2.5 µl.

- Choloroform: Isoamyl alcohol (24:1)
- Ethanol (70%)
- **TE buffer (pH 8) -** 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA.
- Agarose (Sigma, USA)
- **Electrophoresis buffer:** Tris-borate-EDTA (1x)
- **Loading buffer:** Bromophenol blue (0.25%) and glycerol (30%)
- **Fluorescent dye:** Ethidium bromide (10 µg/mL)
- **Marker:** Low range DNA ladder (3 Kb) (Bangalore Genei, India)
- **Enzymes:** RNAase A (10 mg/mL) (Bangalore Genei, India)

Equipments:

1. Milli-RO water system (Millipore, USA):

The instrument is used for obtaining deionized water. There are series of ion exchanging columns. When ordinary water is passed through these columns, it is made free of salts and minerals that are naturally present in tap water. It is important to use deionized water for preparation of reagents as the presence of minerals and salts may alter the final composition.

2. Horizontal electrophoresis unit (Tarson, India):

The basic principle of electrophoresis is, charged ions or molecules migrate when placed in an electric field. The rate of migration of a substance depends on its net charge, size, shape and the applied current. It consists of a power pack and electrophoresis unit. The power pack supplies a stabilized current at controlled or required voltage and current output. The electrophoresis unit contains the electrodes, buffer reservoirs and gel casting assembly.

Table No. 6/44: List of ISSR Primers

UBC Primer Set #9 (Microsatellite)
3 nanomoles/tube

801	ATA TAT ATA TAT ATA TT	851	GTG TGT GTG TGT GTG TYG
802	ATA TAT ATA TAT ATA TG	852	TCT CTC TCT CTC TCT CRA
803	ATA TAT ATA TAT ATA TC	853	TCT CTC TCT CTC TCT CRT
804	TAT ATA TAT ATA TAT AA	854	TCT CTC TCT CTC TCT CRG
805	TAT ATA TAT ATA TAT AC	855	ACA CAC ACA CAC ACA CYT
806	TAT ATA TAT ATA TAT AG	856	ACA CAC ACA CAC ACA CYA
807	AGA GAG AGA GAG AGA GT	857	ACA CAC ACA CAC ACA CYG
808	AGA GAG AGA GAG AGA GC	858	TGT GTG TGT GTG TGT GRT
809	AGA GAG AGA GAG AGA GG	859	TGT GTG TGT GTG TGT GRC
810	GAG AGA GAG AGA GAG AT	860	TGT GTG TGT GTG TGT GRA
811	GAG AGA GAG AGA GAG AC	861	ACC ACC ACC ACC ACC ACC
812	GAG AGA GAG AGA GAG AA	862	AGC AGC AGC AGC AGC AGC
813	CTC TCT CTC TCT CTC TT	863	AGT AGT AGT AGT AGT AGT
814	CTC TCT CTC TCT CTC TA	864	ATG ATG ATG ATG ATG ATG
815	CTC TCT CTC TCT CTC TG	865	CCG CCG CCG CCG CCG CCG
816	CAC ACA CAC ACA CAC AT	866	CTC CTC CTC CTC CTC CTC
817	CAC ACA CAC ACA CAC AA	867	GGC GGC GGC GGC GGC GGC
818	CAC ACA CAC ACA CAC AG	868	GAA GAA GAA GAA GAA GAA
819	GTG TGT GTG TGT GTG TA	869	GTT GTT GTT GTT GTT GTT
820	GTG TGT GTG TGT GTG TC	870	TGC TGC TGC TGC TGC TGC
821	GTG TGT GTG TGT GTG TT	871	TAT TAT TAT TAT TAT TAT
822	TCT CTC TCT CTC TCT CA	872	GAT AGA TAG ATA GAT A
823	TCT CTC TCT CTC TCT CC	873	GAC AGA CAG ACA GAC A
824	TCT CTC TCT CTC TCT CG	874	CCC TCC CTC CCT CCC T
825	ACA CAC ACA CAC ACA CT	875	CTA GCT AGC TAG CTA G
826	ACA CAC ACA CAC ACA CC	876	GAT AGA TAG ACA GAC A
827	ACA CAC ACA CAC ACA CG	877	TGC ATG CAT GCA TGC A
828	TGT GTG TGT GTG TGT GA	878	GGA TGG ATG GAT GGA T
829	TGT GTG TGT GTG TGT GC	879	CTT CAC TTC ACT TCA
830	TGT GTG TGT GTG TGT GG	880	GGA GAG GAG AGG AGA
831	ATA TAT ATA TAT ATA TYA	881	GGG TGG GGT GGG GTG
832	ATA TAT ATA TAT ATA TYC	882	VBV ATA TAT ATA TAT AT
833	ATA TAT ATA TAT ATA TYG	883	BVB TAT ATA TAT ATA TA
834	AGA GAG AGA GAG AGA GYT	884	HBH AGA GAG AGA GAG AG
835	AGA GAG AGA GAG AGA GYC	885	BHB GAG AGA GAG AGA GA
836	AGA GAG AGA GAG AGA GYA	886	VDV CTC TCT CTC TCT CT
837	TAT ATA TAT ATA TAT ART	887	DVD TCT CTC TCT CTC TC
838	TAT ATA TAT ATA TAT ARC	888	BDB CAC ACA CAC ACA CA
839	TAT ATA TAT ATA TAT ARG	889	DBD ACA CAC ACA CAC AC
840	GAG AGA GAG AGA GAG AYT	890	VHV GTG TGT GTG TGT GT
841	GAG AGA GAG AGA GAG AYC	891	HVH TGT GTG TGT GTG TG
842	GAG AGA GAG AGA GAG AYG	892	TAG ATC TGA TAT CTG AAT TCC C
843	CTC TCT CTC TCT CTC TRA	893	NNN NNN NNN NNN NNN
844	CTC TCT CTC TCT CTC TRC	894	TGG TAG CTC TTG ATC ANN NNN
845	CTC TCT CTC TCT CTC TRG	895	AGA GTT GGT AGC TCT TGA TC
846	CAC ACA CAC ACA CAC ART	896	AGG TCG CGG CCG CNN NNN NAT G
847	CAC ACA CAC ACA CAC ARC	897	CCG ACT CGA GNN NNN NAT GTG G
848	CAC ACA CAC ACA CAC ARG	898	GAT CAA GCT TNN NNN NAT GTG G
849	GTG TGT GTG TGT GTG TYA	899	CAT GGT GTT GGT CAT TGT TCC A
850	GTG TGT GTG TGT GTG TYC	900	ACT TCC CCA CAG GTT AAC ACA

3. UV Transilluminator:

For gel visualization under ultraviolet radiation.

4. Gel Documentation System (Biorad):

It is a powerful, flexible package including the hardware and the software for imaging and analyzing 1-D electrophoresis gels, dot blot arrays and colonies. The lane-based functions can be used in determination of molecular weights and other values.

5. Water bath (Julabo):

It is used for maintaining the constant temperature, in which, temperature setting, temperature indicator, and cooling effect are also available.

6. SpinWin (Tarson):

It is a mini centrifuge equipped with continuously variable electronic speed control, speed indicator, Amp meter, timer, dynamic break, zero starting switch and fuse safety device for 230 V 50 Hz AC mains.

7. SpectroPhotometer (Perkin & Elmer):

It is used for quantifying DNA in a solution. The reading is taken at wavelengths of 260 and 280 nm and their ratio provides an estimate of the purity of the sample DNA.

8. PCR Robocyler (Stratagene, USA):

It is microprocessor controlled for block laboratory instrument utilizing a robotic arm to quickly move from one temperature block to another based on user defined program. This system has four separate anodized aluminum temperature blocks (3-heating block &1 cold block) containing 96 precision cut wells that remain at set temperature, where thermal cyclers have a single block that changes temperature during each cycle. In the robocycler tubes are moved from one block to next by the robotic arm. The four-block design decreases cycling time by up to 30% and achieves a well-to-well temperature uniformity of ± 0.1 °C for amplification process.

METHODS:

A. DNA isolation protocol: -

The DNA isolation was carried out based on the basis of isolation protocol without liquid nitrogen by Sharma *et al.,* (2003) with some modification. Fresh leaf samples of five different plants were used for the study. The protocol is as follows:

- 5 gm of material was dipped in chilled fixing solution for 30 minutes in refrigerator at 4^0C.
- Alcohol was allowed to evaporate and tissue was grind in mortor and pestle into fine powder.
- To this, about 50 ml extraction buffer, preheated at 60º C was added and properly mixed.
- Incubated at 60 ºC in a shaking water-bath (100 rpm) for 1 hr.
- Added equal volume of choloform: isoamyl alchol (24:1) and mixed by inversion for 15 min.
- Spinned at 15000 rpm (10000g) for 10 min. at 25-30 ºC.
- Carefully transferred the upper clear aqueous layer to another 50 ml microfuge tube.
- Added 0.6 volume of cold absolute ethyl alcohol and tubes were kept overnight at 4^0C in refrigerator.
- Spool was removed either with pipette or by centrifugation decanting the liquid phase.
- Discarded the supernatant and washed the pellet with 70% ethanol.
- Drained properly by inverting on tissue paper for one hour.
- DNA dried under vacuum. Dried DNA was dissolved in TE buffer (pH 8).
- 2.5 µl RNase was added to 0.5 ml of crude DNA (2.5 µl RNase = 25 µg of RNase). Thus treatment was at 50 µg/ml of DNA.
- After thorough and gentle mixing it was kept for incubation in water bath at 37^0C for one hour.

- 0.2 to 0.4 ml of chilled chloroform: isoamyl alcohol (24:1) was added and mixed thoroughly for 15 min.
- It was then centrifuged at 15000 rpm for 15 min.
- Supernatant was removed and DNA was precipitated by using double volume of chilled absolute alcohol. DNA was removed with a pasture pipette or centrifuged for pelleting.
- 70% alcohol was used for washing and after proper drying it was re-dissolved in TE.
- DNA concentrations were determined by taking the absorbance at 260 nm. The ratio between 260 and 280nm provided an estimate of the purity of the sample DNA. DNA samples with a ratio of approximately 1.8 under spectrophotometer and producing an intact single band without smear on 0.8% Agarose gel electrophoresis were considered as good quality DNA.

B. Quantification of DNA: -

DNA was quantified by using the Ultraviolet (UV) - Visible dual beam spectrophotometer (Hoisington *et al*., 1994). The analysis of UV absorption provides a simple and accurate estimation of the concentration of DNA in a sample. Purines and pyrimidines in DNA show absorption maxima at 260 nm (e.g. dATP- 259 nm, dCTP- 272 nm, dTTP- 247 nm) if the DNA sample is pure without significant contamination from proteins or organic solvents. The ratio of OD_{260}/OD_{280} should be determined to assess the purity of the sample.

This method is limited by quantity of DNA and the purity of the preparation. Accurate analysis of the DNA preparation may be impeded by the presence of the impurities in the sample or if the amount of DNA is too little. In the estimation of the total genomic DNA e.g. the presence of the RNA, sheared DNA etc could interfere with the estimation.

The procedure is described as-

- Take 1 ml of TE buffer or distilled water in an cuvette and calibrate the spectrophotometer at 260nm as well as 280nm
- Add 10 µl of each DNA sample to 900 µl TE or distilled water and mix well.

- Use TE/ distilled water as a blank in another cuvette.
- Note OD at 260 and 208 nm.
- Calculate the OD_{260}/OD_{280}

  The amount of DNA can be quantified using the formula

$$\text{DNA concentration } (\mu g/ml) = \frac{OD_{260} \text{ X } 100 \text{ (Dilution factor) X } 50\ \mu g/ml}{1000}$$

A ratio in between 1.8 - 2.0 denotes that the absorption in the UV range is due to nucleic acids.

A ratio lower than 1.8 indicates the presence of proteins and/or other UV absorbers.

A ratio higher than 2.0 indicates that the samples may be contaminated with chloroform or phenol. In either case (< 1.8 or > 2.0) it is advisable to re precipitate the DNA.

C. PCR protocol using ISSR primers: -

Assessment of the effects of different concentrations of $MgCl_2$, dNTPs and template genomic DNA was carried out and then PCR reactions were run in 25 µl reaction volume that contained 2.5 µl of Taq buffer B (without $MgCl_2$), 2.5 mM MgCl2, 0.2 mM dNTPs, 1 U of Taq polymerase, 0.24 µM of primers and 40ng of template DNA. For the amplifications with thermal cycling conditions were consisted of initial denaturation for 5 min. at 94º C, followed by 40 cycles of 1 min. at 94º C, 1 min. at 48 – 58º C (optimized for different primers), 2 min. at 72º C and 10 min. at 72º C for the final extension. Initially 100 ISSR primers (UBC, Primer set No 9) were screened and optimized for their optimal annealing temperatures on Stratagene's RoboCycler Gradient PCR machine with temperature gradient mode (Fig. 6/21). The amplified ISSR fragments along with low range DNA ladder were then separated electrophoretically on 2.0 % agarose gel in 1.0 X TBE buffer at 100 V for 3 Hrs. Separated ISSR fragments were visualized by staining the gel with ethidium bromide and photographed under UV light in

Gel documentation System. Amplifications with each primer were repeated twice to confirm its reproducibility.

PCR thermal cycling conditions: -

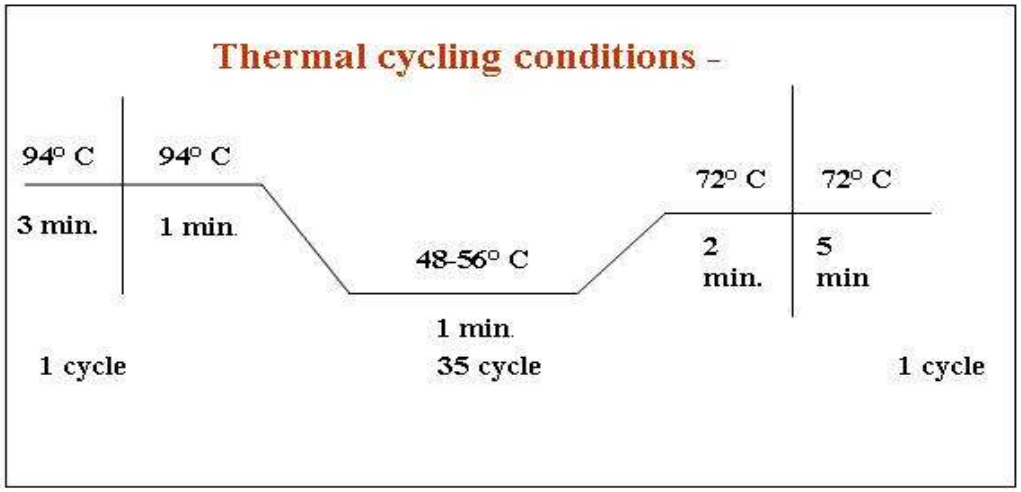


Figure 6/21: PCR thermal cycling conditions

Data analysis:

Amplified ISSR fragments were transformed into a binary character matrix by scoring 1 (presence) and 0 (absence). For statistical analysis, data only from intensely stained and clear bands were considered. For scoring of bands, lane based Band Analysis function of Biorad's Gel documentation system was used. To estimate the genetic relationship among the individuals, the Dice genetic distance coefficient was used to calculate the pair wise band similarity and dendrogram was constructed by Un weighted Pair Group Method of cluster Analysis (UPGMA) using arithmetic average on NTSYS PC version 2.1.

6.3 RESULTS:

Various DNA extraction protocols (de la Cruz *et al*., 1997, Doyle and Doyle, 1990; Barnwell *et al*., 1998; Tel-Zur *et al*., 1999, An Michiels *et al*., 2002) were tried to obtain good quality and quantity of DNA. But not a single one was proved to be working for the purpose. Finally method by Sharma *et al.,* (2003) with some modifications gave the DNA suitable for the PCR reactions and good amplification patterns. This method was modified by increasing the concentration of CTAB and PVP to 0.8% and 2 gm in 100ml respectively. Chilled ethanol was used in place of one at room temperature. Using this method DNA from the fresh leaf samples of the six *Aloe barbadensis* ecotypes and four other species of *Aloe* (*Aloe perryi, Aloe saponaria, Aloe humilis* and *Aloe zebrina*) was isolated. The quality of DNA was confirmed on the agarose gel and then quantified. The DNA quantification was carried out by the spectrophotometric method (Hoisington *et al*., 1994). The concentrations of DNAs obtained by using above methodologies ranged between 40 to 70 µg/ ml.

The PCR conditions were optimized by considering various parameters like template DNA concentration (20, 40, 60, 80 ng / reaction) and *Taq* polymerase (0.2, 0.4, 0.6, 0.8, 1.0 U/ reaction), $MgCl_2$ (1.0. 1.5, 2.0, 2.5, 3.0mM/ reaction), dNTP's (0.1, 0.2, 0.3, 0.4 mM/ reaction). These parameters were tested in five randomly chosen DNA samples using ISSR primer. For clear and reproducible amplification and banding pattern, 2.5 µl of 10X *Taq* buffer B (without $MgCl_2$), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.0 U of *Taq* polymerase, 0.24 µM of primers and 50ng of template DNA were the optimum concentrations per 25 µl reaction volume. For PCR reaction, parameters like extension (synthesizing/ polymerization) temperature and time were optimized (Fig. 6/21).

The rapid and fast optimization of annealing temperatures for each of the ISSR primers was facilitated by Stratagene's RoboCycler Gradient PCR machine. With this machine range of the temperature was selected and used to study the effect of different annealing temperatures on amplification. Thermal cycling conditions that consisted of initial denaturation for 3 min. at 94º C, followed by 35 cycles of 1 min. at 94º C, 1 min. at 48 – 62º C (optimized for different primers), 2 min. at 72º C and 7 min. at 72º C for the final extension resulted in clear and reproducible banding patterns. PCR thermal cycling

conditions have been given in Figure 6/21. Best DNA extension resulted when synthesis temperature was set at 72º C for 7 min.

Out of 100 ISSR primers screened initially, genomic DNA was amplified by 36 ISSR primers, 6 of which did not yield reproducible bands and 3 primers did not show any discernible polymorphism. Among the primers amplified it was observed that the primers amplified by the *Aloe barbadensis* ecotypes (Ecotype 1 to 6, sample no. 1 to 6) and *Aloe perryi* (sample no. 7) didn't show any amplification in the other species (sample no. 8 to 10) i.e. *Aloe saponaria, Aloe humilis* and *Aloe zebrina*. Hence the total number of primers amplified by sample number 1 to 10 was 27. 13 primers were amplified by *Aloe barbadensis*' 6 ecotypes and *Aloe perryi* while 14 primers were amplified by *Aloe saponaria, Aloe humilis* and *Aloe zebrina*. These 27 primers generated 421 bands in total ranging in a size from 285 – 2597 bp, with an average of 15.59 bands per primer. Of these bands 43 % i. e. 183 in total were polymorphic (Table No. 6/45). With all the 27 primers used to distinguish between 10 samples from 8 locations, % polymorphic bands (PPB) ranged from 12.5 – 100%. Of all the primers, UBC 808, 890 and 893 generated highest (100%) PPB.

After screening the 100 UBC primers the results obtained for amplified primers are shown in Figures 6/22 to 6/33.

A. Agarose gel electrophoretic patterns generated with each of 13 primers with genomic DNAs of leaf samples of six *Aloe barbadensis* ecotypes (Ecotype 1 to Ecotype 6, Sample No. 1 to 6 respectively) and *Aloe perryi* (Sample No. 7) have been given in figures 6/22 to 6/28.

B. Agarose gel electrophoretic patterns generated with each of 14 primers with genomic DNAs of leaf samples of six *Aloe saponaria* (Sample No. 8), *Aloe humilis* (Sample No. 9) and *Aloe zebrina* (Sample No. 10) have been given in figures 6/29 to 6/33.

Table No. 6/45: ISSR primers, annealing temperatures optimized, No of bands scored, No of polymorphic bands scored, band size and percent polymorphism per primer

ISSR rimer	Primer Sequence	Annealing Temperature (^{O} C)	No. of bands scored	No. of Polymorphic bands	Band size range (bp)	% polymorphic bands PPB
UBC 808	AGA GAG AGA GAG AGA GC	54	12	12	1169 - 2032	100.00
UBC 810	GAG AGA GAG AGA GAG AT	51	14	3	362 – 1137	21.42
UBC 812	GAG AGA GAG AGA GAG AA	51	19	4	600 – 1557	21.05
UBC 813	CTC TCT CTC TCT CTC TT	48	22	16	900 – 2255	72.72
UBC 815	CTC TCT CTC TCT CTC TG	52	13	7	783 – 1285	53.85
UBC 817	CAC ACA CAC ACA CAC AA	60	13	10	969 – 1656	76.92
UBC 820	GTG TGT GTG TGT GTG TC	56	15	13	850 – 1631	86.66
UBC 823	TCT CTC TCT CTC TCT CC	53	21	0	615 – 1700	50.00
UBC 829	TGT GTG TGT GTG TGT GC	61	13	8	786 – 1290	61.54
UBC 834	AGA GAG AGA GAG AGA GYT	54	24	4	550 – 1800	44.44
UBC 836	AGA GAG AGA GAG AGA GYA	54	29	11	285 - 2299	37.93
UBC 840	GAG AGA GAG AGA GAG AYT	54	20	3	900 – 1300	15.00
UBC 841	GAG AGA GAG AGA GAG AYC	55	13	8	524 – 2597	61.54
UBC 843	CTC TCT CTC TCT CTC TRA	52	15	8	300 – 699	53.33
UBC 844	CTC TCT CTC TCT CTC TRC	52	9	3	780 – 900	33.33
UBC 845	CTC TCT CTC TCT CTC TRG	51	18	5	530 – 2069	27.78
UBC 847	CAC ACA CAC ACA CAC ARC	58	15	8	341 – 629	53.33
UBC 862	AGC AGC AGC AGC AGC AGC	62	21	14	462 – 2154	66.66
UBC 864	ATG ATG ATG ATG ATG ATG	48	32	14	856 – 2460	43.75
UBC 868	GAA GAA GAA GAA GAA GAA	50	20	6	430 – 1556	30.00
UBC 884	HBH AGA GAG AGA GAG AG	49	5	1	570 – 1190	20.00
UBC 885	BHB GAG AGA GAG AGA GA	51	8	1	714 – 1288	12.50
UBC 886	VDV CTC TCT CTC TCT CT	56	15	5	300 – 1126	33.33
UBC 887	DVD TCT CTC TCT CTC TC	51	2	0	1060 - 1100	50.00
UBC 888	BDB CAC ACA CAC ACA CA	58	22	8	415 – 1342	36.36
UBC 890	VHV GTG TGT GTG TGT GT	61	5	5	1204 - 2200	100.00
UBC 893	NNN NNN NNN NNN NNN	45	6	6	787 – 1192	100.00

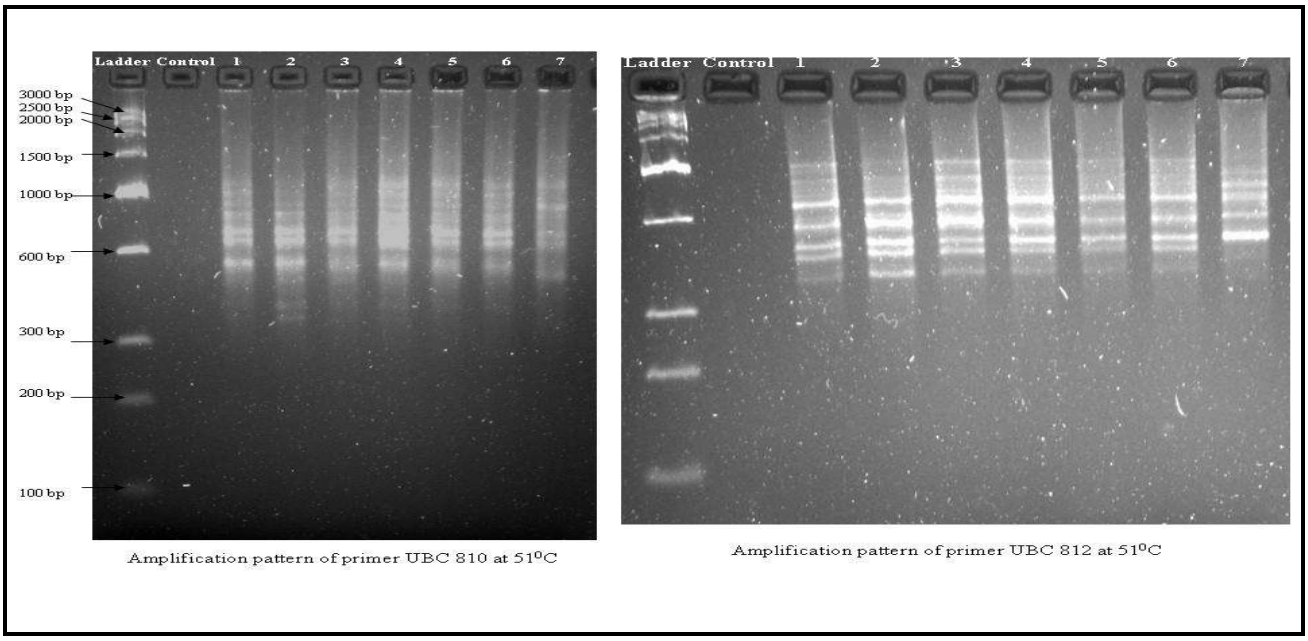


Figure 6/22: Amplification pattern of 7 *Aloe* plants by Agarose gel electrophoresis using the primer 810 and 812 at annealing temperature 51º C. (Sample No. 1 to 6- *Aloe barbadensis* ecotypes and sample no. 7- A*loe perryi*).

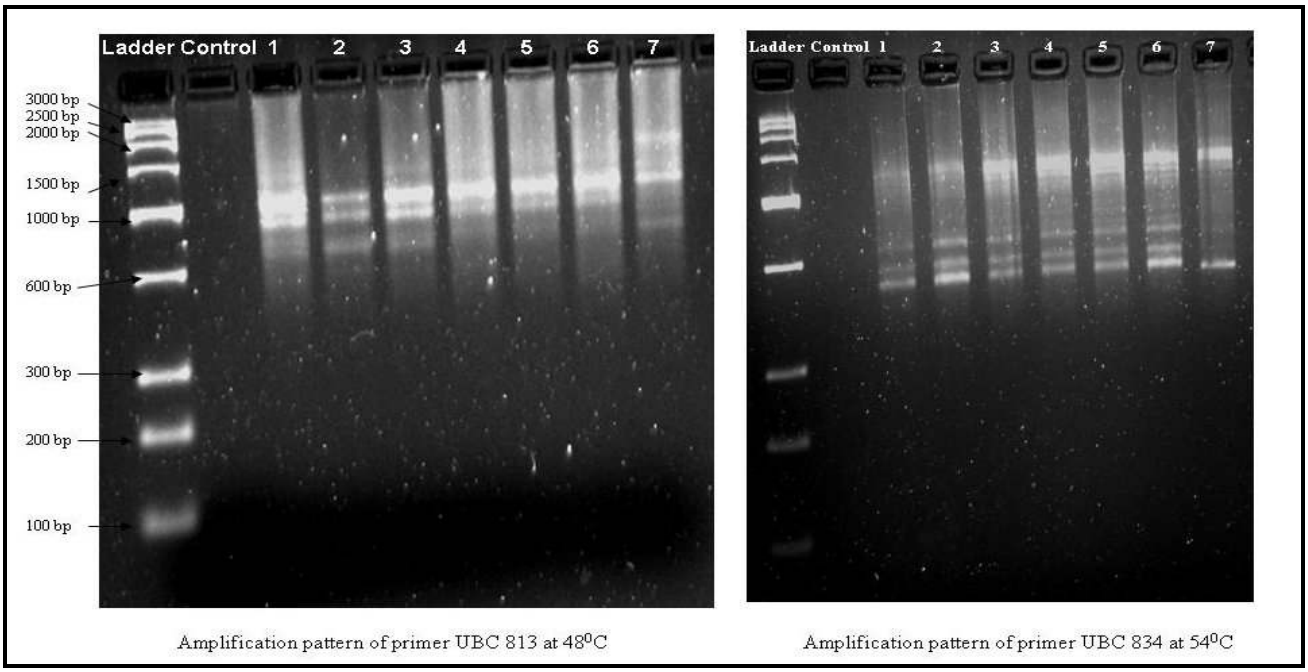


Figure 6/23: Amplification pattern of 7 *Aloe* plants by Agarose gel electrophoresis using the primer 813 and 834 at annealing temperatures 48º C and 54º C respectively. (Sample No. 1 to 6- *Aloe barbadensis* ecotypes and sample no. 7- A*loe perryi*).

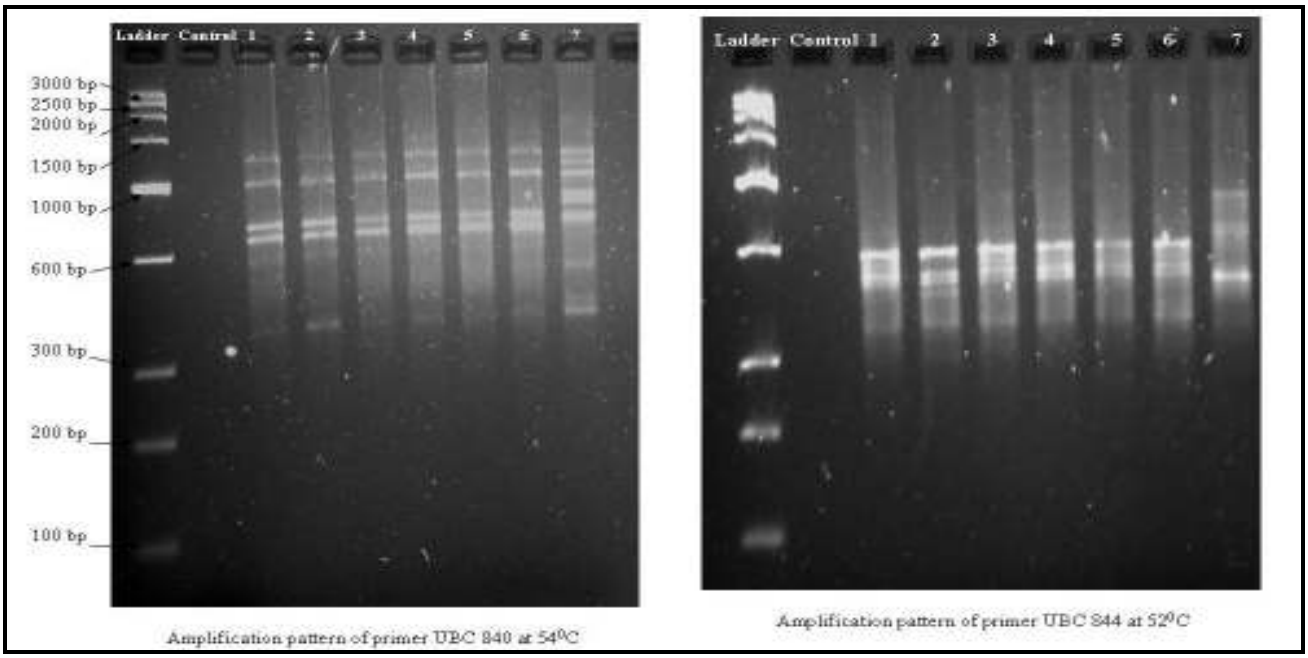


Figure 6/24: Amplification pattern of 7 *Aloe* plants by Agarose gel electrophoresis using the primer 840 and 844 at annealing temperatures 54º C and 52º C respectively. (Sample No. 1 to 6- *Aloe barbadensis* ecotypes and sample no. 7- A*loe perryi*).

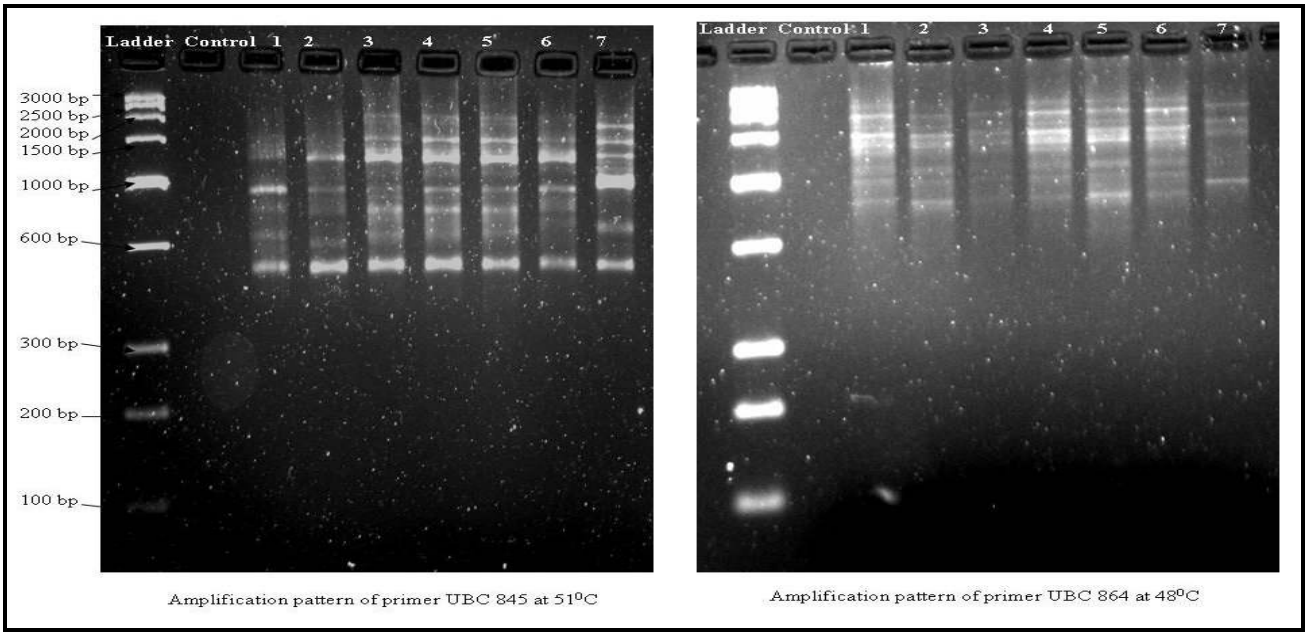


Figure 6/25: Amplification pattern of 7 *Aloe* plants by Agarose gel electrophoresis using the primer 845 and 864 at annealing temperatures 51º C and 48º C respectively. (Sample No. 1 to 6- *Aloe barbadensis* ecotypes and sample no. 7- A*loe perryi*).

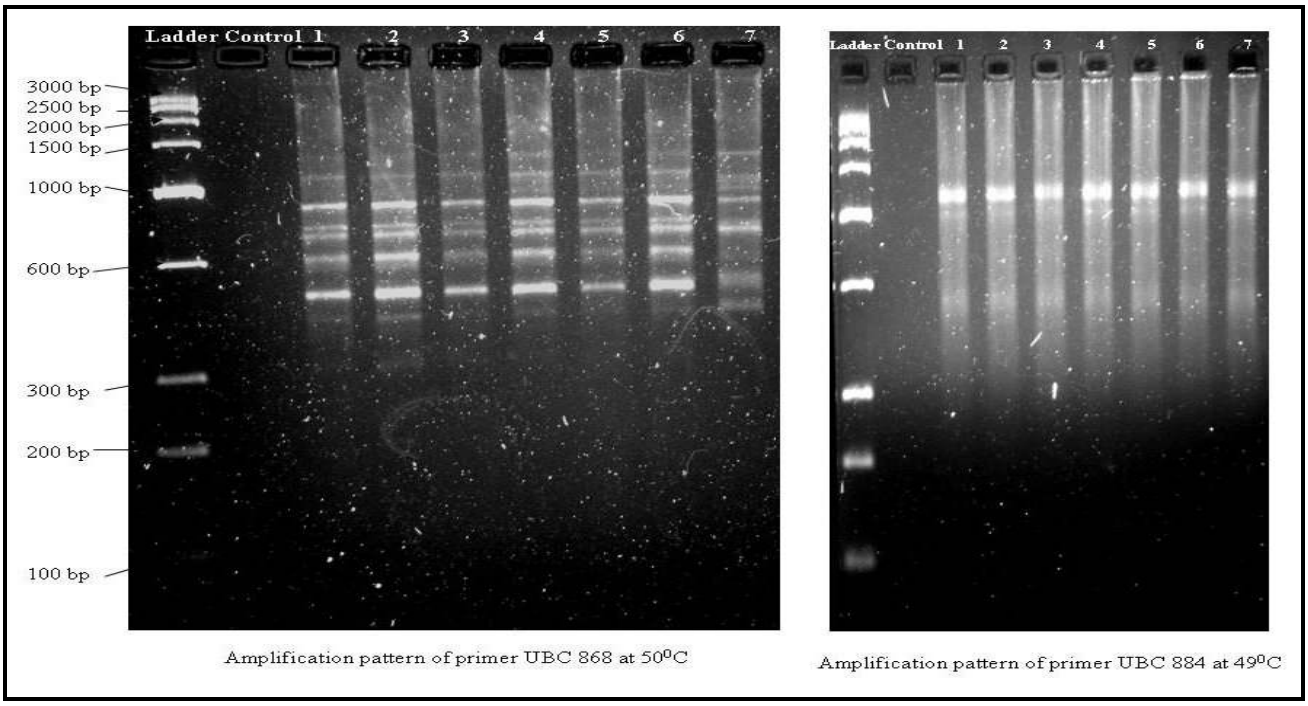


Figure 6/26: Amplification pattern of 7 *Aloe* plants by Agarose gel electrophoresis using the primer 868 and 884 at annealing temperatures 50º C and 49º C respectively. (Sample No. 1 to 6- *Aloe barbadensis* ecotypes and sample no. 7- A*loe perryi*).

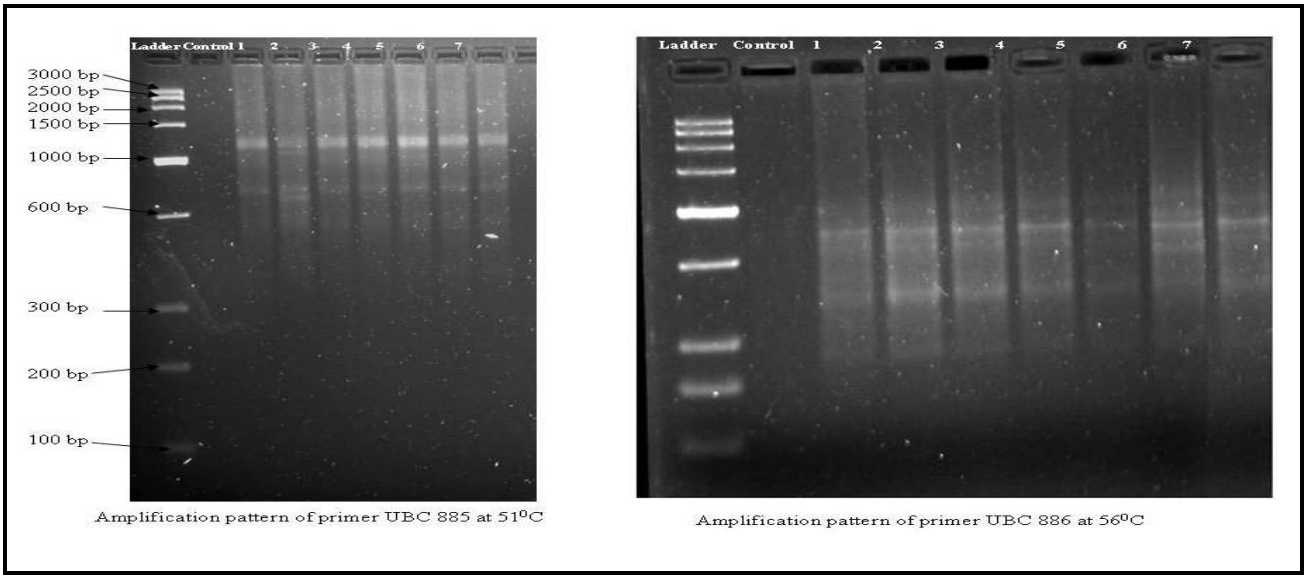


Figure 6/27: Amplification pattern of 7 *Aloe* plants by Agarose gel electrophoresis using the primer 885 and 886 at annealing temperatures 51º C and 56º C respectively. (Sample No. 1 to 6- *Aloe barbadensis* ecotypes and sample no. 7- A*loe perryi*).

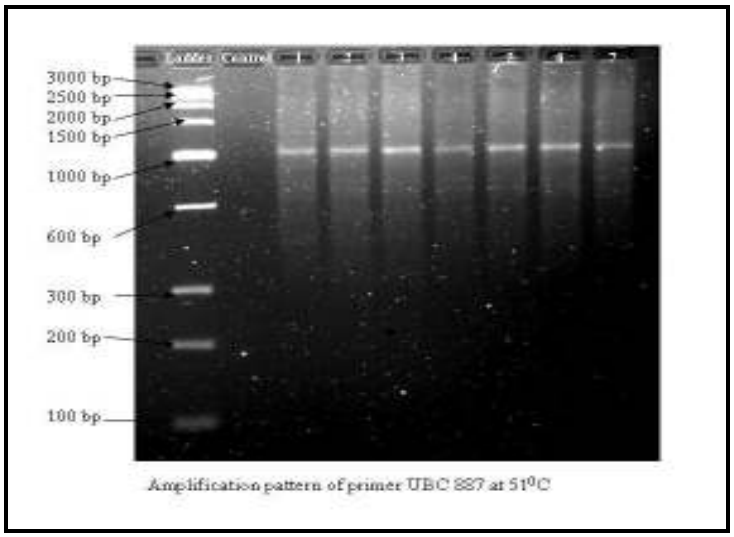


Figure 6/28: Amplification pattern of 7 *Aloe* plants by Agarose gel electrophoresis using the primer 887 at annealing temperature 51° C. (Sample No. 1 to 6- *Aloe barbadensis* ecotypes and sample 7- A*loe perryi*).

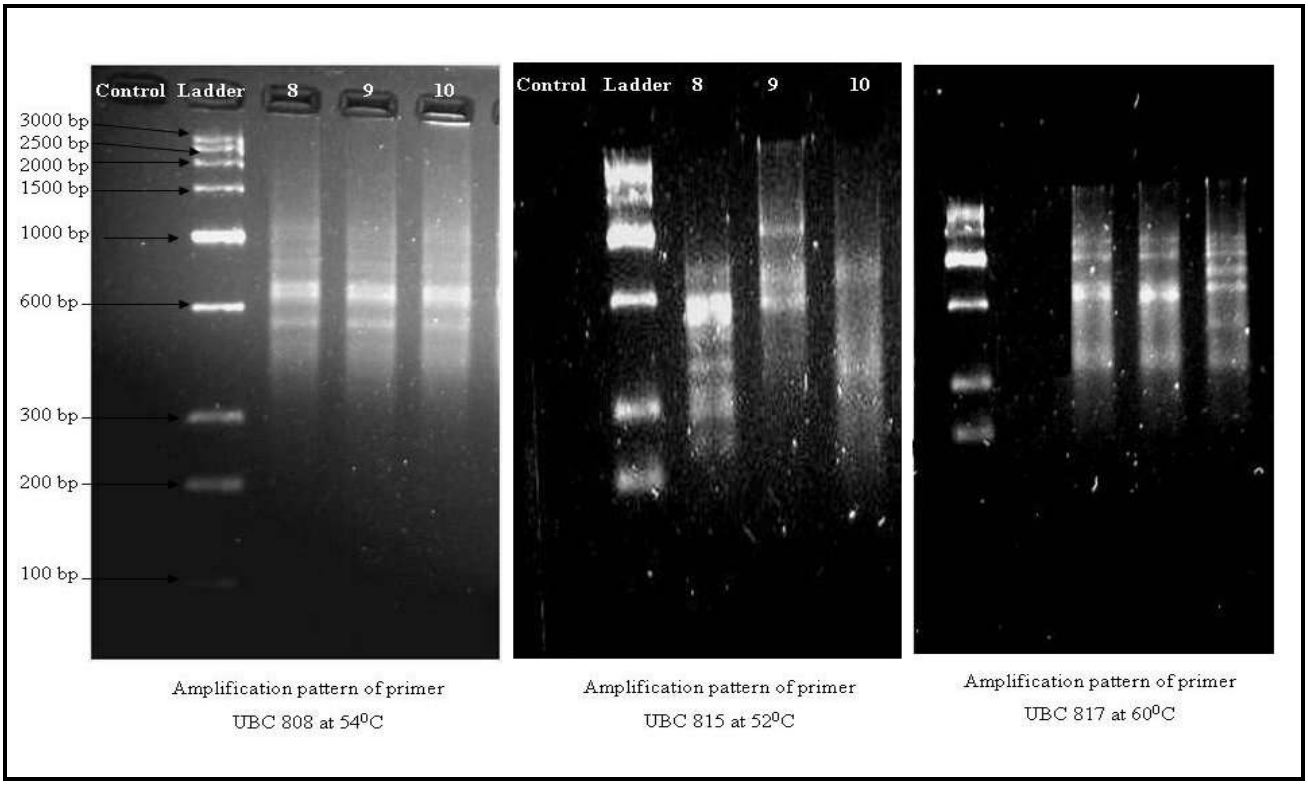


Figure 6/29: Amplification pattern of 3 *Aloe* species by Agarose gel electrophoresis using the primer 808, 815 and 817 at annealing temperatures 54° C, 52° C and 60° C respectively. (Sample No. 8, 9 and 10 - *Aloe saponaria, Aloe humilis* and A*loe zebrina* respectively).

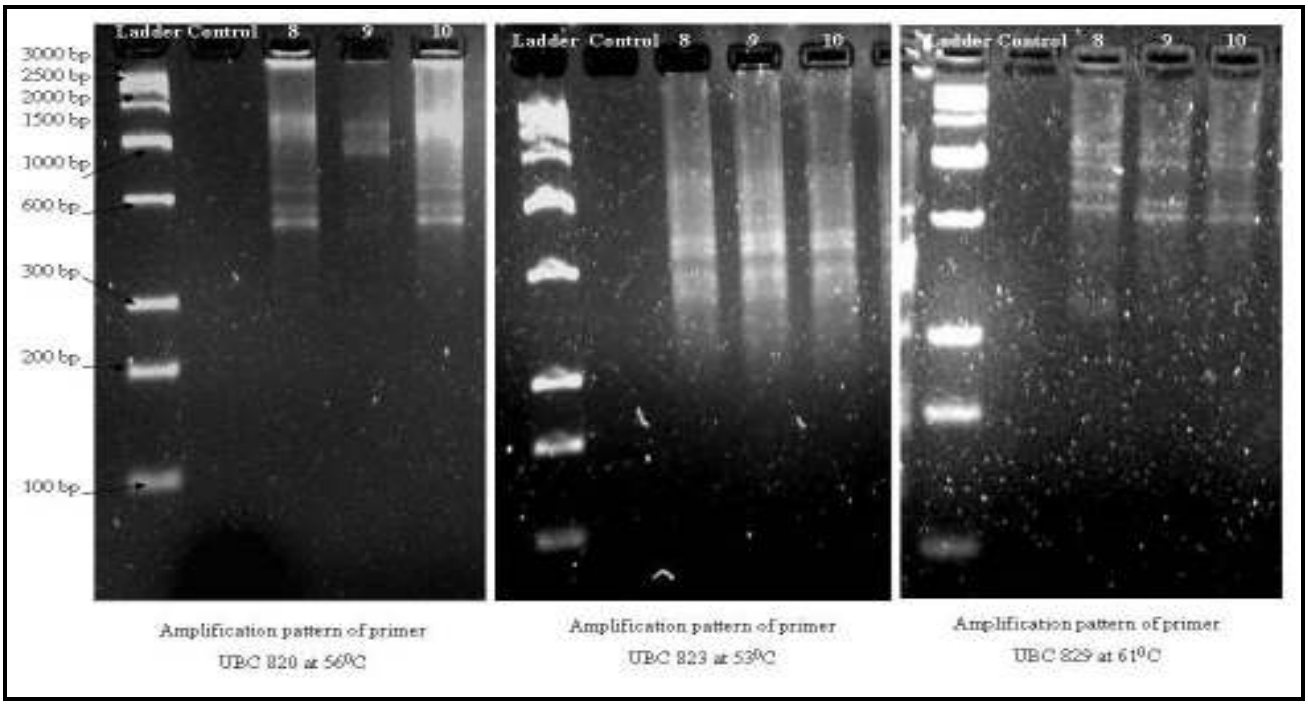


Figure 6/30: Amplification pattern of 3 *Aloe* species by Agarose gel electrophoresis using the primer 820, 823 and 829 at annealing temperatures 56º C, 53º C and 61º C respectively. (Sample No. 8, 9 and 10 - *Aloe saponaria, Aloe humilis* and A*loe zebrina* respectively).

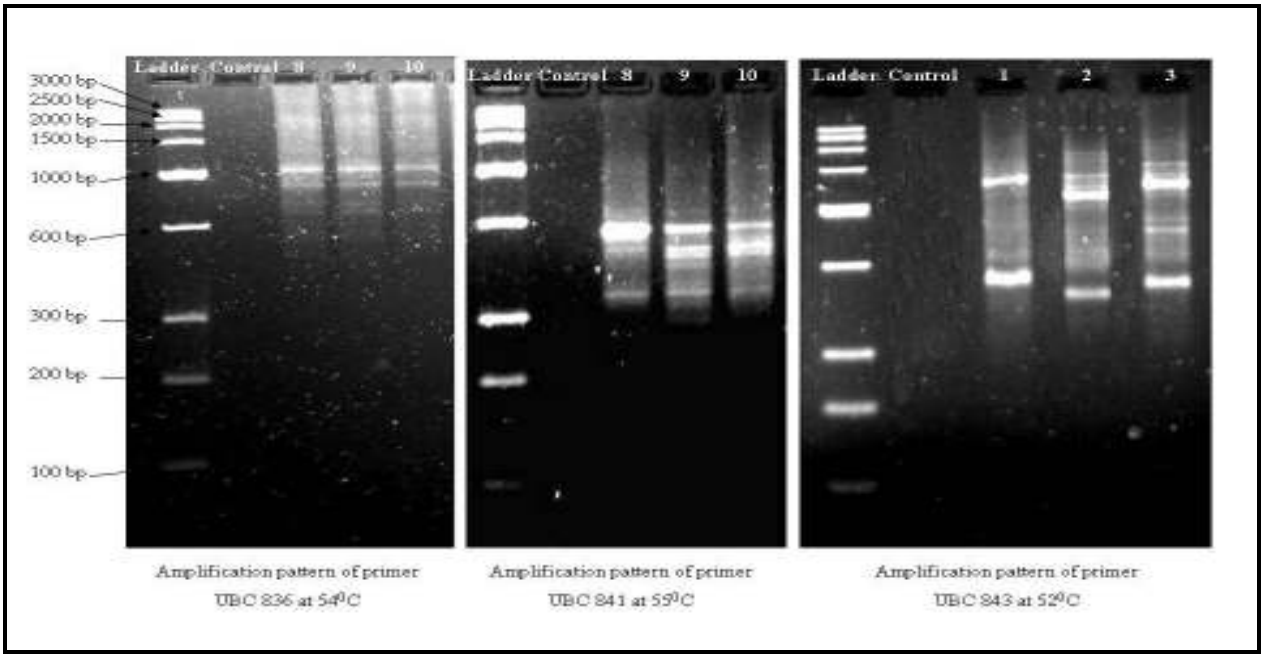


Figure 6/31: Amplification pattern of 3 *Aloe* species by Agarose gel electrophoresis using the primer 836, 841 and 843 at annealing temperatures 54º C, 55º C and 52º C respectively. (Sample No. 8, 9 and 10 - *Aloe saponaria, Aloe humilis* and A*loe zebrina* respectively).

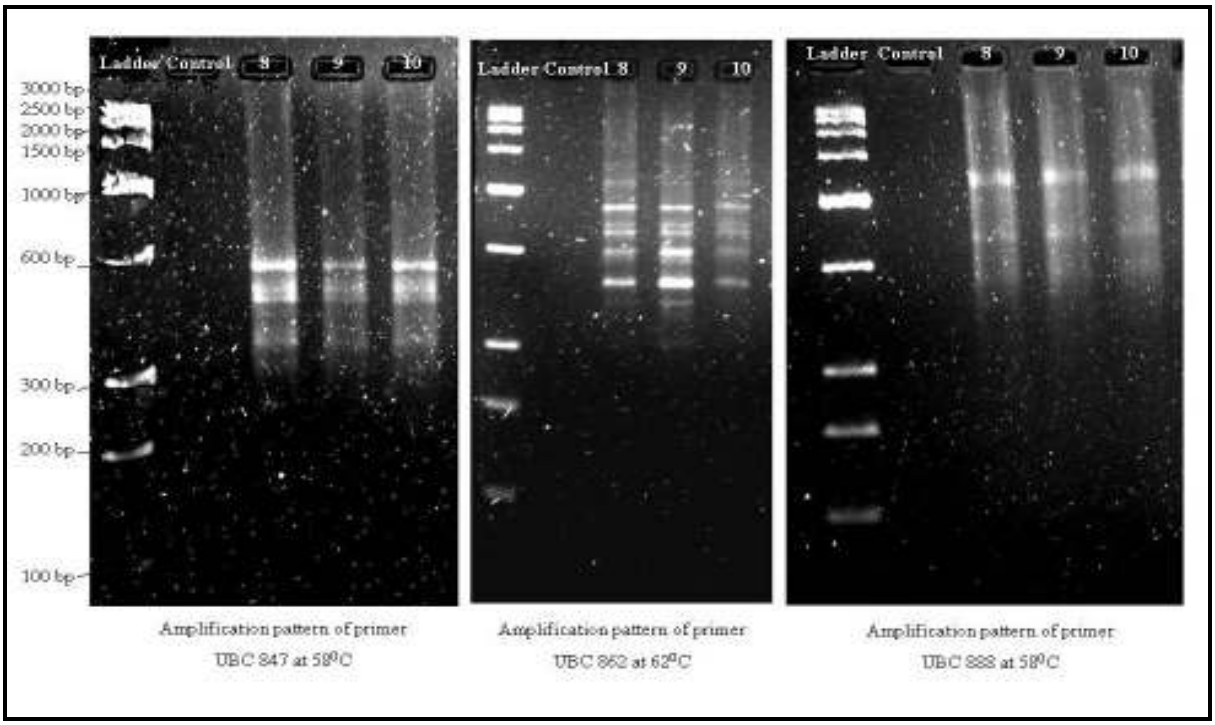


Figure 6/32: Amplification pattern of 3 *Aloe* species by Agarose gel electrophoresis using the primer 847, 862 and 888 at annealing temperatures 58º C, 62º C and 58º C respectively. (Sample No. 8, 9 and 10 - *Aloe saponaria, Aloe humilis* and A*loe zebrina* respectively).

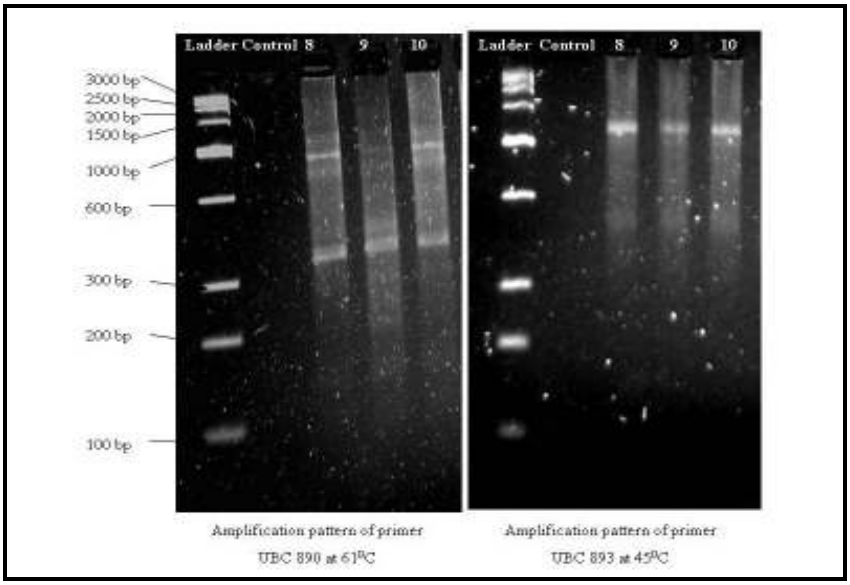


Figure 6/33: Amplification pattern of 3 *Aloe* species by Agarose gel electrophoresis using the primer 890 and 893 at annealing temperatures 61º C and 45º C respectively. (Sample No. 8, 9 and 10 - *Aloe saponaria, Aloe humilis* and A*loe zebrina* respectively).

After assessing the 27 ISSR primers for the 10 *Aloe* plants from the 8 different locations (Chapter No. 2, Table No. 2/7) dendrogram was constructed using Nei's (1978) genetic distance coefficient (Fig. 6/34).

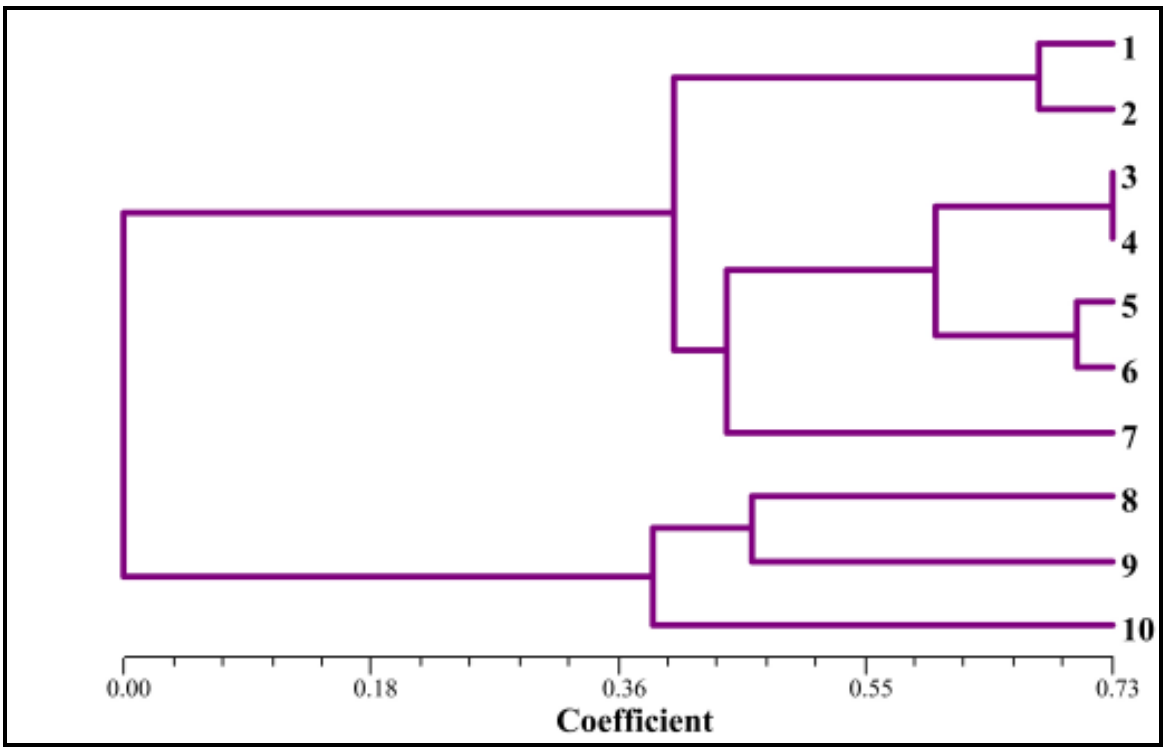


Figure 6/34: UPGMA Dendrogram based on Nei's genetic distance coefficient from ISSR data of 10 *Aloe* plants collected from Uttaranchal region of India. (1. Ecotype1; 2. Ecotype 2; 3. Ecotype 3; 4. Ecotype 4; 5. Ecotype 5; 6. Ecotype 6; 7. *Aloe perryi; 8. Aloe saponaria; 9. Aloe humilis; 10. Aloe zebrina)*

Nei's genetic distance coefficient in the given dendrogram ranged between 0.00 – 0.73. The dendrogram showed the two major groups Ist group consist of the plants of ecotypes of *Aloe barbadensis* (Mill.) Ecotype 1 (Pithoragarh, 5397' MSL), Ecotype 2 (Lamgarha, 5375' MSL), Ecotype 3 (Champawat, 5414' MSL), Ecotype 4 (Betalghat, 1257' MSL), Ecotype 5 (Dehradun, 1105' MSL), Ecotype 6 (Haldwani, 1082' MSL). The IInd group was clearly of the different species of Aloe irrespective of the locations.

This group comprised of *Aloe perryi* (Haldwani, 1082' MSL), *Aloe saponaria* (Dehradun, 1105' MSL), *Aloe humilis* (Joshimath, 7983' MSL) and *Aloe zebrina* (T. Garhwal, 3786' MSL). Even though the trend of clustering was on the basis of the species differentiation, the Sample 7 i.e. *Aloe perryi* showed more similarity towards the group 1 which was of *Aloe barbadensis* ecotypes. The geographical details of the location are already given in Chapter No. 2, Table No. 2/7.

The similarity was found to be the maximum (73%) in the Ecotypes 3 and 4. minimum similarity was found in the different species of *Aloe.* The difference was observed amiong the Aloe barbadensis ecotypes and the other species of *Aloe*. It can be clearly depicted from the UPGMA dendrogram that the two groups were separated on the basis of the genetic and species differentiation irrespective of the agro climatic differentiation and altitude

6.4 DISCUSSION:

Genus *Aloe* is a succulent plant and contains large amount of water (mucilage), phenolics and yellow latex. The major components of latex are anthraquinones which are produced constitutively as secondary metabolites. In addition to these polyphenolics the genus *Aloe* often contain high concentrations of fructan oligo- and large amount of polysaccharides. The presence of polyphenolics together with the high polysaccharide content makes the isolation of high-quality intact nucleic acids problematic (Katia *et al*., 2003). These containments in *Aloe* plants co-precipitates with DNA and makes DNA extraction, purification, and amplification by using PCR difficult (Poresbski *et al*., 1997). In addition to complicating extraction residual polyphenolics and polysaccharides interfere in enzymatic reactions such as PCR and endonuclease restriction digestion (Bryant, 1997).

Some authors advocate the use of hydrolytic enzymes (Rether *et al*., 1993) or of ion-exchange resins (Guillemaut and Marechal-Drouard, 1992; Maréchal - Drouard and Guillemaut, 1995) to remove polysaccharides from nucleic acid solutions. However, in more generally used methods, the key feature in avoiding co-precipitation of polysaccharides with the DNA is to keep one of them in solution and precipitate the

other. Dellaporta *et al*., (1983) did not attempt to avoid co-precipitation of nucleic acids with polysaccharides at the first precipitation step in their method for preparing DNA from 'difficult' species. The most widely used method for avoiding co-precipitation of polysaccharides and DNA is to keep the polysaccharides in solution at the DNA precipitation step, usually by using Cetyl-Trimethyl Ammonium Bromide (CTAB) as a selective precipitant of nucleic acids (Barnwell *et al.,* 1998).

Another limiting factor was liquid nitrogen as Defence Agricultural Research Laboratory (DARL); Pithoragarh is located in the remote areas where availability of liquid nitrogen and its transportation has its own issue to be dealt with. Hence method developed by Sharma et al. (2003) which describes the isolation of DNA without liquid nitrogen was used with some modifications. The concentration of CTAB was increased after optimization as it acts as a detergent and helps in removing the polysaccharides and DNA binding proteins. Concentration of PVP was also increased to deal with the high amount of polyphenolics. Chilled ethanol was used instead of the one kept at room temperature. The weight of leaves used for extraction was 5 gm on contrary to the 1 gm mentioned in the protocol. Thus a good quality of DNA was obtained with the initial fixing solution, which was used for the further ISSR analysis by PCR.

Standardization of PCR was critical as many difficulties were faced like reproducibility, low band intensity. Hence all the parameters were tested carefully from the concentration of the DNA to final annealing temperature. Initially the PCR conditions were optimized for different parameters like concentration of template DNA, $MgCl_2$, Taq polymerase, primers and dNTPs. Thermal cycling conditions like initial denaturation, binding of primers, elongation and final extension were standardized. The final conditions are shown in Fig. 6/21.

DNA based molecular markers offer a way of studying the genome directly without being affected by environmental or phenotypic (epigenetic) variation. Inter-Simple Sequence Repeat (ISSR) amplification have been used for determining genetic diversity of plants for which no prior sequence information is available (Williams *et al*. 1990; Zietkiewicz *et al*. 1994). The reference work shows that ISSR is proved to be the powerful tool for the intra specific and inter specific assessment of the plants (Table No. 6/43). In the present study morphological variation was observed among the 10 plants of

the genus *Aloe* which have already been discussed in Chapter No.2. Hence an attempt was made to assess diversity between them using ISSR molecular markers. Number of amplified primers, total number of bands, percent polymorphic bands and reproducibility reflects the reliability of the ISSR primers for the genetic assessment of the genus *Aloe.*

In the present study it was also found that primers amplified by *Aloe saponaria, Aloe humilis* and *Aloe zebrina* were completely different from the primers amplified by ecotypes of *Aloe barbadensis* and *Aloe perryi.* This may be because of the absence of target microsattelites in the genome of these species or the ecotypes and *Aloe perryi* which could not be amplified by the primers. The distance between the macro satellites is also important because if distance is too much the ISSR region could not be amplified by PCR. Role of Taq polymerase is also considerable because practically it can amplify fragment of about less than 3 kb size. Higher fragments can not be amplified. As reproducibility of the PCR protocol was checked, there is no possibility of failure of PCR reaction.

Thus the ISSR polymorphism has been used to determine the population structure and genetic relatedness of genus *Aloe* from eight different locations, mainly focusing on Uttaranchal (Kumaon and Garhwal) region of India. The UPGMA dendrogram revealed two clusters corresponding to the species differentiation (Fig. 6.14). The Ecotype 3 (5414' MSL) and 4 (1257' MSL) of the *Aloe barbadensis* were found to be genetically very similar irrespective of the large altitudinal differences. While the data showed much more morphological differences among these plants (Chapter No.2). The Ecotype 1 and Ecotype 2 have the genetic similarity and so do the Ecotype 5 and Ecotype 6. *Aloe perryi* showed the much similarity with the ecotypes. The remaining three species *Aloe saponaria, Aloe humilis* and *Aloe zebrina* showed a cluster depending upon the genetic differences among which *Aloe saponaria* and *Aloe humilis* showed some similarity.

There have been no prior studies conducted on the genetic diversity estimates of species *Aloe perryi, Aloe saponaria, Aloe humilis* and *Aloe zebrina* using molecular techniques. It has been reported for the first time. Assessment of the genetic variation in *Aloe* populations is an important step in selection of appropriate germplasm for plantations. It also provides information on the conservation strategies that need to be used for maintaining this variation.

Conservation implications: -

The overall genetic diversity of a genus *Aloe* has great implications for its long-term survival and evolution (Frankel *et al*., 1995; Avise and Hamrick, 1996). Therefore, knowledge of the levels and patterns of genetic diversity is important for designing conservation strategies for threatened and endangered species (Hamrick, 1983; Hamrick and Godt, 1989; Francisco-Ortega *et al*., 2000). The present study, together with the results of earlier investigations (Chapters 2 to 5) has conservation implications for the genus *Aloe*. However, most of the genetic diversity is observed between populations, implying that more populations are needed to maintain genetic diversity of the species when implementing ex situ conservation strategy.

Furthermore, high degree of genetic differentiation among populations suggests that the transfer of germplasm between populations should be avoided to ensure that the genetic material is adapted to local site conditions (Amos and Balmford, 2001). Population, characterized by small population size, severe human impact, and a particularly low level of genetic variation, should be given the highest priority for any ex situ conservation plan. The Himalayan ecosystem have largely been disturbed or destroyed by human activities at an alarming rate. Therefore, in the long term, the most suitable strategy for the conservation should be implemented for the conservation of *Aloe* in Uttaranchal region. A further management measure should aim at increasing the number of plants in small populations. Since Aloes can be spread vegetatively via suckers in suitable habitats, a strategy seems feasible involving propagation via suckers segments and tissue culture techniques, followed by cultivation in garden plots, and subsequent reintroduction into their original wild habitat. Some efforts have already been initiated in this direction. It must be noted, however, that ISSR markers are not expressed phenotypically, therefore probably having no direct relevance to natural selection and adaptation. Nevertheless, like other supposedly ‘neutral markers’, ISSRs may serve as an indicator of local adaptation, e.g., via linkage with other functional gene regions.

6.5 CONCLISION:

DNA extraction protocol by Sharma *et al.,* (2003) i.e. extraction of DNA without liquid nitrogen was found to be efficient as good quality and quantities of DNAs were yielded from fresh *Aloe* leaves.

PCR conditions standardized were the optimum for reproducible and clear amplifications. ISSR successfully identified the variations among the ecotypes of Aloe barbadensis and other species.

From the UPGMA dendrogram it can be depicted that the relative morphological differences in the genus *Aloe* collected from the Uttaranchal region has the basis of the genetic diversity. The clusters of the dendrogram proved the genetic variability among the *Aloe barbadensis* ecotypes (1 to 6) and the species *Aloe perryi, Aloe saponaria, Aloe humilis* and *Aloe zebrina.*

Thus the working and efficient strategies can be implemented for the conservation of the genus *Aloe* and selection of the germplasm in Uttaranchal (Kumaon and Garhwal) region of India.

6.6 APPLICATION:

The feasibility of using the polymerase chain reaction to identify *Aloe* depends on establishing specific primers that detect its DNA and extraction protocols that reproducibly recover the DNA. The proportion of DNA which survives processing and the successful recovery of DNA and elimination of PCR inhibitory substances from DNA extracts will dictate the sensitivity of the PCR reaction necessary to detect *Aloe* DNA and extracts of *Aloe* in industrial products. The sensitivity of the PCR reaction that is required defines the type of PCR-product detection-system that is necessary and the type of containment required to eliminate spurious results and possible contamination (US patent 6001572).

A study of the genetic diversity is required to understand the future prospects of the species. A large diversity will not only give a better adaptability to the changes in the

environment but will also give a wealth of genetic material for genetic manipulations of the species. Estimates of genetic relationship are important in designing crop improvement programs. Information on genetic diversity is also valued for the management of germplasm and for evolving conservation strategies. At the genus level genetic diversity helps to distinguish morphologically similar species. Molecular markers can also be used to study the genetic diversity in a species.

Considering the high genetic differentiation among the wild populations of genus *Aloe*, conservation of only a few populations may not adequately protect the genetic variations within the species in the Himalayan region. At present, the rate of propagation of *Aloe* plant is in budding stage and far less than the rate of its exploitation. This species, or at least a large part of its genetic diversity, may be lost in near future, owing to its importance and consequent exploitations as a medicinal plant, if appropriate conservation measures are not adopted. Since no single, or even a few plants, will represent the whole genetic variability, there appears to be a need to maintain sufficiently large populations in natural habitats to conserve genetic diversity in *Aloe* and avoid genetic erosion.

Thus ISSR markers can be employed to study the genetic variation in *Aloe* ecotypes and species.

Chapter 7

Summary and Future directions

The process of scientific discovery is, in effect,

a continual flight from wonder

7.1 SUMMARY AND CONCLUSION:

Today, *Aloe* is used as an ingredient in a myriad of health and cosmetic products. It is included in large range of skin moisturizers, face and hand creams, cleansers, soaps, suntan lotions, shampoos and hair tonics, shaving preparations, bath aids, makeup and fragrance preparations, and baby lotions and wipes. Topical preparations of Aloe particularly *Aloe barbadensis* are used to treat frostbite. The cosmetic industry has made claims of its rejuvenating, moisturizing, and healing properties.

The aloe plant, being a cactus plant, contains 99 and 99.5 per cent water, with an average pH of 4.5. The remaining solid material contains over 246 different ingredients including vitamins, minerals, enzymes, sugars, anthraquinones or phenolic compounds, lignin, saponins, sterols, amino acids and salicylic acid.

To utilize the available resources of genus *Aloe* in Uttaranchal, the present study "Conservation and Assessment of Physico-chemical and Molecular diversity of the genetic resources of the genus *Aloe* occurring in Uttaranchal, India (Kumaon and Garhwal region)"was undertaken with the following objectives

1. To screen Kumaon and Garhwal region of Uttaranchal for available resource of Aloe.
2. To authenticate, conserve and multiply with development of agro-technology.
3. To evaluate them for their biochemical potentials.
4. To identify them by using molecular marker technique like ISSR.

In the present study Uttaranchal region was screened for the genus *Aloe* where 5 species of *Aloe* were collected from 8 different locations at different altitudes ranging in between 1082' to 7983' MSL in Kumaon and Garhwal regions. *Aloe barbadensis* ecotypes were found in Kumaon region only. While other species were found in Garhwal region. *Aloe humilis* was found at the highest altitude at Joshimath (7983' MSL) from Gharwal region.

The present study indicates that even though Uttaranchal is not the natural habitat for the occurrence of the genus *Aloe,* it was found distributed all over the Kumaon and Garhwal

region. This means these species may have been introduced by humans in this region in the past and in the course of time they have adopted to the climate.

Agro-technology developed in accordance with the norms of WHO for cultivation of medicinal plants proved to be effective as all the species could be gown and maintained successfully in Uttaranchal at Pithoragarh (5397' MSL) and at high altitude (Joshimath, 9000' MSL). The cultivation in glass houses showed improvement in morphological parameters like length, width and number of leaves etc. These parameters can be correlated to proper nourishment of the plants. Rather higher yield was observed at these sites than the yield at the temperate region, which are the natural habitat of the *Aloe.* Covered conditions i.e. glass and polyhouses gave more yield (2400 q/ha) as compared to open fields i.e. 1750 q/ha. In ideal conditions i.e. in natural habitat of the *Aloe barbadensis,* 525 quintals /hectare yield is reported. These cultivation practices can open up the new avenues for commercial cultivation of Aloe in this region.

In the nutraceutical assessment of all the cultivated plants, it was found that the variations in the biochemical parameters were influenced by agro-climate, soil type and its nutrients, altitude and species specific differences. It was observed that all the plants showed increase or decrease in the biochemical constituents relative to their controls which were collected from wild. Ecotype 2 collected from the slopes and water deficient soil showed high moisture content due to its retention. Moisture was found relatively high in its control as compared to other ecotypes. Other species of Aloe collected from the plains i.e. *Aloe saponaria* and *Aloe perryi* showed the maximum moisture content.

All the biochemical parameters like total carbohydrates, proteins, fats, total minerals were found to be increased in the cultivated species as compared to their respective controls. Chlorophyll a, b and total chlorophyll were estimated from fresh leaf sample while other estimations were carried out by using dried leaf powder. This increase was found in all the ecotypes and species under cultivation. In case of phenolics, tannins and proline the concentration was found to be decreased as the cultivated plants were maintained and provided with better irrigation and fertilizers. Ecotype 1 and *Aloe perryi* were found to be the best in biochemical composition among the ecotypes and species respectively.

11 minerals were quantitatively determined from *Aloe barbadensis* ecotypes and other species. It was observed that Ecotypes 4 contains most of the minerals like Na, K, P, Cu and Li in higher concentration than other ecotypes. *Aloe perryi* was found to be the best source of minerals as its content of these minerals were maximum of all the species. In all the plants Co was absent or beyond the detectable limit. Thus it can be concluded that low biochemical composition estimated in control plants which were collected from wild can be improved by these practices, fertilizers and irrigation.

Aloin content was tested for all the species under cultivation from whole leaf powder, fresh latex and lyophilized latex. It was found that aloin content in the all three samples was maximum in *Aloe perryi* as compared to all the species. Among the *A. barbadensis* ecotypes, Ecotypes 1 was found to be the best source of aloin. Thus the plants rich in particular component can be used and cultivated for trading, cottage industry set ups.

High altitude cultivation studies were carried out Auli (9000' MSL) where morphological parameters, biochemicals, minerals and aloin content were studied. Ecotype 1 was cultivated at this location and its wild source was used as a control. It was observed during the study that moisture content was reduced even though the standard agro technology was followed. All the biochemical showed decrease or least significant increase in these plants. Proline was found to be increased as survival molecule. All the secondary metabolites used for the survival in harsh climate were found to be increased. Ecotype 1 at this site can be cultivated for the production of secondary metabolites which constitutes the major portion of the herbal drug and other synthetic products. Cultivation at the high altitude (Auli) of Ecotype 1 was found to be satisfactory but due to high acidic and very low nutrient content of soil along with high degree of slopes, heavy rains and frost conditions the nutraceutical constituents showed reduction.

Prebiotic and synbiotic potential of the *Aloe barbadensis* ecotypes were evaluated by using the gut microorganisms i.e. probiotics. Other species of *Aloe* were not used for the study because they are not recommended for the edible purposes. Normal growth pattern was observed for all the probiotics by using gel of ecotypes, as a source of carbon in media. Ecotype 1 showed best synbiotic potential for all the probiotics- *Lactobacillus plantarum* (2084), La*ctobacillus lactis (*2589*), Lactobacillus acidophilus (*2285),

*Lactobacillus delbrueckii (*2292) *Bifidobacterium bifidum.* Hence, Ecotypes 1 can be used as a rich source of prebiotic for the industrial cultivation of above mentioned microorganisms for various food preparations and medicines.

In antimicrobial activity assessment, water, methanol and hexane extracts of whole leaf powder were used and it was found that methanol extracts were best as it showed minimum MIC and maximum zone of inhibition against all the food pathogens viz *E. coli, S. typhii, S. aureus* and *B. subtilis*. Here also Ecotype 1 showed the maximum zone of inhibition as it may contain higher amount of anti-microbial compounds extracted by methanol. The antimicrobial compounds can be isolated and produced on commercial scale by using these plants and can be used in food preparations also.

DNA extraction protocol yielded good quality and quantities of DNAs from the leaves of all the *Aloe barbadensis* ecotypes and other species. ISSR markers successfully identified the variations among *A. barbadensis* ecotypes and with the species. It was found that the primers amplified by the ecotypes of *Aloe barbadensis* and *Aloe perryi* were different from the primers amplified by other species due to variation in the genetic make up and the microsattelite regions. Clustering in dendrogram based on ISSR variation grouped 10 plants in three groups and indicated correlation between geographical position and genetic differentiation.

Future Directions: -

In recent times, the use of *Aloe* has reached a level of concern, since some herbalists and organizations are promoting oral consumption of it as a prophylaxis and treatment to alleviate a variety of unrelated systemic conditions. Promoters offer a number of whole leaf formulations that are widely available for consumption at various concentrations in liquid, powder, and tablet form.

Aloe is to be uniquely valued for its content of active biochemicals. These are substances which interact with living cells in very small amounts, producing significant changes to cell metabolism and cell behaviour. These substances interact with specialized receptors on the cell surface to produce these changes, in a way which might be described as “pharmacological.” Yet the substances within *Aloe* which are doing this are entirely non-toxic natural substances and they leave no residues in the tissues. *Aloe* itself is not a

food, but pharmacologically active substances of the same general type are well distributed among unprocessed whole foods. None of our foods contain the same range of active cell-stimulating constituents as *Aloe* in the same proportions, but the principles involved in using *Aloe* are much the same as when one uses some foods as medicines.

During the present study at DARL, Pithoragarh some of the products having *Aloe* gel as one of the major ingredients were prepared. Those are given in Fig. 7/36 and 7/37.

There is a vast scope for the development of the new products and food supplements. Nutraceutical evaluation can lead to development of these products using rich source of particular *Aloe* ecotypes for specific constituents. Aloin like compounds can be extracted for the bulk and crude drug manufacturing.New prebiotics can be isolated, synthesized or tailored form these rich prebiotic source and important antimicrobial compounds can be isolated for the food purposes. By evaluating the elite sources they can be conserved by genetic information.

The present work indicates that the *Aloe* genus can be fully utilized for its medicinal importance and development of the new products like anti-frost bite which was the concern of DRDO for this study so that troops can be benefited

Cultivation practices, nutritional evaluation, prebiotic, antimicrobial and genetic diversity studies can lead understanding to mass and commercial scale cultivation, small scale processing and production industries in the Kumaon and Garhwal region. These activities will improve the socioeconomic and nutritional status of the people in Uttaranchal.

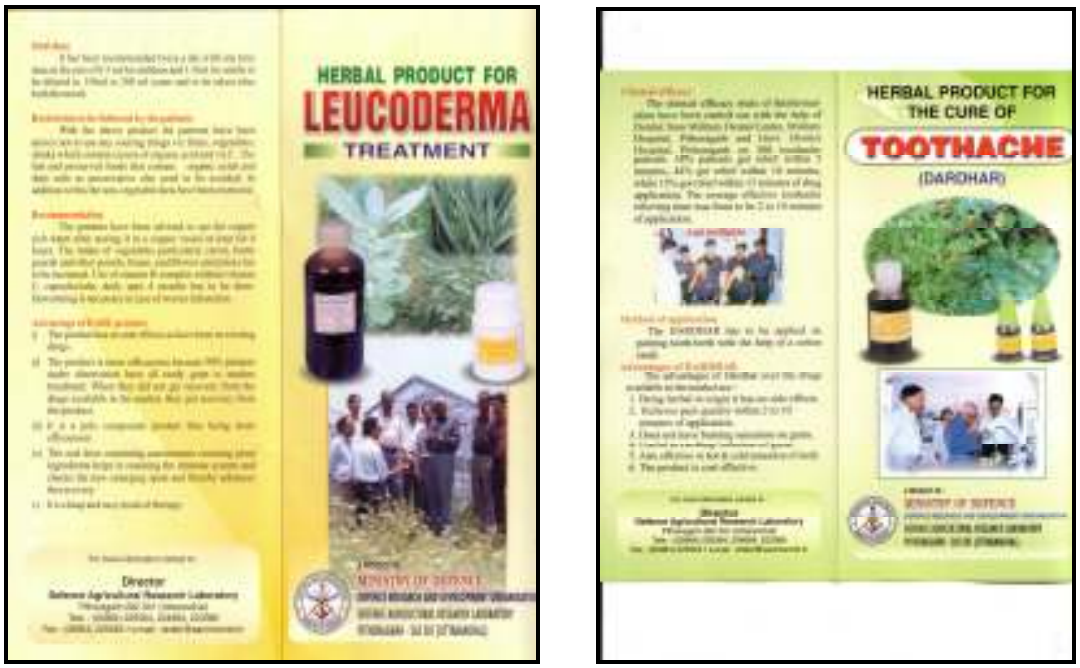


Figure 7/35: Antileucoderma and Anti toothache medicine developed at DARL

Figure 7/36: Anti eczema medicine and honey developed at DARL

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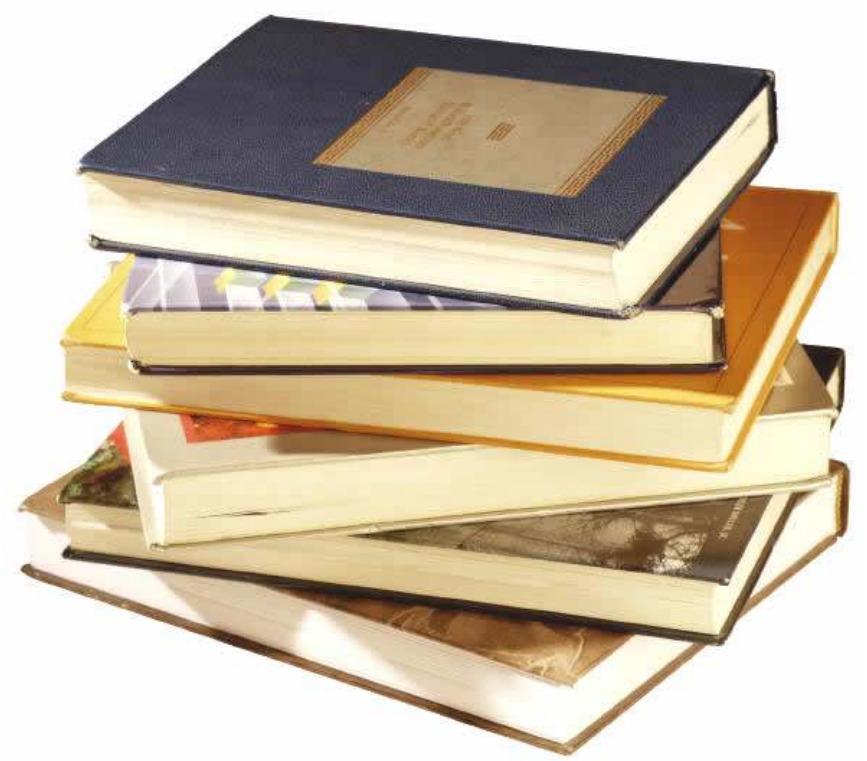

Everything has been said before, but since nobody listens
we have to keep going back and beginning all over again

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AUTHOR'S PAPERS

1. Paper presented at International Conference at GKVK, Bangalore entitled "Micronutrient Composition of *ALOE BARBADENSIS* (MILL) Plants cultivated at Different Altitudes In India".
2. Paper presented at Symposium on Medicinal Plants in Himalayas by NABARD at Pithoragarh (UA) titled "Economic Importance of Chyura (*Bassica butyracea)* and *Aloe vera*.
3. Paper presented at G. B. Pant Institute of Himalayan Environment and Development, Kosi-Katarmal, Almora, Uttaranchal entitled "Threatened Herbal Heritage of Western Himalaya: A need of conservation".
4. Paper presented at IIT Kharagpur, in AFSTI conference on "Anti-microbial activity of *Aloe barbadensis* plants collected from different agro climatic conditions".