Morphological, *in vitro*, Biochemical and Genetic Diversity Studies in *Aloe species*

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B. CHANDRA SEKHAR SINGH

DEPARTMENT OF GENETICS

OSMANIA UNIVERSITY

HYDERABAD - 500007, INDIA

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DECLARATION

The investigation incorporated in the thesis entitled **"Morphological, *in vitro*, Biochemical and Genetic Diversity Studies in *Aloe species''*** was carried out by me at the Department of Genetics, Osmania University, Hyderabad, India under the supervision of **Prof. Anupalli Roja Rani**, Osmania University, Hyderabad, India.

I hereby declare that the work is original and no part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Date: **(Bhaludra Chandra Sekhar Singh)**

DEDICATION

I dedicate this work to my beloved and beautiful wife B. Ananda Sekhar

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List of important abbreviations

%	:	Percentage
AcOH	:	Acetic acid
AIDS	:	Acquired Immuno Deficiency Syndrome
AFME	:	*Aloe ferox* methanol extract
AC	:	Acetone
ALE–EA	:	*Aloe CIM-Sheetal* Leaf Extract With Ethyl Acetate
ANOVA	:	Analysis Of Variance
ATP	:	Adenosine Triphosphate
AFLP	:	Amplified Fragment Length Polymorphism
AT	:	Adenine-Thymine
B.C	:	Before Chris
BHT	:	Butylated Hydroxyl Toluene
BAP	:	6-Benzylaminopurine
b.w.	:	Body weight
^{o}**C**	:	degree at Centigrade
CITES	:	Convention on the International Trade of Endangered Species of Wild Fauna and Flora
CIMAP	:	Central Institute of Medicinal and Aromatic Plants
CH	:	Chloroform
CIMAP	:	Central Institute of Medicinal and Aromatic Plants
CMA	:	Chromosomal Microarray Analysis
CG	:	Cytosine - Guanine
CAM	:	Crassulacean Acid Metabolism
cm	:	Centimeter
CMA	:	Chromomycin A3
Conc.	:	Concentration
CTAB	:	Cetyl Trimethyl Ammonium Bromide
dil.	:	Dilute
DNA	:	Deoxyribonucleic acid

DARL	:	Defense Agricultural Research Laboratory
DAPI	:	4', 6 - diamidino -2- Phenylindole
DGGE	:	Double Gradient Gel Electrophoresis
DM	:	Diabeties Mellitus
DPPH	:	1, 1-Diphenyl-2-picryl-hydrazy
EACC	:	Ehrlich Ascite Carcinoma Cell
EAC	:	Ehrlic Acites Carcinoma
EDTA	:	Ethylene Diamine Tetra Acetic Acid
Fig	:	Figure
^{o}F	:	Fahrenheit
FRAP	:	Ferric reducing antioxidant power
FYM	:	Farm Yard Manure
g	:	Gram
GBC	:	Glibenclamide
GRASE	:	Generally Recognized Safe And Effective
GST	:	Glutathione-S-Transferase
H_2O	:	Water
HCl	:	Hydrochloric acid
HIV/AIDS	:	Human Immunodeficiency Virus/Acquired Immuno-Deficiency Syndrome
HNO_3	:	Nitric Acid
HPLC	:	High-Performance Liquid Chromatography
hr	:	Hour
IBA	:	Indole-3-butyric acid
IASC	:	International Aloe Science Council
ITS	:	Internal Transcribed Spacers
ITIS	:	Integrated Taxonamic Information System
ISSR	:	Inter Simple Sequence Repeat
IAA	:	Indole-3-acetic acid
i.e	:	That is
K	:	Potassium

KCl	:	Potassium chloride
Kg	:	Kilogram
KIN	:	Kinetin
KOH	:	Potassium hydroxide
LAV	:	Large *Aloe vera*
lbs	:	pounds
LDH	:	Lactate dehydrogenase
LUX	:	A unit of illumination (lumen per square meter)
M	:	Molar
mAU	:	Milli Absorbance Units.
MET	:	Methanol
µm	:	Micrometer
MI	:	Mitotic Index
Meq/l	:	Milli equalence per litre
MF	:	Molecular Formala
mg	:	Milligram
min	:	Minutes
ml	:	Milli litre
mm	:	Milli meter
mp	:	Melting point
MBC	:	Minimum Bactericidal Concentration
MIC	:	Minimum Inhibitory Concentration
MS	:	Mass Spectrum
MS medium	:	Murashige and Skoog medium
Mwt	:	Molecular Weight
MRDC	:	Medicinal and Aromatic Plant Research Development Centre
µg	:	Microgram
µl	:	Microliter
NAA	:	Naphthaleneacetic acid
Na_4 EDTA	:	Tetra Sodium Ethylene Diamine Tetra Acetic acid

Na	:	Sodium
Na+	:	Sodium ion
NaCl	:	Sodium chloride
NaOH	:	Sodium Hydroxide
NAD	:	Nicotinamide Adenine Dinucleotide
NBPGR	:	National Bureau of Plant Genetic Resources
NH_4OH	:	Ammonium Hydroxide
NIN	:	National Institute of Nutrition
nm	:	Nanometer
NMR	:	Nuclear Magnetic Resonance
NK cells	:	Natural killer Cells
No.	:	Number
ORAC	:	Oxygen radical absorbance capacity
P	:	Phosphorous
ppm	:	Parts per million
PAS	:	Periodic Acid Schiffstain
PCO	:	Principle coordinate analysis
PMCs	:	Pollen Mother Cells
PVP	:	Poly Vinyl Pyrrolidone
RAPD	:	Random Amplified Polymorphic DNA
Rf	:	Relative front
RFLP	:	Restriction Fragment Length Polymorphism
rpm	:	Rotation per minute
ROS	:	Reactive Oxygen Species
RSA	:	Radical Scavenging Activity
SAV	:	Small *Aloe vera*
SANBI	:	South African National Bio Diversity Institute
SD rats	:	Sprag Dawly rats
SD	:	Standard Deviation
SOD	:	Superoxide Dismutase Assay
SPF	:	Sun Protection Factor

SSR	:	Simple Sequence Repeat
STZ	:	Streptozotocin
sq.crn	:	Square Centimeter
tGPx	:	Total Glutathione peroxidases
TLC	:	Thin Layer Chromatography
UV	:	Ultraviolet
US $	:	US Dollar
UPGMA	:	Unweighted Pair Group Method with Arithmetical Averages
Vc	:	Volume in Control
Vt	:	Volume in Test
WHO	:	World Health Organization
w/v %	:	weight/volume percent

CHAPTER - 1

INTRODUCTION

1. INTRODUCTION

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural resources. Since ancient days medicinal plants have been playing a pivotal role in primary health care. They are nature's gift to human being for disease free healthy life. About 80% of people in developing countries relay on plants and animal based medicine for their primary health care. It is estimated that 25% of all prescriptions distributed with in the USA contain a plant extract or active ingredients derived from plants. It is also estimated that 74% of the 119 currently used drugs contain active ingredients from plants used in traditional medicine (Sahu et al., 2013). Another study on the most prescribed drugs in the USA indicated that a majority contained either a natural product or drug synthesized from natural product.

In the present scenario, there is an imperative and continuous need of exploration and development of low cost, effective new plant based drugs with higher bioactive potential and least facet effects. Hence, attention has been paid on biologically active extracts and compounds from plant species. Nature is the world's best combinational chemist. All the lead compounds in drug discovery are derived from natural sources, but there is relatively little exception whether this lead compounds will find its way to market. Synthesis of natural products is another important aspect in recent years. Hence, recent attention has been paid to biologically active extracts and compounds from plant species (Essawi and Srour, 2000; Joshua et al., 2010).

***Aloe vera (Aloe barbadensis* Miller)**:

It is an important, ayurvedic, medicinal, ornamental and succulent plant commonly known as *Aloe vera*, *Aloe vulgari, Aloe indica* Royle, *Aloe perfoliata* (L.) and *Aloe vera.* It is used worldwide in drug and cosmetic industry. It is originated in Northern Africa and has been used by humans for almost 6,000 years. The name *Aloe vera* was derived from the Arabic word 'alloeh' meaning 'shining bitter substance'. It belongs to the family Xanthorrhoeaceae (Liliaceae). The genus Aloe contains over 450 different species. Among them *Aloe barbadensis* Miller gained worldwide recognition and has been intensively used from the oldest of times due to its extraordinary features. The Egyptians call Aloe as "The plant of immortality'' (Sikarwar Mukesh et al., 2010).

Aloe vera is a xerophytic perennial, ornamental plant with a short, cylindrical, simple stem of 30-60 cm. The leaves are succulent, 40-70 cm long, erect, broad, thick and fleshy that are throwned in a rosette and glaucous-green in color, narrow lanceolate in shape with long acuminate tip with little thorns on both edges (Fig 1).

Fig 1: *Aloe vera* (*Aloe barbadensis* Mill) Plant

Flowers are yellow to orange in color and are borne in dense racemes terminating within the cylindrical spike. The folk name of this plant is “Kanniedood”, which means “can-not die”. Most formularies and reference books regard *Aloe barbadensis* Miller as the correct species name and *Aloe vera* (L.) Burm. f. as a synonym. According to the International Rules of Botanical Nomenclature, *Aloe vera* (L.) Burm. f. is the legitimate name for the species (Bradley, 1992 ; Newton, 1979).

Scientific classification (Based on ITIS Report – Taxanomic serial No: 182653)

Kingdom	:	Plantae
Division	:	Tracheophyta
Infradivision	:	Angiosperms
Class	:	Magnoliopsida
Superorder	:	Monocots
Order	:	Asparagales
Family	:	Xanthorrhoeaceae
Subfamily	:	Asphodeloideae
Genus	:	Aloe
Species	:	*vera* (L.) Burm.f. - Barbados aloe
Botanical name	:	*Aloe vera* (Lin) Burn.f.
Chromosome number	:	2n = 14

Various regional names for *Aloe vera* are Kalabanda (Telugu), Gheekanwaar (Hindi), Kuwaargandal (Punjabi), Ghrita Kumari, Kumaari, Ghrit Kumaarika (Sanskrit), Indian Aloe (English), Kumari (Malyalam, Oriya), Katarazhai, Kilimukan, Chirukuttali (Tamil), Lolisara (Kannada), Kumarpathu (Gujrati), Ghrit Kumaari (Bengali), Korepharh (Marathi) *Aloe barbadensis* (Malik et al., 2013).

The medicinal use of Aloe was mentioned more than 4000 years ago in a collection of Sumerian clay tablets dated 2100 BC. Aloe has had a long historical use as a strong laxative and purgative. Aloe was also mentioned as a laxative in the Egyptian Papyrus Ebers from 1552 BC (Taylor et al., 1965). The legend also claims that famous

Queen Cleopatra of the 1st century BC also used Aloe gel as a beauty aid. *Aloe vera* has been used medicinally for many thousands of several cultures from Egypt, Greece, Rome to China and India (Marshall, 1990). There are anecdotal references to its use in ancient Egypt in 1500 BC and it is mentioned in the pharmacopoeia made by Dioscorides with in the 1st century AD (Castleman, 1991; Steenkamp, et al., 2007).

Aloe plant is native to the Mediterranean region of Southern Europe and to Canaries. It has been extensively cultivated in the Caribbean Islands and in Mexico since early1800s. In the United States, most of the *Aloe vera* is grown in Southern California, Florida and the city Rio Grande Valley of South Texas. Internationally, Aloe crops will be found in Mexico, South America, Pacific Rim Countries, India, Central America, The Caribbean, Australia and Africa. It grows readily in hot, dry climates and currently as a result of demand, is cultivated in large quantities (Newall et al., 1996).

In India it grows wildly in Maharashtra and Tamil Nadu states where as Andhra Pradesh, Gujarat and Rajasthan states are known for its cultivation. Major areas of Aloe production are Rajasthan, Andhra Pradesh, Gujarat and some parts of Tamil Nadu, Maharashtra and Kerala. Besides Europe, the product is additionally exported to Australia, Costa Rice and Malaysia (Brihans natural products). Washed *Aloe vera* leaves are sold in the United States for about $6.00 per kg (Waller et al., 2004). The current global turnover of raw Aloe leaves costs up to US $80–100 million dollars. USA provides the major bulk of Aloe in world market having a share of 60 - 65%, whereas Latin American countries 20 - 25% and Australia, India and China have a market share of only 10%. It is estimated that about 40 - 50 thousand rupees could be earned annually by a plant grower from Aloe cultivation (Das and Chattopadhay, 2004).

There is a big list of therapeutic claims made from *Aloe vera* depending on its pharmacological activities. Most of these claims are based on historical use than hard evidences. Different parts of the plant are employed in traditional management of diverse veterinary and human diseases. The herb is used internally to combat most digestive problems, including constipation, poor appetite, colitis, irritable bowel syndrome, asthma, diabetes, immune system enhancement and peptic ulcers. Aloe is used externally for the treatment of skin irritation, burns, scalds, sunburn, wounds, eczema, psoriasis, acne, dermatitis, ulcers and to stimulate cell regeneration. The plant is also used in the treatment of skin exposed to UV and gamma radiation. The cytotoxic power of *Aloe vera* has been proved which gives a base that it can be used against peptic ulcers and different types of cancers. Apart from that it helps in curing cardiovascular diseases. The plant has many medicinal values like antioxidant, anti-inflammatory, antidiabetic, anti-microbial, anti-fungal, anti-viral, anti-tumor, anti-aging and antiseptic properties (Paoulomi Chatterjee et al., 2013).

Various parts of the plant have different effects on the body. Major value added products from aloe are gel and juice. Gel is the pulp of the leaf and juice is obtained by homogenizing and diluting the gel. Aloe juice has been shown to lower cholesterol and triglycerides while demonstrating antidiabetic activity. The most often used substance from this herb is the aloe gel, found in the interior of the leaves. The leaves are used in the treatment of burns and it contains the Barbaloin (aloine) - a bitter milky yellowish liquid is used as a laxative. Today there are hundreds of different medicinal, cosmetic and food products on the market that contain various amounts of non laxative aloe juice/gel.

This glorious plant is reportable to be used as purgative, laxative, analgesic, tonic, painkiller, antiasthmatic, antileukopenic, antipyretic, antihelmintic, decoagulant, demulcent, diuretic, emollient, worms expellent, hair stimulant, hypoglycemic, insecticides, liver stimulant, anesthetic, rejuvenative, craving stimulant, stomachache, uterine stimulant and parasite killer (Kawai et al.,1998 ; Lee et al., 2001; Grover et al., 2002; Ferro et al., 2003; Cock Ian Edwin, 2008). *Aloe vera* has been used for centuries for the above discussed properties. This plant can be said as a "pharmacy in a pot".

A list of diseases cured by *Aloe vera* include allergies (related to plant/insects), abrasions, acne, arthritis, anemia, AIDS, burns, boils, bits, blisters, coughs, cold, corneal ulcers, cuts, cataracts, conjunctivitis, genital sores, chronic ulcers, constipation, dermatitis, dandruff, dry skin, dental sores, diabetes, digestive disorders, dysentery, edema, eczema, eye infection, fever, gingivitis, glaucoma, gastrointestinal ulcers, high blood pressure, infected pierced ears, insect stings, joint pain, kidney infections, lowers blood lipid levels, minor sore throats, mouth irritations, megrim headaches, mouth and gum diseases, muscle cramps, nausea of all kinds, psoriasis, rashes, ringworm, scars, scalds, skin cancers, swelling, tuberculosis, ulcers, vaginitis, genital sores, wounds of every kind, warts and yeast infections (Krinsky et al., 2003; Yagi et al., 2002; Mantle et al., 2001; Chithra et al.,1998; Tarro, 1993; Fulton, 1990; Davis et al., 1987).

In addition to pharmaceutical importance, *Aloe vera* is widely used in food and cosmetic industry. In the food industry, Aloe is used as an ingredient for health drinks, desserts and beverages. *Aloe vera* gel is used as an ingredient in commercially available yogurt, beverages and a few desserts (Reynolds, 2004). Recently, the plant has emerged as a vital source for production of industrial products. Aloe gel is surprisingly new

antiageing agent. Gel is widely used in numerous medical, cosmetic and neutraceutical applications in industry (Ni et al., 2004). The Aloe products are widely adopted as main ingredients in cosmetic and hair care products. Large numbers of Aloe containing beauty product, lotions, creams, facial cleaners, soaps, moisturizers, shampoos, oils, etc. are available worldwide. Owning to its utile utility, *Aloe vera* has been introduced into cultivation as ornamental, medicinal and household plant.

Aloe vera is grown worldwide in temperate climates and can be found in commercial operations in USA, Central America, South America, China, India, Africa, The Caribbean, Australia and Asian tropics. Recent, request for assistance in setting up greater commercial cultivation and processing operations in countries such as Greece, Iran and other middle-eastern areas have been noted (Nejatzade et al., 2012). There is a lack of expressions of reproductive characters in some of the species due to which it is impossible to distinguish morphological variations in some economically important *Aloe species*. It is essential to characterize the medicinally and economically important genus because morphochemical characters help in giving proper guidance to the farmer who wants to cultivate the best species for commercial purpose. Morphological characterization of *Aloe vera* (*Aloe barbadensis* Miller) germplasm was carried out on plant collected from different places such as National Bureau of Plant Genetic Resources (NBPGR), New Delhi. The experimental material comprised of twelve accessions of *Aloe vera*. Micropropagation is the process of vegetative growth and multiplication from plant, tissues or seeds. The advantages being production of large number of plants under controlled or alter environment to meet specific needs of plant. Tissue culture also helps in making the availability of plant all over the year, identification and production of

clones with desired characteristics, production of secondary metabolites, conservation of threatened plant species and preservation of genetic material. Cultivation of high value medicinal plants had created new dimension in the field of agriculture. Regeneration of *Aloe vera* in nature is slow due to its male sterility which forms a barrier in rapid propagation (Dwivedi et al., 2014). Aloe is exclusively propagated crop using lateral buds or off shoots produced by donor plant. A single plant produces 2-3 offshoots in a year which is not sufficient for undertaking commercial cultivation and to meet the industrial demand. Additionally a higher incidence of the disease is expected to happen to the donor plant after withdrawing the lateral roots. Increased demand of Aloe biomass to meet the demand of food and cosmetic industry had generated the need to undertake large scale cultivation of the plant.

The information of *in vitro* propagation of *Aloe vera* has been noticed by several reports (Ahmed, 2007; Aggarwal and Barna, 2004; Gui et al., 1990; Hosseini and Parsa, 2007; Fattachi et al., 2004; Tripathi and Bitallion, 1995; Albanyl, 2006). Researchers obtained various results by using plant growth regulators in different formulation. Different genotypes require different hormonal requirement for *in vitro* differentiation. The main aim of the study was to develop a rapid, efficient, less expensive and easy method of micro-propagation of *A. vera* at commercial level. We have standardized new combinations of growth regulators for efficient and rapid micropropagation of *Aloe vera* using shoot tip explant.

Phytochemicals are naturally occurring compounds present in various parts of the medicinal plants that have defense mechanism and protect from various diseases. They are primary and secondary compounds. *Aloe vera* has a high enzyme content (about

92 enzymes) which makes it rare and valuable resource. It contains 90-99.5% water. Other potentially active constituents of *Aloe vera* include sugars, lignin, saponins, anthraquinones, salicylic acid and amino acids (Antherton, 1998). Reynolds and Dweck (1999) listed 16 different polysaccharides that have been extracted from the *Aloe ver*a leaf. A variety of minerals like aluminium, boron, barium, calcium, magnesium, phosphorus, sodium, silicon, iron and strontium has been detected in Aloe gel (Yamaguchi et al., 1993). The *Aloe vera* leaf contains over 75 nutrients and 200 active compounds including 20 minerals, 18 amino acids and 12 vitamins (Park and Jo et al., 2006). It contains all the eight essential amino acids and eleven of the fourteen secondary amino acids useful for human health.

There are many natural medicinal herbs, but *Aloe vera* is granted with large array of healing benefits. It is used for external application and perhaps taken internally because of its medicinal importance. Some of the compounds present in it are acemannan, Aloe-Emodin, Aloins, enzymes, Mucopolysaccharides, Galactomannans. Phytochemical analysis of the following compounds was done by different procedures. Confirmation for alkaloid, carbohydrates, proteins, amoinoacids, steroids, saponins, phenolic compounds and tannins was basically done in our prilimary screening.

It is well known that in past years infection rates have increased enormously and antibiotic resistance has become an increasing therapeutic problem. In addition a greater interest in the antioxidant activity of plant extracts exists because of free radicals (e.g., reactive oxygen species) that can be responsible for several diseases, like heart stroke, arteriosclerosis and cancer, as well as the aging process. We have selected the activities

like antimicrobial, anti-inflammatory and antioxidant activity because of their great medicinal relevance.

It has an extended history of chemical analysis from Biblical times. International Aloe Science Council (IASC), a non-profit organization certifies *Aloe vera* products by measuring total solids, magnesium, calcium, malic acid and polysaccharide content (Anirban Ray et al., 2013). Aloe has more nutritional values and biological active compounds i.e. alkanes, aldehydes, alcohols, ketones, dicarboxylic acids, fatty acids, organic acids, sterols, alkaloids, indoles, pyrimidines, phenolic acids/polyphenols. The aloe herb contains twenty minerals among which is calcium, magnesium, zinc, chromium, selenium, 12 vitamins: A, B, C, E, folic acid and twenty amino acids. Out of 22 amino acids required for the human body, 8 essential aminoacids are found in *Aloe vera*.

According to the world health organization, diabeties mellitus (DM) is one of the most common metabolic disorders all over the world. Many studies indicate, oxidative stress is one of the major route of pathophysiological condition during DM. Scientific investigations on traditional medicinal plants indicates that medicinal plants play a significant role in treatment of diabetes worldwide. WHO has recommended that the evaluation of traditional medicinal plants treatment for diabetes were effective, non-toxic with less or no side effects and is considered being excellent candidates for oral therapy (Yogesha Mohan et al., 2013). During the past two decades, *Aloe vera* is used as beneficial therapeutatic agents which protectively act as a free radical scavenging and other antioxidant properties on diabetic patients, by controlling elevated anions in an alloxan or STZ induced diabetic animal models. The liver is the largest organ in the body

and the centre of all metabolic activities. Drugs and other foreign substances are metabolized and inactivated in the liver. Essentials functions of the liver tend to be lost in the development of hepatic disease or disorder. Drugs and toxins could cause hepatic cell damage (Ajayi et al., 2011). The damage to the hepatocytes will lead to release of intra cellular constituents into circulation. Effect of *Aloe vera* extract on STZ induced diabetes in male SD rats was studied by comparing with standard drug glibenclamide. Our studies were continued on the histological changes of liver, kidney and pancreas after the treatment of diabetic rats with *Aloe CIM-Sheetal* leaf extract.

Natural products have inspired many developments in organic chemistry leading to advances in synthetic methodologies in developing several analogues of lead compounds with therapeutic potential. A variety of techniques can be used to determine and estimate the presence of such phytochemical compounds. Various chromatography methods like High Pressure Liquid Chromatography (HPLC) and Thin layer Chromatography (TLC) were commonly used. For further evaluating the more purified compound NMR has been used by which the presence of novel compounds was confirmed.

The identification of the amount and distribution of genetic diversity in the gene pool of the concerned plant leads to success of any genetic conservation and breeding programme. The knowledge on the genetic diversity and relationships among plant varieties is important to recognize gene pools, to identify gaps in the germplasm collection and to develop effective conservation and management strategies. Morphochemical evaluations can provide insights into the genetic structure and diversity within and among varieties from different geographical origins, producers and

distributors. Without this information there is no means of selecting appropriate plant material for the participation in screening programs with a view to the introduction of the novel varieties for the industrial purpose (Radha Mahavi et al., 2012). Due to vegetative mode of replication, spontaneous biological and genetical aberration acquired by the plants, they got fixed up in their genome.

In order to improve the medicinal value of *Aloe vera* and to fill the gap between the demand and supply of the elite planting material for pharma and cosmetic industry, there is a need to conserve this species sustainable use in future. Among the various molecular markers the random amplified polymorphic DNA (RAPD) is most widely used because it allows a rapid and inexpensive assay compared with different primers. Due to the technical simplicity and speed of RAPD methodology it has been successfully used for the assessment of the genetic structure and phylogenetic analysis. It has been successfully applied to study the genetic differentiation in some genera of *Mangifera* and *Asparagus* (Nayanakantha et al., 2010). Inter-simple sequence repeat (ISSR) markers, developed by Zietkiewicz et al., (1994) is based on the amplification of a single primer containing a microsatellite 'core' sequence anchored at the′or5 3′ end by a set of 2 -4 purine or pyrimidine residues and offers a high degree of reproducibility with the detection of rich level of polymorphism in a relatively simple procedure. Hence, it has been widely used in assessments of genetic diversity and cultivar identification (Bhattacharya, 2010). We used 64 RAPD primers and 25 ISSR primers to evaluate the genetic diversity of different accessions of *Aloe vera*.

Aloe ferox

Aloe ferox originated from South Africa and it is widely distributed throughout the tropics and sub tropics. It is grown as ornamental and medicinal plant. It is known as grandfather of 'Aloes' common species for cultivation in South Africa. Many plants of *Aloe ferox* grow up to 5 meters tall, but there is variability in size over the distribution in different areas (Shackleton and Gambiza, 2007). *Aloe species* can be identified by their characteristic leaf structure and are generally recognized by their rosettes of succulent leaves and tall, candle-like inflorescences, which are also seen in several other succulent genera, such as Agave. Twenty two *Aloe species* are listed in CITES (Convention on the International Trade of Endangered Species of Wild Fauna and Flora) Appendix I (Eggli, 2001; Mc Gough, 2004) it includes the genus of *Aloe ferox* but excluding *Aloe vera*. *Aloe vera* is listed in CITES Appendix II **(**Mc Gough, 2004). *Aloe ferox* is similar to *Aloe vera*, but has many times more nutritional and medicinal value than *Aloe vera (Aloe ferox* US, 2013; Fig 2).

There is an increased international demand for *Aloe ferox* due to its effectiveness, availability and safety in medicinal use, worldwide population of developing countries continues to rely heavily on the use of traditional medicine as primary source of healthcare. Hence, various communities in different parts of the world would use the species of aloe indigenous to their immediate surrounding as medicine. In South Africa various traditional communities and local industries are using a variety of *Aloe species* for example, *Aloe ferox* in the Eastern and Western Cape provinces (Shackleton and Gambiza, 2007) in the treatment of arthritis (Shelton, 1991), skin cancer, burns

(Barrantes and Guinea, 2003; Zhang and Tizard, 1996), eczema, psoriasis, digestive problems, blood pressure problems and diabetes (Loots et al., 2007).

Fig 2: *Aloe ferox* plant with red flowers

Taxonomy: *Aloe ferox* falls under the scientific classification as follows (Based on ITIS Report – Taxanomic serial No: 505880; DAFF, 2013).

Kingdom	:	Plantae
Division	:	Tracheophyta
Infradivision	:	Angiosperms
Class	:	Magnoliopsida
Sub class	:	Magnolidae
Superorder	:	Monocots
Order	:	Asparagales
Family	:	Xanthorrhoeaceae (Liliaceae)
Sub family	:	Asphodelodeae
Genus	:	*Aloe*
Species	:	*ferox* Mill.
Chromosome number	:	2n = 14.

Vernacular names: *Aloe ferox* has been granted tree status and its national tree number in South Africa is 29.2. The species name ferox means ‘ferocious’ and it refers to the spiny leaves.

Common names: Lily of the desert, Cape aloe, bitter aloe, bergaal wyn, Tap aloe, Red aloe

Local names: Zulu, Xhosa, Sotho- umhalba, Africans-Bitteralwin, Tapaalwyn, English-Bitter Aloe, Red Aloe, French-Aloes du Cap.

Another names: The Doctor of the sky, The plant Doctor, The plant which cures, The plant miracle, The plant of first aid, The plant of burns, The remedy of harmony, The vegetable Doctor, The green Doctor, Doctor aloes, The Doctor out of pot, The quiet healer, The fountain of youth and Elixir of long life.

Synonyms: *Aloe candelabrum, Aloe berger*.

Geographical Distribution *Aloe ferox* has an extensive distribution in South Africa (Street and Prinsloo, 2013). A number of herbs belonging to the genus Aloe are noted for their medicinal and cosmetic benefits in traditional system of medicine (Maduna, 2006). Aloe comprises about 450 species in Africa and Arabia, of which 315 occur in mainland Africa, 100 are endemic to Madagascar or the Indian Ocean islands (including the former Lomatophyllum) and remaining 50 occur in Arabia.

Botanical description: *Aloe ferox* Mill. is well known as Cape Aloe or bitter Aloe. It is a single stemmed robust and succulent plant. The height of stem reaches around 10 m tall, leaves reach around 1m in length. It refers to the thorny sharp red brownish spines on the leaves. The beautiful bright red or orange flowers are bloomed between May and August. They attract birds and numerous insects.

The yellow bitter sap and white gel are present in Aloe leaves. Among these gels, the bitter sap lies underneath the green peel and is part of the peel (Wasserman et al., 2002). Aloe bitters is largely used in purgatives for the past 200 years. The inner fleshy portion of aloe leaves consists of a mucous material, called aloe gel, which is not bitter. It was suggested that the pregnant women should not take the bitter sap (Brinker, 1998; Steenkamp and Stewarta, 2007) as it can stimulate the uterus. It can pass through the breast milk from mother to baby resulting in colicky babies (Nusko et al., 2000).

In this study we worked on morphological, *in vitro*, biochemical, antidiabetic and genetic diversity studies of *Aloe vera* which can be useful as a potential drug in pharmaceutical industry. We have studied *in vitro*, antioxidant, anti-inflammatory and biochemical properties of *Aloe ferox*.

CHAPTER -2

RE VIEW OF LI TERA TURE

2. REVIEW OF LITERATURE

The plant kingdom is the best remedy for curing a variety of diseases. Medicinal plants play a key role worldwide in maintenance of health. Natural products of higher plants are an important source of therapeutic agents; the demand on plant based therapeutics is increasing in both developing and developed countries due to their recognition as: non- narcotic properties, easily biodegradable, minimum environmental hazards, no adverse effects and affordability. For the successful introduction of a new synthetic product about 3000-4000 compounds are to be synthesized, screened and tested, who's cost of development ranges from 0.5 to 5 million dollars which is highly expensive. On the contrary, many medicines of plant origin had been used since years without any adverse effects. It is essential that efforts should be made to introduce new medicinal plants to develop cost effective drugs (Duganath et al., 2010).

Aloe vera has been used for its medicinal value for several thousand years. It is being used worldwide for pharmaceutical, food, and cosmetic industries due to the plethora of biological activities of some of its metabolites. Its applications have been recorded in ancient cultures of India, Egypt, Greece, Rome and China. In biblical times the Egyptians hailed *Aloe vera* as the plant of immortality. The Chinese called it their elixir of youth. The *Aloe vera* has many common names and often referred to as burn plant, first aid plant or medicine plant. Its name is most likely derived from the Arabic word "Alloeh" meaning shining bitter substance. Aloe was originated in tropical Africa and it is now cultivated in warm climatic areas of Asia, Europe and America. Presently, the use of *Aloe vera* has gained popularity because of herbal movement initiated by

naturopaths, yog gurus, alternative medicine promoters and holistic healers (Kulveer Singh and Bhupender Singh, 2011).

Aloe vera is extensively used in industry due its enormous medicinal properties. The industry size for aloe raw material is estimated about $125 million dollars. The volume of the industry for finished products containing *Aloe vera* is about $110 billion dollars. Recent market analysis indicates that in 2008 Americans have spent almost 40 billion dollars on functional foods, drinks and supplements for the improvement of their appearance as well as to provide energy and added nutrition to handle health issues such as hypercholesterolemia and diabetes. *Aloe vera* products are among the popular ones for these applications. Today, the *Aloe vera* industry is flourishing and the gel is used in many products such as fresh gel, juice and other formulations for health, medicinal and cosmetic purpose. However, the fast expanding *Aloe vera* industry urgently needs reliable testing protocols to assess the quality and quantity of bioactive chemicals present in the final products (Bozzi et al., 2007). The product claims must be tested by intensive clinical trials, verified and certified by the Government regulatory authorities to built consumer confidence and safety of the *Aloe vera* products. Basically we worked on two plants i.e I. *Aloe vera* II. *Aloe ferox.*

I. *Aloe vera (Aloe barbadensis* Miller)

The name comes from the Arabic “alloeh” (means bitter substance), from the Greek word “aloe” (mean aloe) and from the Latin “vera” (means truth).

Origin: The species “*Aloe barbadensis* Miller”, originated from North Africa (Bozzi, 2007).

Leaf: It consists of long thick and brittle leaves with thorns at both the edges grow in a spiral rosette around the stem. They have short, thin prickles along the sides of the leaves. They measure 30 to 60 cm large, and 5 to 9 cm wide. The leaf is composed of the outer epidermis from above and inner epidermis underneath. Under the leaf peel there is a layer, with concentrated aloin (bitter substance, laxative with yellow colour). After this layer, there are internal ducts of vegetable tissue, which contains, the mucilage that is pulp from which the gel is made.

Plant stem: There is no stem; leaf grows from the ground level. Stem appears only after harvest.

Roots: There is a main wide root, from which tiny secondary roots are produced. It is a shallow rooted plant with 30-60 cm root length. The plants have multiple tuberous roots and lots of supporting roots penetrating into the soil.

Infloresence: Inflorescence is arranged in dense, large and elongated racemes with 50-80 cm long peduncle and 5-8 spreading branches, bracts are 2-5 mm × 2-4 mm broadly ovate.

Flower stems: Flower is between the leaves, not from the centre, it is 30 to 60 cm large.

Flowers: Resemble a small trumpet yellow colour, with six petals which are 2 to 4 cm large. Flowers can be described as bisexual, regular, trimerous, pedicel 1-1.5 cm long tubular, 2.5–3.5 cm long, lobes 6, 12-17 mm long, red color, sometimes white or yellow stamens-6 in number, exerted ovary superior, 3-celled, style filiform, stigma head-shaped, exerted.

2. Germplasm Characterization

There are about 400 species of *Aloe* grown worldwide. However, only two species are grown commercially i.e. *Aloe vera* (*Aloe barbadensis* Miller) and *Aloe aborescens*. There are at least two other species that have medicinal properties namely *Aloe perry* Baker and *Aloe ferox*. By visual observation of the Aloe plant, it is commonly noticed that a number of differences occur particularly in their morphology perhaps due to the differences in the environmental factors, or gene re-arrangements or due to speciation. None of the studies attempted till now provided answer for the differences in the vegetative features of various *A.vera* plants grown at different locations. The difference in the vegetative morphology inturn can be related to the chemistry, nutritional and the medicinal property of the plant (Akinyele and Odiyi, 2007). For the above reasons to establish the best commercial variety it is important to morphologically characterize different accessions of the *Aloe species*.

In the International Cosmetic Ingredient Dictionary and Handbook (Gottschalk and McEwen, 2004), different kinds of *Aloe species* are marketed for different Aloe products. The important species contributing to the world market of Aloe products are *Aloe andongensis*, *Aloe arborescens* (Kidachi Aloe), *Aloe vera* (*Aloe barbadensis*

Miller), *Aloe ferox* (Cape Aloe). Out of these species, *Aloe vera* scores high in quantity and quality.

In India, 2 or 3 easily recognizable varieties are found, but their exact delimitations are not clear. In *Aloe vera* var. chinensis Baker, common all over the Deccan, the leaves have a distinct purple colour towards the base and the spines are not sharp. Another species *Aloe variegata* Linn, a close to kin of *Aloe vera*, is found in parts of Maharashtra. It has giant, fleshy, green leaves with sharp spines and white specks at the base of the leaves. A variety which thrives on the Kathiawar coast is called *Aloe abyssinica* is the source of the Jaffarabad aloes. Based on the morphology of accessions of *Aloe vera* germplasm were divided into two morphotypes viz. Small *Aloe vera* (SAV): plant size up to 20 cm and plant size ranged between 21 to 40 cm and Large *Aloe vera* (LAV): plants with leaves more than 40 cm in size. The LAV type of accessions show highest incidence.

Reproduction: It is possible by seeds from the flowers or the most popularly by off shoots which grow at the bottom of the short plant stem.

3. Cytological Studies

Aloe vera acquired the attention of many cytologists throughout the world. Botanists have investigated the chromosome numbers in plants for more than 75 years and the results have been published in a number of books and journals. Chromosome research has made immense contribution in the elucidation of the systematic relationship of many closely related taxa, species, families etc. Sutaria (1932) is the probable pioneer worker who reported 2n=14 for the Aloe. Vig (1968) investigated commercial American

Aloe and reported 2n=14. He also observed the meiotic abnormalities like asynapsis, bridges, delayed disjunction, lagging chromosomes. Interestingly he found 2n=10 in the root tip cells.

Matos and Molina (1997) carried out cytogenetic study on root tip cells of *Aloe vera* in order to determine the time of mitosis and mitotic index (MI). The highest frequency of dividing cells was determined at 7.00 hr, selected as mitotic hour with MI of 15.24%. The karyotype composition was bimodial and consists of 14 sub-metacentric chromosomes (eight large and six small) ranging from 5.55 - 17.76 µm of length. Seedling of *Aloe vera* L. treated with colchicine to form polyploid plants and the variant of chromosome number is 28 while the normal diploid plant is 14. A dicentric chromosome has been reported in root tip cells of *Aloe vera* by Umesh and Ranganath (2003). They provided ultra structural evidence for the flawless transmission of dicentric chromosomes in the root tip cells and proposed the presence of two functional kinetochores.

Alam and Khanam (2005) carried out fluorescent karyotypic analysis of four *Aloe species*. All the species have eight large and six small chromosomes. Based on CMA and DAPI banding properties of the chromosomes of Aloe species they considered CG and AT rich base sequences in chromosomes are probably involved in karyotypic diversification of these species. The analysis of PMCs at metaphase-I showed 34.74% showing precocious disjunction of one, two or all the little bivalents. As many as 32.16% of PMCs at A-I showed abnormalities with laggards, bridges, unequal distribution etc. According to them all the chromosomal aberrations are most likely be correlative with some metabolic disturbances in the cells.

Clonal fedility of the two years old micropropogated plants were established by studying mitotic and meiotic chromosomal behavior and also considered the chromosome number and structural organization. There were alterations in the chromosome phenotypes, somatic haploid (pollen mitosis) and Diploid chromosome count (n = 7; 2n =14) or meiotic behavior. Randomly amplified polymorphic DNA analysis revealed there were no somaclonal variations among these regenerates (Moqummel Haque and Biswajit Ghosh, 2013).

4. Tissue culture Studies

Tissue culture research began nearly four decades ago with the first report on production of test tube fertilization (Kanta and Maheshwari, 1963). Plant cell and tissue culture is defined as the capability to regenerate and propagate plants from single cells, tissues and organs under sterile and controlled environmental conditions (Murashige and Skoog, 1974). This method also helps to raise pathogen free plants. Majority of plants used for medicine are harvested from the wild species which make the species extinct. In such conditions, tissue culture plays major role in propagation of particular species.

Cultivation of *Aloe vera* for commercial purpose has been adopted in many countries due to its therapeutic and commercial importance. This crop is vegetatively propagated where young side branches are used to propagate the plant. Single plant may produce 2-3 side shoots per year making the availability of planting materials limited quantitatively and qualitatively. Tissue culture techniques for micro-propagation are being used profitably to overcome such problems in various horticultural crops and ornamental plants like *Aloe vera*. Many serious problems like insufficient supplies of

plant material, depletion of resources, extinction of rare species, seasonal collections, adulterations in dried materials, incorrect identification of plant material and collection from the wild variety can be minimized by systematic cultivation of *Aloe species*.

A number of protocols for micropropagation of Aloe plants have been developed using a variety of explants like shoot tip, stem cuttings, auxiliary bud, etc. by various researchers. *Aloe vera* has been cultured by various researchers Gui et al., 1990; Abrie and Staden, 2001; Hosseini and Parsa, 2007 successfully regenerated the adventitious buds by the stem tissue culture and organogenesis and found that zeatin was better than kinetin. Roy and Sarkar (1991) reported the rapid propagation by inducing shoot formation from *in vitro* callus cultures produced by auxiliary shoot segment explants taken from the underground rhizomatous stem. Polyvinylpyrrolidone (PVP) was used to reduce the secretion of phenolic substances from the explant. Zhou et al., (1999) suggested that number of regenerated buds could be increased 8-9 times following bud splitting before culturing on growth media. For induction of these buds they used MS media with BA at 3 mg/l and for rooting NAA at 0.3 mg/l.

Hirimburegama and Gamage (1995) found that high rates of shoot proliferation were obtained from axillary and apical buds of *Aloe vera* cultured on MS media supplemented with 0.18 mg/l IAA+ 2.25 mg/l BA and rooting was achieved on MS media with 0.18 mg/l IAA+ O.226 mg/l BA after 3 weeks. Richwine et al., (1995) reported that the induction of shoot cultures of Aloe, Gasteria and Howorthia species from immature inflorescence. They used modified MS medium containing zeatin and later maintained on zeatin and BA containing medium. Sanchez et al., (1988) performed micropropagation on vegetative meristems and found that callus induction was difficult

in *Aloe barbadensis*. Budhiani (2001) demonstrated that the best combination for initiation of shoot was MS medium supplemented with 0.2 mg/l BAP + 0.002 mg/l NAA. Chaudhuri and Mukundan (2001) reported that best multiplication of shoots was obtained on medium containing 10 mg/l BA + 160 mg/l Adenine Sulphate + 0.1 mg/l IBA.

A rapid micropropagation protocol was established by Liao et al., (2004) for Chinese Aloe and recommended that sucrose of explants was the most important for the bud initiation on semi solid MS medium and MS medium supplemented with 2 mg/l BA + 0.3 mg/l NAA as the best medium for micropropagation of *Aloe vera*. Aggarwal and Barna (2004) provided a micropropagation protocol for an elite selection of *Aloe vera* and suggested that citric acid at 10 mg/l and liquid medium improved the shoot multiplication and produced 100% rooting on hormone free agar medium.

Campestrini et al., (2006) reported the development of a cloning protocol of *Aloe vera* to provide propagation material with superior quality to the private sector in southern Brazil. Studies conducted by Velcheva et al., (2005) and Debiasi et al., (2007) indicated that BA is more effective than NAA for shoot proliferation Hashemabadi and Kaviani (2008) suggested supplementation of MS medium with various concentrations of Benzyladenine and α-naphthaleneacetic acid for rapid micropropagation of *Aloe ver*a L. via shoot multiplication. Best rooting was obtained on MS media with 0.5 mg/l benzyladenine + 0.5 mg/l α-naphthaleneacetic acid. Here, the explant used was shoot tip and used MS media supplemented with 1 mg/l 2, 4-D and 0.2 mg/l kinetin for callus formation.

According to Wang Li et al., (2001) auxins and cytokinins are necessary for shoot proliferation. The results showed that the best induction rate could reach 50% after treatment with 0.06% colchicine in 12 hrs. A variety of aloe soil mixtures were also suggested for raising tissue cultured generating seedlings for hardening in poly house. Natali et al., (1990) proposed a variety of mixture of soil, sand and perlite or vermiculite soil. Farm yard manure was formulated by Aggarwal and Barna (2004), coconut peat and perlite was proposed by (Hashemabadi and Kaviani (2008).

In vitro regeneration and propogation protocols have been proposed by different researchers in different varieties of *Aloe species* (Ahmed et al., 2007; Ujjwala 2007; Michael et al., 2007; Balraj Singh and Neelu Sood, 2009; Kalimuthu et al., 2010; Das et al., 2010; Gantait et al., 2010; Saggoo and Ramandeep Kaur, 2010; Arvind Kumar Bhandari et al., 2010; Jayakrishna et al., 2011; Ashish kumar Choudhary et al., 2011). Nodal portion of rhizomatous stem of *Aloe vera* was cultured on MS medium supplemented with various cytokinins and *Aloe vera* leaf gel was used as organic supplement. The results for the above experiment confirmed a reliable method for large scale production of true to type plantlets of *Aloe vera* which can be used for commercial purpose (Moqummel Haque and Biswagit Ghosh, 2013; Dwivedi et al., 2014) standardized new composition of growth regulators for rapid and efficient micro propagation of *Aloe vera* using young auxiliary shoot.

5. Phytochemistry or Biochemistry of *Aloe vera*

In the present scenario, there is an urgent and continuous need of exploration and development of affordable, effective new plant based drugs with better bioactive potential

and least side effects. Racio et al., (1989) reported that the traditional medicine is an important source of potentially useful new compounds for the development of chemotherapeutic agents. Hence, recent attention has been paid to biologically active extracts and compounds from plant species (Essawi and Srour, 2000).

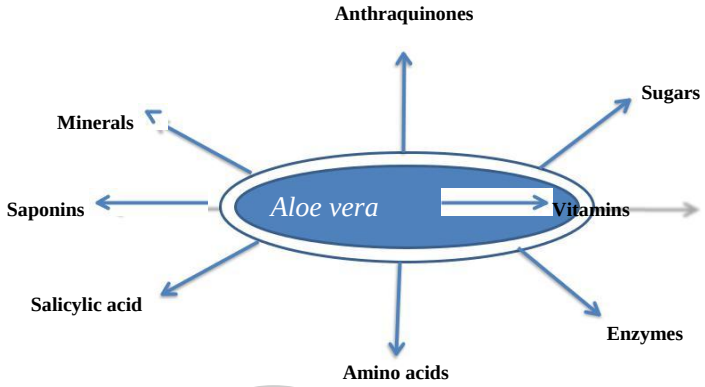

Fig 3: Components of *Aloe vera*

Aloe vera is a unique and amazing medicinal plant with rich source of many chemical compounds (Fig 3) which plays a vital role in the international market Chauhan et al., (2007) reported the chemistry of the plant that revealed the presence of more than 200 different biologically active substances in which anthraquinones, saccharides, vitamins, enzymes non- essential amino acids, essential aminoacids and miscellaneous compounds like saponins, sterols, vitamins, minerals, enzymes and salicylic acid etc. were present. It is reported that *Aloe vera* contains as many as 75 nutrients and 200 active compounds (Dureja et al. 2005; Park and Jo, 2006). *Aloe vera* contains both mono and polysaccharides. Mucilage of Aloe consists especially of the mucopolysaccharides, glucose, mannose and uronic acid. Sugars (muco-polysaccharides) are derived from the mucilage layer of the plant, surrounding the inner gel.

Table 1: Constituents of *Aloe vera* (Shelton, 1991; Vogler and Ernst, 1999**)**

Anthraquinones	**Saccharides**	**Vitamins**	**Inorganic Compounds**
Aloin / Barbaloin	Cellulose	B1	Calcium
Isobarbaloin	Glucose	B2	Sodium
Aloe-emodin	Mannose	B6	Chlorine
Emodin	L-RhamnoseAldopentose	Choline	Manganese
Aloetic Acid		Folic Acid	Zinc
Ester of Cinnamic Acid		Ascorbic Acid	Chromium
Anthranol		α-Tocopherol	Copper
Chrysophanic Acid		β–Carotene	Magnesium
ResistannolAnthracene			Iron
Ethereal oil			
Enzymes	**Nonessential Amino Acids**	**Essential AminoAcids**	**Miscellaneous**
Cyclooxygenase	Histidine	Lysine	Cholesterol
Oxidase	Arginine	Threonine	Triglycerides
Amylase	Hydroxyproline	Valine	Steroids
Catalase	Aspartic Acid	Leucine	β-Sitosterol
Lipase	Glutamic Acid	Isoleucine	Lignins
Alkaline phospahatase	Proline	Phenylalanine	Uric Acid
Carboxypeptidase	Glycine	Methionine	Gibberellin
	Alanine		Lectin like substances
			Salicylic Acid
			Arachidonic Acid

Chemical analysis of the gel has shown that it contain various carbohydrate polymers notably either gluco-mannans or pectic acid along with other organic and inorganic components (Table 1). According to Mabusela (1990) primary constituents are glucomannoglycan polysaccharides containing acetylated monosaccharides (70-80%). In *Aloe vera* five saccharides namely galactose, mannose, glucose, arabinose and xylose are present. Acemannan, a complex of mannose carbohydrate, derived from the *Aloe vera* plant has an inherent viscosity (Tello et al., 1998). Reynolds and Dweck (1999) reported 16 different polysaccharides extracted from *Aloe vera* leaf gel. Anthraquinones

are the phenolic compounds which are found in the sap were first to identify the principal active substance of the plant and Smith (1851) named it 'Aloin'. Danhof (1998) stated that the aloe polysaccharides consist of linear chains of β-1-4 linked glucose and mannose molecules, known as glucomannans.

The major leaf exudate compounds identified were aloeresin, aloesin, aloin A and aloin B, aloinoside B (Viljoen et al., 2001). The major (25-40%) constituent of aloe is the Hydroxyanthraquino derivatives aloin (barbaloin, a mixture of aloin A and
B, the diastereoisomeric 10-C glucosides of Aloe-emodin anthrone) and 7-hydroxyaloin isomers. Other constituents present in minor quantities include Aloe-emodin, chrysophanol, derivatives aloresin B (aloesin, upto 30%) with its p-coumaryl derivative aloeresins A and C and the aglycone aloesone Dagne et al., (2000). Seven of the eight essential amino acids required by human body are also present in aloe gel Chauhan et al., (2007) were reported that in aloe gel includes campesterol, sterols, β-Sitosterol and Lupeol.

The barbaloin content of latex from different *Aloe species* was assessed by a number of methods and found to be between 10-25% on a dry weight basis of the latex and about 1% on a leaf dry weight basis (Groom and Reynolds, 1986). Aloin/barbaloin, saccharides, vitamins, inorganic compounds, cellulose, calcium, iso-barbaloin, glucose, sodium, aloe-emodin, mannose, chlorine, emodin, L-Rhamnose, choline, manganese, aloetic acid, zinc, ester of cinnamic acid, ascorbic acid, chromium, aldopentose, folic acid, anthranol, anthraquinones, α-Tocopherol, copper, chrysophanic acid, β–Carotene, magnesium, resistannol, anthracene, iron, ethereal oil, enzymes, nonessential amino acids, essential amino acids, miscellaneous cyclooxygenase, histidine, lysine, cholesterol

oxidase, arginine, threonine, triglycerides, amylase, hydroxyproline, valine, steroids, catalase, aspartic acid, leucine, β-Sitosterol, lipase, glutamic acid, iso-leucine, lignins, alkaline phospahatase, proline, phenylalanine, uric acid, carboxypeptidase, glycine, methionine, gibberellin, alanine, lectin like substances, salicylic acid, arachidonic acid, the main crystalline glycoside and barbaloin are found in all the commercial varieties. Aloin A and B are collectively known as barbaloin and these are most outstanding members of this class (Rauwald, 1989; Mannitto et al., 1990). The leaves contain several free anthraquinones like aloin, Iso-barbaloin, barbaloin, anthranol, anthracene, aloetic acid, ester of cinnamic acid, aloe-emodin, emodin, chrysophanic acid, ethereal oil and resistannol (Vogler and Ernst, 1999; Peter, 2007).

Mineral composition of *Aloe vera* juice and reported that potassium and chloride concentrations appeared to be excessive for most plant products. Lignin, a pulp like substance existing in a formation with cellulose comprising the leaf gel in *Aloe vera*, was first discovered in *Aloe vera* gel by Wang (1993). Duke and Beckstrom-Sternberg (1994) stated that the flavor and extract manufacturers association accepted level for *Aloe vera* was 5-2,000 ppm. According to IASC (2003) (International Aloe Science Council) the maximum aloin content in Aloe-derived material for non-medical use is 50 ppm or lower. UAE and Switzerland legislation gave a limiting value for the maximum content of aloin in food stuffs.

Aloin A and B, aloeresin E, aloe-emodin, aloenin and aloenin B in *Aloe barbadensis* Miller and *Aloe arborescens* were separated and quantified by the HPLC method. Phenolic anthraquinones were separated and characterized using TLC, HPLC and column chromatography by Rajendran et al., (2007). HPLC has been used to

quantitate barbaloin in Aloe capsules with an LOD of 0.02 µg (Chen et al., 2002; Chauhan et al., 2007) reported that *Aloe vera* contains many vitamins i.e A, B1, B2, B6, C, E and F excluding D. Some authors suggested the presence of vitamin B12 (Cynocobalamin) in trace amount (Antherton 1998; Dureja 2005). Sambhav Jain and Rohith Rai (2014) reported that aloe gel have enzymes such as alkaline phosphatase, amylase, phosphatase, lactic acid, dehydrogenase and lipase.

6. Properties of *Aloe vera*

6.1. Medicinal Properties

The *Aloe vera* plant has been used in folk medicine for over 2000 years. The earliest known documentation of Aloe in medicinal reference is from 1500 B.C. Many nations such as Greece, China, Mexico and United States have historical documentation of *Aloe vera* being used as a trestment for different alimments. It helps to fight against different health ailments as given below (Fig 4).

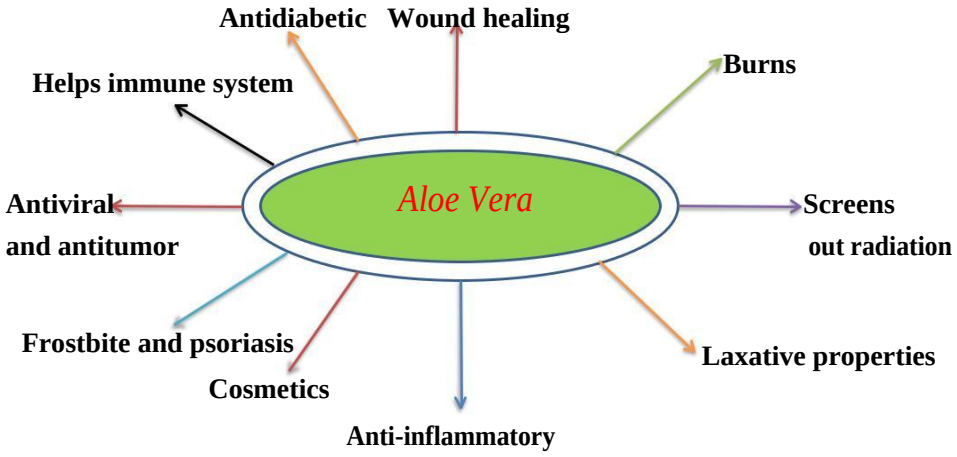

Fig 4: Medicinal uses of *Aloe vera*

The plant products can be divided into two products:

(I). Aloe latex and (II). Aloe gel.

I). Aloe latex or aloe juice is the bitter yellow exudates from the peri cyclic tubule in the outer skin of the leaves. The major and the active constituents of the aloe latex are hydroxy anthracene derivatives.

II). Aloe gel is the colourless gel present in the inner part of the fresh leaves, the gel consists primarly more than 98% of water, polysaccharides (pectins, cellulose, Hemicellulose, glucomannan, acemanan, and mannose derivatives).

6.2. Medicinal properties of Aloe latex:

It consists of

A. Endocrine system effects
B. Cathartic effects
C. Antibacterial/antiviral activicity
D. Antioxidant or prooxidant activity
E. Cytotoxicity / antitumoral effects

A. Endocrine system effects: There is strong evidence that aloe has blood glucose lowering effect. The oral administration of aloe has shown to lower plasma glucose levels in non-insulin dependent diabetic patients and in alloxin- induced diabetic mice (Kavishankar et al., 2011).

B. Cathartic effects: The major C-glycosides of *Aloe vera* latex, barbaloin and iso-barbaloin are the principal compounds responsible for the pergative and laxative effect in humans and animals (Patel et al., 2012).

C. Anti-bacterial/antiviral activity: The phenolic and aloins of *Aloe vera* were found to be dose dependent non competitive and showed inhibitory effects on *Clostridium hystolyticum* metalloproteinases and collagenase (Kathuria et al., 2010). The anti-microbial activity of *Aloe vera* juice was investigated by Cock Ian Edwin (2008) by agar disc diffusion against a panel of bacteria, fungi and yeast. *Aloe vera* juice showed anti-bacterial activity against the Gram-negative bacteria hydrophilia and E.coli and not against any fungi or yeast tested. Similar results have been obtained by Alemdar and Agaoglu (2009) and established the anti-microbial activity of the *Aloe ve*ra juice against Gram-positive bacteria (*Mycobacterium smegmatis, Staphylococcus aureus, Enterococcus faecalis, Micrococcus luteus and Bacillus*

sphericus), Gram-negative bacteria (*Pseudomonas aeruginosa, Klebsiella pneumonniae, E.coli and Salmonella typhimurium*) and *Candida albicans.*

D. Antioxidant or pro-oxidant activity: The antioxidant activities of anthraquinone and anthrones of *Aloe vere* have been evaluated using different model systems (Pulok et al., 2014).

E. Cytotoxicity / antitumoral effects: *Aloe vera* in general and Aloe-emodin in specific have been reported to have *in vitro*-cytotoxic effects against tumor and abnormal cells. Aloe-emodin was shown to have specific dose dependent cytotoxic effects on non-epithelial tumors in particular neuroblastoma cells (Teresa Pecere et al., 2000).

6.3. Medicinal properties of leaf gel:

It has been claimed that the poly saccharides in *Aloe vera* gel have therapeutic properties such as immunostimulation, anti-inflammatory effects, wound healing, promotion of radiation damage repair, antibacterial, anti-viral, anti-fungal, antidiabetic and anti-neoplastic activities, stimulation of hematopoiesis, cosmetic effect, dentistry and antioxidant effects (Gomathi Periasamy et al., 2014).

A. Immunostimulation:

Aloe products can modulate and stimulate both humoral and cellular immunity and it also stimulates proliferation of murinepluri potent hematopoietic stem cells, granulocyte macrophage colony-forming cells and cells forming myeloid and erythroid colonies. It is demonstrated that immunomodulating and immunostimulating effects of these products are dependent on the activation of the innate immune cells (macrophages, neutrophils, lymphocytes and NK cells), synthesis and release of cytokines (TNF-α, IFN-α, IFN-γ, IL-1, IL-2, IL-6, IL-8), generation of enhanced cell-mediated responses, and

induction of nitric oxide production. Besides the role in cellular immunity, researchers also found that *Aloe vera* and β-Glucan induced antibody responses. The effects on antibody responses were proved by the significant increase in the number of B lymphocytes forming both IgM and IgG antibodies. It is reported that Aloe products can be used as an effective aduvant when administered with antigen (Nuri Altug et al., 2010). Masood Akhtar et al., (2012) has worked on immunostimulatory and protective effects of *Aloe vera* against coccidiosis in industrial broiler chicken and proved that *Aloe vera* can be used as immunotheurapitic agent coccidiosisin industrial broiler chickens.

B. Anti-inflammatory activities:

Chronic inflammatory diseases remain one of the major health problems. Inflammation is the response of living tissues to injury. It involves a complex array of enzyme activation, mediator release and extravasation of fluid, cell migration, tissue break down and repair inflammation is one among them. Conventional drugs used to ameliorate this phenomenon are either too expensive or toxic and not commonly available to real folks that constitute the major population of the world *Aloe vera* promotes a variety of anti-inflammatory responses in the body reducing swelling from injuries and promoting recovery from infections. Such anti- inflammatory responses in the body, not only aid in the relief of pain and discomfort, but also enhances the overall wound relief process (Sampath kumar et al., 2010). The presence of small amounts of barbaloin has an antiseptic effect which may help the healing process by reducing the risk of infection (Hans B. Juneby, 2009).

There are many reports showing the anti-inflammatory effect of *Aloe vera* gel, (Yagi et al., 2002). Aloe contains the enzymes like carboxypeptidase and bradykinase both of which have been shown to relieve pain and decrease inflammation and swelling (Duke, 1997). Sturm and Hayes (1984) stated that Aloe may be used as an anti-inflammatory agent after oral surgery or brushing. Bradykinase, isolated from Aloe is known to break down the bradykinin, an anti-inflammatory substance that induces pain (Ito et al., 1993). A new hormone isolated from *Aloe vera* exhibited topical anti-inflammatory activities (Hutter, 1996). Mantle et al., (2001) studied the adverse and beneficial effects of plant extracts on skin. Preparations of *Aloe vera* have been used to treat frostbite (Miller, 1995), ulcers (Thomas et al., 1998) and psoriasis (Seyger et al., 1998). Aqueous extracts of whole leaf of *Aloe vera* at various concentrations (100, 200, 400, and 600 mg/kg of body weight) was investigated for its anti-inflammatory and analgesic activities in albino *Wistar* rats in which oedema was induced by carrageenan and formaldehyde. Whole leaf aqueous extract showed dose dependent tolerance on anti-inflammatory and analgesic activities (Aruna and Thirunethiran, 2011).

C. Wound healing properties:

Aloe vera has an ancient history for healing wounds and found in folk medicine for treatment of burns and chronic wounds (Saleem et al., 1997). *Aloe vera* gel is widely used as a natural remedy for burns (Haller, 1990). Aloe gel is widely used for treatment of inflammatory skin disorders. According to De Azeved et al., (2001), Aloe accelerates the wound healing while according to Heggers (1996) Aloe gel treatment accelerates wound contraction. Thompson (1991) reported that topical application of the *Aloe vera* derived allantoin gel stimulated fibroblast activity and collagen

proliferation. Study showed that topical application of aloe gel re-established the property of vascularity of burn tissue for a guinea pig, though no specific constituents were recognised (Heggers et al., 1993). Heggers et al., (1995) found a commercial preparation of *Aloe vera* gel that reduced the healing time wounds in rats. Davis et al., (1989) noted that *Aloe vera* gel improved wound healing by increasing blood supply which increased oxygenation of blood vessels.

Zawahry et al., (1973) detected that the active healing principle existed in the mucopolysaccharides of the aloe gel. A mannose-6-phosphate component of the gel has been credited with a wound healing effect (Davis et al., 1994). Yagi et al., (1997) reported that *Aloe vera* gel contains a glycoprotein which has cell proliferating promoting activity. The *Aloe vera* gel polysaccharide acemannan was shown to activate macrophages, an effect that improved wound healing in rats (Tizard et al., 1994; Maxwell et al., 1996). Mantle et al., (2001) studied the adverse and useful effects of plant extracts on skin. The average healing time was shorter in aloe gel treated wounds (11.89 days) when compared to Vaseline gauze treated wounds (18.19 days) (Visuthikosol et al., 1995). Scientific evidences that support the use of *Aloe vera* as effective wound healer are limited. Some studies show that aloe promotes the speed of healing (Vogler and Ernst 1999). Recently, Feily and Namazi (2009) found that oral administration of aloe in mice is effective on wound healing will reduce the number and size of papilomas and also reduce the incidence of tumors and leishmania parasitemia by 90%.

D. Promotion of radiation damage repair:

All though Aloe gel has an old history protection against radiation injury, it was scientifically proved by Williams et al., (1996). The juice of aloe made as an ointment in

Vaseline has been found to fasten healing of wounds of thermal burns and radiation injury in albino rats (Singh et al., 1973). A positive impact additionally was documented in mouse skin exposed to soft x-irradiation (Sato et al., 1990). Pande et al., (1998) studied that treatment with the Aloe extract reduced radiation induced damage to germ cells and loss in weight. Studies show that aloe gel contains immunomodulator that prevent ultraviolet B induced damage on epidermal cell (Fox, 2003).

Acemannan containing topical gel was demonstrated to reduce skin damage following exposure to gamma radiation in mice (Roberts and Travis, 1995).

E. Anti- bacterial properties of Aloe:

Aloe vera has a long history of medicinal usage and its biological activities have been well documented in different forms of bioassays. *Aloe vera* extracts are being widely used in anti-bacterial and anti-fungal products (Farnsworth, 1984). Many scientific studies support anti-bacterial activity of *Aloe vera* extracts (Klein and Penneys, 1988). Heggers et al., (1995) suggested that the anti-bacterial effect of the *Aloe vera* gel could enhance the wound healing process by eliminating the bacteria that contribute to inflammation. Heggers et al., (1979) tested *Aloe vera* gel and Dermaide Aloe (a commercial purified extract) against ten bacterial strains viz. *Streptococcus pyogenes, Staphylococcus aureus, Streptococcus agalactiae, Escherichia coli, Serratia marcescens, Klebsiella sp., Enterobacter sp., Citrobacter sp., Bacillus subtilis* and *Candida albicans.* At 90% concentration *Aloe vera* gel was effective against all the organisms but at 70% concentration only against *S. Pyogenes*. It was found that preserved *Aloe* gel extract was more effective in controlling bacterial growth than unpreserved one *Aloe vera* gel was shown to inhibit the growth of gram positive bacteria, *Shigella*

flexneri and *Streptococcus pyogenes* (Ferro et al., 2003). Roboson et al., (1982) studied the anti-bacterial effects of *Aloe vera* extract and found that concentration as low as 60% were bactericidal against seven of the 12 species of organisms studied. Heck et al., (1981) tested preserved Aloe gel extract and an unpreserved aloe extract against *Pseudomonas aeruginosa, Enterobacter aerogenes, Staphylococcus aureus* and *Klebsiella pneumoniae*.

A variety of isolates from *Aloe vera* were shown to inhibit microbes like *Staphylococcus aureus* (Martinez et al., 1996; Servet et al., 2005; Gaurav Kaithwas et al., 2008), *Pseudomonas aeruginosa* (Servet et al., 2005), *Klebsiella pneumoniae* (Heggers et al., 1979). The anti-bacterial activity of leaves in *Aloe vera* is attributed to anthraquinones (Boateng, 2000; Dabai et al., 2007) and saponins present in it (Reynolds and Dweck 1999). Acemannan, a polysaccharide component from aloe has been proposed to have indirect anti-microbial activity through its ability to stimulate phagocytic leukocytes (Lawless and Allan, 2000).

The comparative study on anti microbial screening was carried out by Yebpella et al., (2011) found that the minimum inhibitory concentration (MIC) of the extracts against organisms ranged from 6.25-25 mg/ml while the minimum bactericidal concentration (MBC) was within the range of 12.5-50 mg/ml. This study revealed that the green rind methanol extract and gel have greater medicinal potential against the *B.subtillis.*

A work on antimicrobial and immunomodulatory effects of *Aloe vera* peel extract was carried out by Ka Hee Kwon et al., (2011) which proved that it was significantly apparent as seen by the decreased colony counts in the Aloe treated groups compared with the untreated groups for the entire tested gram positive and gram negetive bacteria. Antimicrobial activity of ethonolic extracts of *Aloe vera* plant against five microbial

cultures namely *Pseudomonas aeruginose, staphylococcus aureus, Escherichia coli, Bacillus subtillis and Aspagillus niger* was studied by agar well diffusion method which showed inhibition of the above bacteria at different levels (Pratibha Prashar et al., 2011). Effect of Pomegranate and *Aloe vera* extract on streptococcus was evaluated by Priya Subramaniam et al., 2014 who proved that a significant inhibitory effect was seen with extracts of *Aloe vera*.

F. Antifungal activities of *Aloe vera*:

Antifungal activity of leaf powder of *A. barbadensis* has been established by many workers. Ali et al., (1999) found that the extracts of fresh leaves of *Aloe barbadensis* and *Aloe arborescens* have anti-fungal potential against *Aspergillus niger, Cladosporium herbarum* and *Fusarium moniliforme*. *Aloe vera* extract have been shown to inhibit the growth of fungi that cause tinea (Sumbul et al., 2004). Antifungal activity of *Aloe vera* was analyzed against *Aspergillus flavus* and *Aspergillus niger* by disc difussion method which showed that the maximum anti fungal activities was observed in acetone extract disc than aqueous and ethanol extracts on *Aspergillus flavus* (Arun kumar and Muthuselum, 2009). The leaf extract of *Aloe vera* had an inhibitory effect against *Pseudomonas auruginosa* which is known to cause skin infection especially at burns site, wounds, pressure sores and ulcers (Pratibha Prashar et al., 2011).

G. Antiviral Properties

Womble and Helderman (1988) published the first study indicating that Acemannan isolated from *Aloe vera* had an anti viral effect on human cells. Kahlon et al., (1991) tested acemannan for anti-viral activity against HIV-I. Glycerine extract of *Aloe barbadensis* inactivated a variety of viruses, including *Herpes simplex* virus (HSV-1 and

HSV-2), *Pseudorabies virus*, *Varicella zoster* virus, influenza virus, rhinovirus and adenovirus (Sydiskis et al., 1991). Yates et al., (1992) demonstrated that the anti-viral activity of acemannan in a pilot study of clinically symptomatic immuno-deficiency virus infected cats. Zandi et al., (2007) tested the anti-viral activity of a crude hot glycerine extract of *Aloe vera* gel against HSV-2 replication in *in vitro* cell line (African green monkey kidney cell line). Aloe leave extracts have been reported to have anti-viral activity by a number of workers (Andersen et al., 1991 ; Semple et al., 2001; Malvy et al., 2005). Antiviral activities of flowers, flower peduncles, leaves and roots of *Aloe hijazensis* against hemagglutinating viruses of avian paramyxovirus type-1 (APMV-1), avian influenza virus type A (AI-H5M1), newcastle disease virus (NDV) and egg-drop syndrome virus (EDSV) a specific pathogen free chicken embryos were performed by Howaida et al., (2012).

H. Anti-oxidant effects

The importance of reactive oxygen species (ROS) and free radicals has attracted increasing attention over the past decade. ROS includes free radicals such as superoxide anion radicals (O_2^-), hydroxyl radicals (OH^-) and non-free radical species such as H_2O_2 and singlet oxygen (O_2) are various forms of activated oxygen. ROS is continuously produced during normal physiologic events and they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. Under pathological conditions, ROS is overproduced and results in oxidative stress. There are a lot of antioxidants that are introduced to minimize actions of ROS. Phenol compounds can trap the free radicals directly or scavenge them through a series of coupled reactions with

antioxidant enzymes. Thus, efforts have been made to search for novel natural antioxidants from fruits, vegetables, herbs and spices.

Evaluation of anti oxidant potential of *Aloe vera* extracts were carried out by Yun Hu et al., (2003). Attempts were made to investigate the effect of *Aloe vera* leaf gel extract on tissue lipid peroxides and enzymatic oxidants in rats with STZ induced diabetes which proved that *Aloe vera* leaf gel extracts acts as an effective anti-oxidant (Subbaiah Rajasekaran et al., 2005) Anti oxidant capacities of two different *Aloe species* were confirned by Lisa Botes et al., (2008). The antioxidative properties of extracts of *Aloe vera* gel made in methanol (MEAG), 95% ethanol (EEAG), hexane (HEAG), acetone (AEAG) and chloroform (CEAG) were investigated employing various *in vitro* systems viz. 1, 1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anion radicals scavenging, metal ion chelation, reducing power, hydroxyl radicals scavenging and total antioxidant activity in linoleic acid emulsion system. The results showed that MEAG and AEAG possessed maximum DPPH free radical and superoxide radical scavenging activities. All the extracts were effective in scavenging the hydroxyl radicals in non site-specific assay as well as in site specific assay (Saritha and Anil Kumar, 2010). The antioxidant activity of the Aloe extract was evaluated spectrophotometrically following the DPPH method with different concentrations of the extracts (5, 10, 15, 20 and 25 μg/ml) in methanol and ethanol. The result was in agreement with the statement, *A.vera* gel made in methanol and ethanol possessed maximum DPPH free radical scavenging activities (Tin Khaing, 2011).

I. Antidiabetic Properties

Diabeties mellitus refers to a group of metabolic disorders that results in increased blood glucose concentrations, either because the pancreas does not produce enough functional insulin (type-I diabeties) or because cells donot respond to the insulin which is produced (type-II diabeties). The causes of diabetes mellitus include the autoimmune destruction of pancreatic cells. Number of studies has indicated the beneficial effect of *Aloe vera* extracts in diabetic patients. Antidiabetic potentials of Aloe plants have been established by various workers (Bunyapraphatsara et al., 1996; Yongchaiyudha et al., 1996; Okayar, 2001; Grover et al., 2002; Jones, 2004). Oral administration of *Aloe vera* was helpful for lowering blood glucose in diabetic patients (Yongchaiyudha et al., 1996; Bunyapraphatsara et al., 1996). These positive effects are thought to be due to the presence of compounds such as polysaccharides, mannans, anthraquinones and lectins. There are some evidences that shows *Aloe vera* extract may be useful in the treatment of diabetes (Boudreau and Beland, 2006). Significant decrease in blood-glucose level after oral administration of ethanol extract of *Aloe vera* was observed in streptozotocin induced diabetes in rats (Rajasekaran et al., 2005). Noor et al., (2008) reported that *Aloe vera* extract possesses antidiabetic and hypoglycemic activity. Ghannam et al., (1986) reported the anti-diabetic effect of *Aloe vera* in alloxan induced mice. Extracts of Aloe increases glucose tolerance in both normal and diabetic rats (Al-Awadi and Gumaa, 1987).

Evaluation of hypoglycemic and anti atherogenic effect of *Aloe vera* in diabetes mellitus induced albino rats was carried out by Anupama Gupta et al., (2011). It was proved that the *Aloe vera* as potential antidiabetic drug. The outcome of the study showed

that after 30 days of treatment with Aloe leaf extract, the diabetic rats showed significant reduction in blood glucose level (Saghir et al., 2011). Saif-Ur-Rehman et al., (2011) proved that the blood glucose levels of treated groups of rats showed significant reduction after 30 days treatment with *Aloe vera* water extract.

I. Anti-neoplastic activities or anti-cancer activity

Cancer continues to represent the largest cause of mortality in the world and claims over 6 million lives every year. Plant vegetables and herbs used in the folk and traditional medicine have been accepted currently as one of the main source of cancer chemoprevention, drug discovery and development (Naveena et al., 2011). The induction of oxidative stress has been linked with several types of cancers. Currently used anti cancer agents eg. doxorubicin, daunorubicin, mitomycin C, etoposide, cisplatin arsenic trioxide, ionizing radiation and photodynamic therapy depend exclusively or in part on the production of ROS for cytotoxicity, sensitivity of tumour cells to oxidative stress or apoptosis may affect treatment success. *Aloe vera* product Acemannan was found to be effective on fibroscsrcomas (King et al., 1995). Treatment of (Ehrlich Ascite Carcinoma Cell) EACC tumors with *Aloe vera* leaf active principles isolated *via* supercritical carbondioxide extraction active principles resulted in a significant elevation in activity of key antioxidant enzymes (SOD, GST, tGPx, and LDH). Naveena et al., (2011) proved that ethanolic extract of *Aloe vera* was effective against Ehrlic acites carcinoma (EAC) tumor in mice which exhibited antitumor effect by modulating lipid peroxidation and augmenting antioxidant defense system in EAC bearing mice.

K. Stmulation of hematopoesis:

The major active components of Aloe gel include minerals, aminoacids, poly phenols and poly saccharides, glucose and mannose are the main polysaccharides in the gel. The gel has been reported to possess various biological activities such as hepato protective and hematopoitic activity (Min-cheol Kang et al., 2014).

L. Cosmetic Properties:

Aloe has a long historical use as a cosmetic agent. There are historical claims which state that famous queen cleoptera of the 1st century BC used aloe gel as a beauty aid. *Aloe vera* is being used in a large scale in cosmetics such as skin moisturizers, face creams, body lotions, sunscreen, soothing night cream, shampoos, soaps, cleansers, hair tonics, shaving creams, bath aids, make-up, fragrance preparations and wipes (Gallagher and Gray, 2003). The clear pulp that is additionally referred to as gel is widely used in cosmetic, medical and neutraceutical applications (Ni et al., 2004). Hu Q et al., (2005) noted that higher antioxidative activities of *Aloe vera* extract is due to components in its rind. *Aloe vera* has been used externally to treat various skin conditions such as cuts, burns and eczema (Serrano et al., 2006). Aloin and its gel are used as skin tonic against pimples.

Gels used in cosmetics have been increased by claims that it has anti-aging effects similar to vitamin - A derivatives (Saeed et al., 2004). *Aloe vera* is used for soothing the skin, and keeping the skin moist to help avoid flaky scalp and skin in harsh and dry weather. The Aloe sugars are also used in moisturizes preparations. Mixed with selected essential oils, it makes an excellent skin smoothening moisturizer, sun block lotion plus a

whole range of beauty products. Due to its soothing and cooling qualities, Maharishi Ayurveda recommends *Aloe vera* for a number of skin ailments (Pankaj Sahu et al., 2013)

M. *Aloe vera* in Dentistry

Hayes, (1999) discussed the useful effects of *Aloe vera* treatment during a case study of patient diagnosed with 'Lichen Planus', an illness that is brought by emotional distress and cause lace-like lesions in the oral cavity and on the skin. Similar impact has been reported by Choonhakarn et al., (2008) ; Uzma et al., (2008) in their study stated that Aloe gel was safe and effective treatment for patients with vulval lichen planus, a chronic inflammatory disorder of mucosal surfaces. Dilip George et al., (2008) describesd an *in vitro* investigation that compared the antimicrobial effectiveness of *Aloe vera* tooth gel with two popular, commercially available dentifrices. The preliminary results showed that *Aloe vera* tooth gel and the tooth pastes were equally effective against *Candida albicans*, *Streptococcus* mutans, *Lactobacillus acidophilus*, *Enterococcus faecalis*, *Prevotella intermedia* and *Peptostreptococcus anaerobius*. The Aloe seems to promote more rapid healing and pain relief.

Aloe vera has shown multiple uses in dentistry. Some of its extreme uses have been observed in the treatment of gum diseases by reducing bleeding of the gums, acting as antiseptic in gum pockets and its antifungal properties help greatly in the problem of denture stomatitis (Sambhav Jain and Rohit Rai, 2014).

7. Other Therapeutic uses

Aloe vera plants have been used for the treatment of hepatitis (Kim et al., 1999). Awang and Dennis, (2009) postulated some of the beneficial effects of Aloe which stated

that a caboxypeptidase inhibit the pain producing agent bradykinin. Some of the *Aloe species* are used around the world for conditions ranging from dermatitis to cancer (Kemper and Chiou, 1999). Lee et al., (2000) shown that the aqueous ethanol extract of *Aloe vera* powder have anti-mutagenic and anti-leukemic activities.

Aloe is used in several other purposes like reduction of burns and incisions, adjuvant arthritis, frost bite, psoriasis, edema, decreasing cholesterol levels, lowering plasma levels of calcitonin and parathyroid hormone etc. (Reynold and Dweck, 1999). Larvicidal activity of *Aloe vera* leaf extract was tested on *Culex alinarius* (Ramesh Kumar et al., 2013). Pankaj et al., (2013) in his review revealed that *Aloe vera* is anthelmintic, aperients, carminative, depurative, diuretic, stomachic and emmena-gogue. Aloe juice is used in skin care medicine, dyspepsia, amenorrhea, burns, colic, hyperadenosis, hepatopathy, splenopathy, constipation, span menorrhea, abdominal tumors, dropsy carbuncles, sciatica, lumbago and flatulence. The elio, a product made by juice of this plant, is used for helminthiasis in children and is a purgative, anthelmintic and emmenagogue. It is said that acemannan is also used on patients affected by AIDS.

8. *Aloe vera* products

Aloe vera is being used in a large scale in cosmetics such as skin moisturizers, face creams, body lotions, sunscreen, soothing night cream, shampoos, soaps, cleansers, hair tonics, shaving creams, bath aids, make-up, fragrance preparations and wipes (Gallagher and Gray, 2003). *Aloe vera* has number of commercial uses mainly as a food preservative and medicine. Commercially, aloe is found in pills, sprays, ointments, lotions, liquids, drinks, jellies and creams (Karkala Manvitha and Bhushan Bidya, 2014).

List of some Aloe products, their uses which are commercially available are given below with the company name and purpose.

1. Shampoo: prevents hair loss, anti dandruff, revitalizes hair roots, chemical free and suitable for all kinds of hair (Besure Health Pvt. Limited, New Delhi).
2. Gel: for skin diseases, burns, cuts, wounds, ulcers, eruptions etc. (S.S Life Sciences Pvt. Limited).
3. Body wash: it hydrate and nourish the skin, protects the skin from UV rays and other skin infections. (Besure Health Pvt. Limited, New Delhi).
4. Body lotion: it hydrates, moisturizes and revives the skin. Repair damage cells, gives soft and healthy skin (Forever living products company).
5. Sunscreen lotion: it has high SPF for more sun protection, safe and gentle, sooth, lubricate, moisturize and protects the skin again sunlight and wind (Forever living products company).
6. Tea: low calorie, no caffeine, refreshing (Forever living products company). Soothing night cream, moisturizes, comfort, calm and sooth the skin (Shop on line).
7. Cosmetics: highly effective for maintaining skin healthy. No chemicals are used. (Skin care naturals (P) Ltd. Delhi).
8. *Aloe vera* cream: highly effective to protect skin from atmospheric pollution and blemishes, freshness to the skin. (Kapoor herbal products).
9. Soft Gels (5000 mg): helps in treating ulcers, heal external wounds, including burns. (Nutri - Force Nutrition).

10. Arthritis cream: *Aloe vera* is a powerful anti - inflammatory and an analgesic (Besure Health Pvt. Limited, New Delhi).

9. Toxicity of Aloe

Excessive use of Aloe extract causes diarrhoea, nephritis, gastritis and vomiting (Capasso et al., 1998; Goodman and Gilman 1990). Aloe gel has few side effects. In doses of 0.6 gm per day is unsafe for pregnant women and is likely to cause abortion. It is contraindicated in pregnancy and in individuals afflicted with hemorrhoids and can cause kidney irritation (Duke and Wain, 1981). It should not be used during pregnancy except under medical supervision. Mixed *Aloe vera* gel with prednisolve and indomethacin and incorporated it into cell maintenance medium to look for cytotoxicity in He La cells and rabbit kidney fibroblast. Winters et al., (1981) found *Aloe vera* gel was cytotoxic for human normal and tumor cells. It should not used by nursing mothers (Brinker, 1998). Danof and Analley, (1983) tested four commercial stabilized *Aloe vera* gel samples and yellow Aloe sap for cytotoxicity. A dose of 0.5g/day could produce a strong purgative and oxytoxic properties provoking uterine contractions in females (George, 1999). They discovered sap was lethal to human fibroblasts and two gel products were cytotoxic to human endothelial cells and fibroblasts. Tello et al., (1998) stated that formulations of acemannan had significant cytotoxicity against human fibroblasts.

Hypersensitivity and allergic conditions to aloe preparations have been reported (Morrow et al., 1980). There have also been several reports of aloe gel lowering plasma glucose levels in laboratory animals and in humans (Ghannam et al., 1986). There are some reports of burning sensations and development of dermatitis on the face of

patients who applied gel (Hunter and Frumkin, 1991). The Handbook of Medicinal Herbs (Duke and Fulton, 2002) has given the lowest ranking for toxicity in Aloe. It was also reported to have genetic toxicity (Muller et al., 1996) and besides that the first case of acute hepatitis due to ingestion of this compound was described by Rabe et al., (2005); Yang, (2010).

10. Molecular Studies

The most common uses of the technique for scientific and commercial purposes include the discrimination among species for post harvest purposes, identification of crop cultivars to settle lawsuits, marker assisted selection and establishment of phylogenetic position of natural populations. The multipurpose use of *Aloe vera* attracted global attention which prompted for the conduct of molecular studies. The availability of the variety of the DNA markers such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and inter simple sequence repeat (ISSR) has enabled researchers to investigate genetic diversity among various plant species across natural populations (Sanjay Lal et al., 2011).

Hiroko et al., (2003) used RAPD analysis for identification of three species of Aloe i.e *Aloe vera*, *Aloe arborescence* and *Aloe ferox*. Adams et al., (2000) investigated the physical organization of 18S-5.8S-26S and 5S ribosomal DNA (rDNA) using fluorescent *in situ* hybridization (FISH) of 13 Aloe species. A team of workers at CIMAP, Lucknow carried out the assessment of genetic diversity in Aloe using RAPD and AFLP analysis (Darokar et al., 2003). Yagi et al., (2006) compared

ribosomal DNA sequence analysis of different geographically distributed *Aloe vera* plants which were clonally regenerated.

Eleven *Aloe* germplasm accessions; *A. vera*, *A. perryi*, *A. lotus*, *A. zeylanicum* and seven strains of *A. vera* available at the Defense Agricultural Research Laboratory (DARL), Pithoragarh, and Medicinal and Aromatic Plant Research Development Centre (MRDC), Pantnagar, Uttaranchal, India were subjected to Random Amplified Polymorphic DNA (RAPD) analysis in relation to morphometric parameters for estimating the extent of diversity within and between species. Morphological evaluation of the 11 accessions for selected characters showed qualitative variation among the accessions studied (Nayanakantha et al., 2010). Assessment of genetic stability and instability of tissue culture-propagated plantlets of *Aloe vera* L. by RAPD and ISSR Markers was carried out by Rathore et al., 2011). Cytogenetic assessment of two years old micropropagated plants and mother plants was analysed by RAPD profiles. These results confirm the reliable method for large scale production of true-to-type plantlets of *A. vera*, which can be used for commercial purpose (Moquammel Haque et al., 2013).

11. Cultivation of *Aloe vera*

Aloe vera is widely used in the commercial industry which makes that cultivatation of *Aloe vera* very important. *Aloe vera* is grown worldwide in temperate climates and can be found in commercial operations in US, Central America, South America, China, India, Africa, the Caribbean, Australia and the Asian tropics. Recent request for assistance in setting up greater commercial cultivation and processing operations in countries such as Greece, Iran and other middle-eastern areas have been noted by IASC (International Aloe science council 2009-2012). Though, Aloe is native to

North Africa and Spain, the plant is now also grown in the hot dry regions of Asia, Europe and America.

Aloe plant survives for more than 7 years without water. It takes water it needs for survival and growth from dew collected on the surface of its leaves. It avoids attacking insects, rodents and snakes by means of the bitter Aloin (the yellow colored part of the sap) just beneath the rind (Karkala Manvitha and Bhushan Bidya, 2014). *Aloe vera* is found as wild herb along the coast of South India. It is under cultivation fairly in large areas in many parts of India Viz. Chatisgarh, Maharastra, Madhya Pradesh, and Gujarath etc.

Climate: Aloe plant due to its stiff, rugged and harsh habit they grown in desert and dry situations. Hot and dry climate suits for its cultivation.

Soil: It grows well on variety of soils, light, muram, loam, black, hill slopes, sea coasts, desert areas etc. It grown well in all kind of soils but the soil should be drained with high organic matter. It needs in bright sun light. Shady conditions may lead to infestation. Plant is highly sensitive to water stagnation. Therefore, lands with 50 cm of rain annually well drained high lands rich in nitrogen and alkaline soil are suitable for its cultivation. Soil nitrogen should ideally be maintained at 0.40%–0.50% while most species of Aloe typically grow in sandy soils.

Tillage: One shallow ploughing followed by harrowing to bring the soils to fine tilth. Mix 5-20 cart loads of FYM (Farm yard Manure) in soil. This will facilitate better moisture holding capacity improve soil texture and structure.

Propagation: Through suckers.

Spacing: 60 x 45 sq. cm (between rows and between plants) in flat beds or by opening ridges 60 cm apart.

Planting: Onset of monsoon is the best time for planting of Aloe. The crop can be planted throughout the year under irrigated conditions. Planting with 60 x 45 sq.crn will require 14800 - 15000 suckers / acre.

Interculturing: The crop should be kept weed free. Suckers planted in flat beds should be supported at the bottom with adjoining soil.

Fertilizer: Lighter, soils should be fertilized with compound fertilizer @ 50 kg N + 25 Kg P + 25 kg K per hectare in order to facilitate good growth of the crop.

Irrigation: During rainy season there is no need to irrigate the crop; however, during summer crop should be irrigated with an interval of 15 days.

Diseases: Appearance of black brown spot can be well controlled by any sulphur containing insecticides.

Harvesting: The leaves can be harvested after 10-12 months of planting by cutting the leaves with sharp sickle close to the stem. Cut leaves were kept aside for some time till the viscous gel from the cut end dries up. Then collect the leaves for packing.

Yield: One year old plant will produce about 3-4 kg leaves. It means 40-45 tonnes of leaves can be harvested from 1 acre planting.

Market: As per the contract Aloe leaves can be solid in India @ Rs. 2.50 to 3.00 per kg.

II. *ALOE FEROX*:

The *Aloe ferox* is one of the species of Aloe originated from South Africa.

1. Growth and development: It can grow to 10 feet (3.0 m) in height, and can be found in grassy fynbos, on rocky hills and on the edges of the Karoo. It was observed that it grows at selected sites in the Makana region of the Eastern Cape (Shackleton and Gambiza, 2007).

Leaf: *Aloe ferox* leaves are thick and fleshy, arranged in rosette shape with reddish-brown spines, on its margins contains smaller spines on both upper and lower surfaces (*Aloe ferox* Floridata, 2009).

Flower: Its flowers are orange or red, and stand between 2 and 4 feet (0.61 and 1.2 m) above the leaves. *Aloe species* follow the Crassulacean acid metabolism (CAM). Minimizing of water loss in CAM plants caused by closed stomata during day time. It results photosynthesis to fix CO_2 at night. The succulent leaves and stems and the presence of a thick cuticle, makes them well adapted to dry conditions. Severe drought stops exudates production from the leaves.

Stem: It has a stem surrounded with a persistent layer of dead leaves (Bond, 1983) that insulate the stem in the case of bush fires.

Flower: The flower morphology of *Aloe ferox* suggests pollination by birds. However, honey bees also play a role in the pollination. It is self incompatible and only a few flowers per raceme. The stamens produce pollen in the morning and wither in the afternoon, whereas the style is exerted on the second day of anthesis.

Seeds: Fruit is an ovoid capsule upto 3 cm long, many seeded. The seeds are about 9mm long broadly winged the seed storage of *Aloe ferox* orthodox. The dry seeds survive without significant reduction in their viability and thus can be store by long term freezing.

2. Ecology: *Aloe ferox* is one of the dominant species in the 'succulent bush land' vegetation in South Africa. It is especially abundant on arid rocky hill sides up to 1000 m altitude, where mean temperatures range from 27 - 31°C and annual rainfall ranges from 50 - 300 mm. It grows in a wide range of climatic conditions. It occurs in land with moderate or less rains and dry, less fertile soil, water requirement is moderate during the growing season and springy in dormant stage. Seed can be sown at 70^{o}F or separate offsets in late spring. Though the root system is shallow, the plant can grow under dry conditions. Water logging should be avoided.

3. Geographical distribution: *Aloe ferox* is indigenous in South Africa. It is distributed from the Dutch East India Company's garden to the Cape throughout the tropics and subtropics. It is grown as an ornamental and medicinal plant. Although not treated or mentioned in regional floras, its occurrence is quit familiar in several countries of tropical Africa.

4. Genetic resources and breeding: *Aloe species* are potentially at risk of extinction as a result of plant collection from the wild. *Aloe ferox* is cultivated widely as an ornamental plant. Harvesting from the wild is still considered sustainable but warrants monitoring. *Aloe ferox* does not produce suckers but can be propagated by seed and planting of the tops of old plants. Plant regeneration from root and embryo tissue is successful as well.

5. Breeding in *Aloe ferox*: There is no existed information on *Aloe ferox* breeding programmes. Therefore an enormous scope is required for breeding of *Aloe ferox* in future (Protologue, 2013).

Management: Commercial cultivation becomes a profitable option now that not only the exudates but also the gel has become interesting. Details on cultivation have not been published.

6. Cultivation practices: *Aloe ferox* prefers dry tropical climates, sandy loamy soils, open areas, full sun (Nema et al., 2013) and moderate watering with good drainage system. *Aloe ferox* plants are propagated mainly from seed and head cuttings (Sahu et al., 2011). The plants are sowed one meter apart from each other in rows and columns. It takes about 4 to 5 years for the plants to reach the first harvest, from the seed stage. At the time of harvest, each leaf weighs about 1.5 kg to 2 kg.

7. Harvesting: Harvesting of leaves from wild plants of *Aloe ferox* is thought to be sustainable. *Aloe ferox* exudates is often collected by cutting off the leaves transversely close to the stem and positioning them in such a way that the exudates drains into pots, tubs, vessels or even a simple canvas placed over a depression in the ground. The exudates may also be obtained by squeezing the leaves or by warm or cold water retting. In South Africa *Aloe ferox* is preferably tapped during the rainy season, because then the exudates are more abundant, but tapping is also carried out in other periods of the year. The leaves are usually cut in the morning and it takes 4-5 h for the exudates to drain from a pile of leaves. Only older leaves are cut younger ones growing tips are spared.

8. Handling after harvest: Collected exudates are usually concentrated by boiling and then cooling. On cooling, a solid, amorphous extract is formed and this constitutes the

drug. Its appearance varies with the concentration process used. If the exudates have been concentrated slowly, in the sun or over a low fire, the cooled extract is opaque, waxy and liver colored (hepatic aloe) and aloin crystals are visible under the microscope. An option for adding value is to produce dried and ground leaf powder.

The youngest leaves less than 25 cm are not suitable for gel extraction because of their small amount of gel. Gel quantity and quality may declines if the leaves are too old. After the leaf exudates are extracted, the leaves are pulped and squeezed dry under high pressure. The liquid is settled and treated with chemicals to flocculate the jelly fraction. The gel can be obtained by removing the outer tissues of the leaf. In Aruba, gel is obtained by cutting open the leaves lengthwise and scraping the gel from the leaf blade. The dead leaves of *Aloe ferox* have an insulatory function and are used against fire for survival in South Africa (Bond, 1983).

9. Aloe sap crystals: Leaves are stacked in a circle around two to three hundred, cut surfaces facing inwards and overlapping, so that the sap drains and collects in the hollow (below middle). After some hours the sap of several draining sites are collected and processed to produce bitter crystals and bitter powder. The bitter sap is poured in a metal drum and then heated over an open fire. The contents are continuously stirred while hot, until the volume is reduced to half. The warm sap is then decanted into a tin and allowed to cool and solidify. The solid hard block can be broken and splintered into crystal of varying sizes. The leaves, from which the bitter sap has been tapped, are then further processed to make use of the aloe gel. The crushed leaves are used to prepare an aloe jelly. A wholesome fruit drink is made from this and the jelly is also a much sought after component in cosmetic products (Anonymous, 2013; Gerrylocs, 2011).

Yield: Two tons of *Aloe ferox* leaves yield about 1 kg of gel powder, which is a higher ratio than for *Aloe vera* (Protologue, 2013).

10. Diseases and pests: *Aloe species* are susceptible to some of the worst plant disease in the succulent plant world.

a) **Aloe cancer:** It is a viral infection, well known as Witches Broom. Aloe cancer is spread via mites and grows irregularly on different sights of plant. In case of rare Aloes you can cut out infected areas but the plant must be isolated as it is highly contagious and probably infects the neighboring Aloes.

b) **Aloe scale or white scale:** Aloe scale or White scale is a sap suckers about 1mm-2mm in length and they are white in color. White scale starts of as a small infestation on aloe leaves but it can turn into a serious infestation in a short period of time that will probaby result in the death of the Aloe.

c) **Ants and aphids:** Ants on their own do not cause any damage to Aloes but due to their propensity for all thing sweat they can cause severe damage when they partner up with mealy bugs and aphids. Ants and aphids have a symbiotic relationship; the ants afford protection to the mealy bugs in exchange for sweet and sticky honeydew that they excrete.

d) **Snout beetle or aloe beetle:** The presence of adult Snout beetles which feed off on Aloe leaves can be detected by the presence of circular lesions with a transverse slit in the center. Snout beetles lay their eggs at the base of Aloe leaves, the larvae, after hatching; bore into the stem just below the crown which results in the death of the plant.

e) Rot: Rot in Aloes is usually caused by humans as inexperienced growers tend to over water them. It can also be caused by Aloe beetle and mealy bugs etc.

11. Nutritional values in *Aloe ferox*: So far, more than 130 biological active compounds were isolated from *Aloe ferox* (Kambizi et al., 2005; Mabusela et al., 1990). The *Aloe ferox* leaf contains substances such as minerals, amino acids, polysaccharides, vitamins, glycosides, glycoproteins, enzymes, anthraquinones, chlorophyll, lignin, saponins (Alessandro and Stefano, 2005), sterols and other plant chemicals with numerous medicinal activities (Nema et al., 2013; Shackleton and Gambiza 2007; Dagne et al., 2000).

12. Medicinal uses: The yellow bitter sap of *Aloe ferox* is used as laxative and white aloe gel is used in health drinks and skin care products. When compared to *Aloe vera,* it has more amino acids and polysaccharides. In southern Africa Cape aloe, is used as a purgative in human and veterinary medicine. Analgesic activity, (Shelton,1991) anti-inflammatory and oxidative stress (Devaraj and Jialal 2006; Fawole et al., 2010), antiulcer (Koo,1994) wound and burn healing (Chithra et al., 1998; Jia et al., 2008), Immune modulating (Strickland, 2001; Nema et al.,2013; Zhang and Tizard, 1996), antitumor/cancer activities (Saito, 1993; Bradford and Awad, 2007), antimicrobial activity (antiviral, antibacterial, antifungal) (Reynolds and Dweck,1990, antidiabetic, (Bunyapraphatsara et al., 1996; Lichtenstein and Deckelbaum, 2001; Loots et al., 2011; Norme et al., 2000), skin protecting (Syed et al., 1997), psoriasis control (Syed et al., 1996), liver damage controlling (Kuo et al., 2002) HIV/AIDS treatment (Yamamoto,1973, antiseptic (Okyar et al., 2001; Wang et al., 1998) antioxidant activity

(Rice-Evans, 2004), cardiovasicular disease (Patch et al., 2006), Tuberculosis and bronchitis (Ferro et al., 2003).

Numerous scientific studies on Aloe gel demonstrated its analgesic, anti-inflammatory, wound healing, immune modulating and anti-tumor activities as well as antibacterial, antiviral and antifungal properties. The aloe juice has been shown to decrease cholesterol and triglycerides while demonstrating anti diabetic activity. Its medicinal properties can be attributed to the synergistic effect of the combined nutritional elements producing a more powerful effect than the individual components (Nema et al., 2013). It is used as a shampoo to promote hair growth and against dandruff and as a cosmetic to improve the complexion and to smooth the skin. It is also used as a hydrating and skin-protecting agent in creams and liquids such as shaving cream, lip balm, sun lotion and healing ointments (Protologue, 2013).

13. Internal uses: Aloe juice appears to be safe and there is no reported toxicity and toxicological evaluation of aqueous extract of *Aloe ferox* Mill (Wintola et al., 2011; Wintola et al., 2010). The mucilage in the aloe juice may interfere with the absorption of other oral medications taken concurrently. It is clinically proven that the use of anthranoid laxatives, even in the long term, does not cause cancer (Nusko et al., 2000). Aloe gel is also used for the preparation of Aloe bitter powder, Aloe drinks, Aloe bitter crystals, cosmetics, and hair and skin care products.

14. Toxicity: Internal use of aloe bitter and anthraquinones can lead to dehydration, potassium loss in patients taking corticosteroids or thiazide diuretics, potentiate digitalis and other cardiac glycosides due to low potassium levels and intestinal dependence on laxatives. Aloe bitters is not recommended for people (Nusko et al., 2000) with intestinal

inflammation and intestinal obstruction (eg. crohn's disease, ulcerative colitis, appendicitis and abdominal pain of unknown origin).

15. Phytochemical properties: Phytochemicals and their quantities identified include various phenolic acids or polyphenols (Table 1**)**. (Liu, 2002; Herraiz and Galisteo, 2004; Azam et al., 2003). The exudate of *Aloe ferox* contains 15–40% anthrone 10-C-glucosides (anthraquinone derivatives) such as hydroxyaloins and aloin. Aloin is a mixture of the stereoisomers aloin A (barbaloin (Fig 5) and aloin B (isobarbaloin- (Fig 6). Furthermore, the exudate contains the pyrone derivative aloenin and glucosylated 2-acetonyl-7-hydroxy-5-methylchromones. (e.g.aloesone, furoaloesone, aloe resin a, aloe resin B (aloesin Fig 7) and aloeresin C). *Aloe ferox* also contains glycosylated feroxidin (a tetralin) and feralolide (a dihydroisocoumarin). Aloin is an inactive laxative compound, it becomes an active aloe emodin (Fig 8**)** anthrone by Eubacterium sp. and responsible for laxative properties. In 2002 the United States Food and Drug Administration generally recognized safe and effective (GRASE) status of aloe exudates over-the-counter drugs. Anthraquinone containing laxatives such as aloe may play a role in colorectal cancer as they have genotoxic potential and tumorigenic potential in rodents (Chandra Sekhar et al., 2013).

Aloe emodin, chrysophanol and aloin A, isolated from *Aloe ferox* leaf extract, have significant antibacterial activity. Aloeresin A and B both reduce the oedematous response induced by croton oil in the mouse ear by 40%. Aloe resin B also modulates melanogenesis via competitive inhibition of tyrosinase, thus showing promise as a pigmentation-altering agent for cosmetic or therapeutic applications (Mabusela et al., 1990).

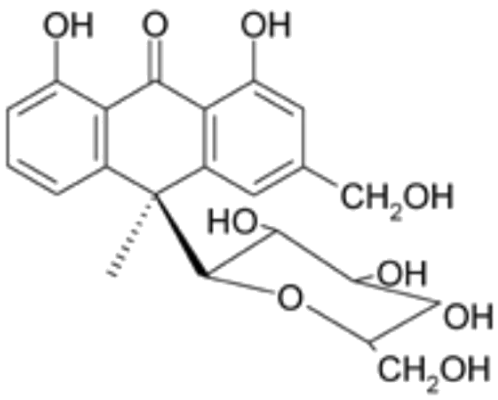

Fig 5: Aloin (Barbaloin)

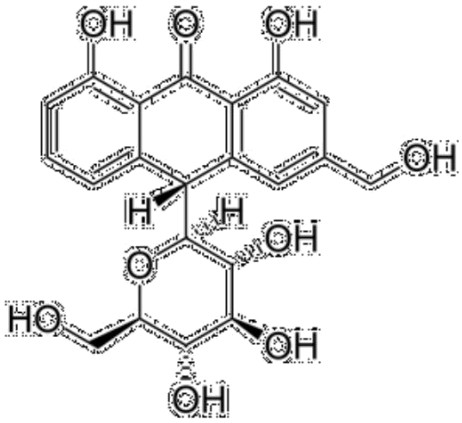

Fig 6: Isobarbaloin

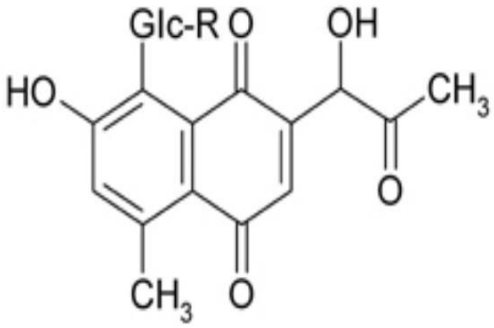

Fig 7: Aloesin

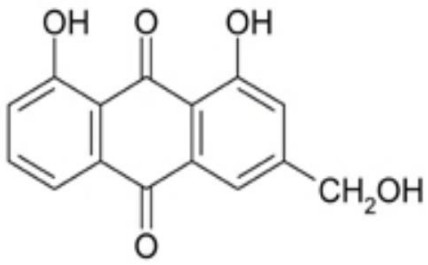

Fig 8: Aloe emodin

16. Anti oxidant and Anti-inflammatory activities of *Aloe ferox*:

Ethano pharmacological research findings showed that *Aloe ferox* and *Aloe vera* has been used in the treatment of many diseases (Loots et al., 2007; Chen et al., 2012; Celestino et al., 2013). Oxidative stress causes many diseases such as tumor growth, cardiovascular disease, cancer, wrinkled skin; alzheimer's and even a decline in energy and endurance. In recent years, there has been an increasing interest in finding natural antioxidants, which may define the human body from free radicals and retard the progress of many chronic diseases (Milady and Damak, 2008). Inflammation is a traditional protective response to tissue injury caused by physical trauma, noxious chemicals and triggered by the discharge of chemical mediators from the migrating cell and injured tissues.

The particular chemical mediators vary with the sort of inflammatory process. Some of the compounds are amines, histamine, serotonin, lipids and prostaglandins and small peptides such as Kinins (Cotran et al., 2001;Lucas et al., 2006) has suggested that many anti-inflammatory drugs might exert a number of their effects by scavenging oxidants and decreasing formation of reactive oxygen species (ROS) by activated phagocytes. Inflammati on has deferent phases and the 1st phase was caused by an increase in vascular permeability, second one by infiltrate of leucocytes and therefore the third one by tumor formation. Thefirst phase was attributed to the release of serotonin, histamine and kinins, and the second part was associated with the discharge of prostaglandins and bradykinins (Vane and Botting, 1987). It has been reported that the second phase of edema is sensitive to both clinically useful steroidal and non-steroidal anti-inflammatory agent (Patel Pinal et al., 2011; Katzung, 1998).

The anti-inflammatory activity exerted by *Aloe ferox* methanol extract (AFME) suggests that it could affect by bradykinin, kinnin, prostaglandin and synthesis of lysozymes. The association of antioxidants and inflammation stems from the recognition that, free radicals are produced during the inflammatory process by phagocytosis cells and generated as by-products of the oxidative degradation of arachidonic acid (Swingle et al., 1985). From this it can be inferred that the anti-inflammatory activity of AFME may be due to its antioxidant activity.

17. Biological activities: *Aloe ferox* plant has a greater concentration of biologically active compounds (Reynolds and Dweck, 1999; Choi and Chung 2003; Chen et al., 2012). Its healing properties are much more powerful than those of the *Aloe vera* plant.

The presence of antioxidant polyphenols, alkaloids and indoles in the *A. ferox* leaf gel showed antioxidant capacity as confirmed by Ferric reducing antioxidant power (FRAP) and Oxygen radical absorbance capacity (ORAC) analyses. Both analytical methods used show the non flavonoid polyphenols to contribute to the majority of the total polyphenol content. Due to its phytochemical composition *Aloe ferox* leaf gel may show promise in alleviating symptoms associated with or prevention of cardiovascular diseases, cancer, neurodegeneration and diabetes (Loots et al., 2007; O'Brien et al., 2011).

Prospects: *Aloe ferox* is a potential crop in arid regions. It will remain beneficial as a household remedy. Fresh gel can easily be prepared and applied to wounds. The Aloe gel industry has a bright future. Other *Aloe species* producing suckers, such as *Aloe*

turkanensis Christian and *Aloe flexilifolia* Christian from East Africa, may prove better candidates for commercial cultivation.

18. Production and international trade: Though considerable quantities of 'Cape aloe' are marketed and used locally, most of the exudates produced in South Africa are exported. Total legal harvest is about 400 tonnes per year, although an additional 300 tonnes is presumed to go undocumented. Exports are destined for Europe, Asia and North America, with the main importing countries being the United States, Japan and Germany. Production of *Aloe ferox* gel has been hampered by lack of processing facilities in South Africa. Most gel is bought by the cosmetic industry, which demands high quality. *Aloe ferox* products have less demand unlike that of *Aloe vera* products. The products of *Aloe ferox* are merely confined to South Africa, United States and few European Countries. Asian markets are mainly dominated by *Aloe vera* products.

CHAPTER-3

MATERIALS AND METHODS

OBJECTIVES

1. **Morphological characterization of different accessions of *Aloe species*.**

2. **Standardization of *ex-situ* conservation protocols for *Aloe vera* and *Aloe ferox*.**

3. **Identification of bioactive compounds through phytochemical screening *Aloe vera* and *Aloe ferox* crude extract**

4. **Screening of antidiabetic and anti-inflammatory properties of *Aloe*.**

5. **Assessment of genetic diversity using RAPD and ISSR molecular markers.**

3. MATERIALS AND METHODS

The present study was carried on germplasm of *Aloe vera* (*Aloe barbadensis* Miller) and *Aloe ferox* an important medicinal and ornamental plant collected from different places such as National Bureau of Plant Genetic Resources (NBPGR), New Delhi and other species of *Aloe ferox* seeds (50gm) were imported from South African National Bio Diversity Institute (SANBI), South Africa (Fig 18). For collection of plant materials intensive surveys have been carried out during 2008-2009 in different places of India and South Africa. The experimental material comprised of twelve accessions of *Aloe vera*.

A total of 12 *Aloe vera* (*Aloe barbadensis* Miller) accessions were collected among which 10 accessions (each 10-12 samples were collected from National Bureau of Plant Genetic Resources (NBPGR), New Delhi. The 10 accessions are IC111267, IC111269, IC111271, IC111272, IC111279, IC111280, IC471882, IC471883, IC471884 and IC471885 respectively for the evaluation of morphological characters. 2 accessions *Aloe CIM-Sheetal* and wild *Aloe vera* (local) were collected from Central Institute of Medicinal and Aromatic Plants (CIMAP), Boduppal, Hyderabad, Telangana State. *Aloe ferox* seeds (50gm) were imported from South Africa National Bio Diversity (SANB), South Africa before four years respectively for the evaluation of tissue culture, biochemical and genetic studies.

Plants have been raised at Indian Immunologicals Ltd, Hyderabad, Telangana State. The details of the accessions are presented in Table 4. The germplasm thus maintained was characterized for morphological, *in vitro*, biochemical at molecular studies.

1. Morphological studies

All the accessions were examined for a number of morphological parameters, from each accession five mature plants were randomly selected for recording observations (Table 5 a**,** b) and mean data were used for statistical analysis. Based on the morphology, the present collection of accessions of *Aloe vera* germplasm have been divided into two morphotypes viz. Small *Aloe vera* (SAV) (plant size up to 40 cm) and Large *Aloe vera* (LAV) (plant size above 40 cm). The LAV type of accessions show highest incidence.

(a) Number of leaves

Number of leaves was counted which are attached to base of the stem. The leaves may have white spots arranged in lines (Striations).

(b) Number of Suckers

Number of suckers produced around to base of the stem was counted.

(C) Number of leaves

Number of leaves produced from base of the stem was counted.

(d) Leaf width / breadth (cm)

Measured the breadth of the lamina at the widest part of the leaf.

(e) Stem length (cm)

Stem length was measured from the base of matured older leaves to the tip of younger leaves around the stem.

(f) Peduncle length (mm)

Peduncle length was measured (Ten samples) from base of attachment from middle of leave to the tip of the Peduncle.

(f) Leaf thickness (Density of leaf) (mm)

Thickness of the lamina was measured by taking the widest part of the leaf. Width of leaves was measured with vernier calipers and average mean values (per plant) were recorded.

(g) Gel fresh weight (gm)

The epidermis of fresh leaves (No. 10) was peeled and white gel present the between upper and lower layers of leaves was removed and fresh weight were recorded. Mean values of the fresh gel weight was recorded.

(h) Leaf dry weight (gm)

Collected fresh green leaves of 10 numbers were cut into small pieces, dried in shade and pulverized and made into fine powder. Dry leaf weight was recorded by taking the mean values.

(i) Biomass (gm)

Collected fresh green leaves (10 numbers) and were cut into small pieces, dried in shade and dry leaf weight mean values were recorded.

(j) Root length (cm)

Root length was measured from base of attachment of stem to the tip of the root.

(k) Aloin Conc. (%)

Collected the aloin from fresh Aloe leaves and exudates of aloin was determined by TLC, HPLC.

2. *In vitro* Studies

2.1. *Aloe vera*

A. Collection of Plant Material

Twelve accessions of *Aloe vera* germplasm collected from NBPGR, New Delhi, India vide Voucher No: NBPGR/2011/1771 dated 11.07.2011) representing totally from different geographical regions of India were obtained. The accessions were from Rajasthan (IC111267, IC111269, IC111271, IC111272), Gujarat (IC111279), Haryana (IC111280), Delhi (IC471882, IC471883, IC471884 and IC471885) and Central Institute of Medicinal and Aromatic Plants (CIMAP), Boduppal, Hyderabad, Telangana State, India (*Aloe CIM-Sheetal* and wild *Aloe vera* (local) vide Voucher No: CIMAP/63/6222 dated 20.07.2011. All the plants utilized in the present study were about two years of age. In all cases, the predominantly grown accession was used. In case of some states, where variation has been reported different provenances were collected. Accordingly, the accessions collected from the different regions were maintained at the research farm of Indian Immunologicals Ltd, Hyderabad, India and the details of the accessions are presented in Table 4. Shoot tips with young leaves were collected from the elite plants. They were trimmed to size of 2-3 cm for further work.

B. Sterilization of explants

The shoot apices were washed thoroughly under running tap water for 30 minutes to remove all the adhering dust particles and microbes from the surface. The explants were then washed with 0.1% liquid detergent (Labolene) for another 30 minutes and then rinsed several time with distilled water to remove detergent. The explants were then treated with bavistin (Systemic fungicide) for 30 min, washed with distilled water. Then the explants were treated with ampicilin (Systemic bactericide) along with streptomycin for 30 minutes to eliminate fungus and bacteria, allowed to wash with sterilized distilled water to remove the residues of fungicide and bactericide. Under the sterile conditions, explants were rinsed with isopropyl alcohol for 45 seconds, than treated with 5% Sodium hypochlorite (10% Clorox) for 30 minutes. The explants were then thoroughly washed (4-5 washings) with sterilized distilled water to remove the traces of Sodium hypochlorite. After sterilization, shoot apices were directly inoculated in to MS medium with different concentrations of hormones.

C. Culture media

AR Grade Chemicals were obtained from Fischer Scientifics and Hi Media Laboratories. The basal medium containing MS (Murashige and Skoog, 1962) salts, vitamins, 3% sucrose and agar. The basal media was supplemented with various concentration and combination of growth regulators 2, 4-D (2, 4-dichlorophenoxy acetic acid), BAP (6-benzyl amino purine), NAA (naphthalene acetic acid), KIN (Kinetin), IBA (Indole -3-btyric acid) and IAA (indole-3-acetic acid). The medium was adjusted to pH 5.8 with acid or base and dispensed into culture bottles and conical flasks of 100 ml

capacity. The media was sterilized by autoclaving at 121^{o}C and 15 lbs pressure for 20 minutes.

D. Media sterilization

Media sterilization was done using an autoclave which was operated at 121^{o}C and 15 lbs pressure for 20 minutes. The media sterilization was done before adding growth hormones into it.

E. Inoculation of explants

After sterilization of explants, explants were inoculated into culture bottles aseptically. For inoculation explants were transferred on to sterile paper with the help of sterile forceps under aseptic conditions. The explants were further trimmed and extra outer leaves were removed to make them in suitable sizes (2-3cm). After cutting the explants they were vertically inoculate into 50 ml capacity bottles containing MS medium with 0.3mg/l of IAA and 3 mg/l of BAP. Then bottles were tightly capped and properly sealed with wrap to avoid entry of external air. The bottles were transferred to culture room.

F. Culture conditions

All the cultures were incubated at 30±2^{o}C and at photoperiod of 16 hrs provided by cool-white fluorescent light with the intensity of 2000 LUX.

G. Shoot proliferation

After 25 days explants devoid of contaminations were then cultured on the basal MS medium supplemented with kinetin (0.5mg/l) and 6-BAP (1.5 mg/l) in combination with NAA either 0.1mg/l or 0.2mg/l and with IAA (0.1mg/l) and IBA (0.2mg/l) for shoot

amplification. Cluster of shoots amplified from initial lateral shoot explants were sub-cultured as it is without separation from the explants on the same regeneration media after one month from initial establishment stage. Data were recorded after 30 days of culture and only shoots greater than 2cm were considered for taking data. After another 5 weeks of incubation the proliferating cultures were transferred to different media for shoot elongation.

H. Rooting of micro shoots

Newly formed shoots measuring 2-3cm in length were excised individually from the parent explants were transferred to rooting media. Two types of rooting media were used one is MS basal media with 3 types of hormones NAA, IAA, IBA and other half strength MS media. Data were recorded after 30 days of culture.

I. Acclimatization

After 30 days of culture on rooting media, the plantlets were successfully acclimatized. Pots (8×6 cm) were kept readily filled with garden soil, compost and sand in the proportion of 2:1:1 respectively. The plants were then transplanted into the pots then thoroughly watered and kept under plastic house having 80% humidity and 31°C temperature for ten days. Then the plants were shifted to shade house with less humidity and indirect sunlight. After 15 days, the hardened plantlets were transferred to the soil. The plants were watered periodically and upper layer of the soil mulched occasionally whenever necessary. The flow diagram of overall procedure of micro propagation is given Fig 9.

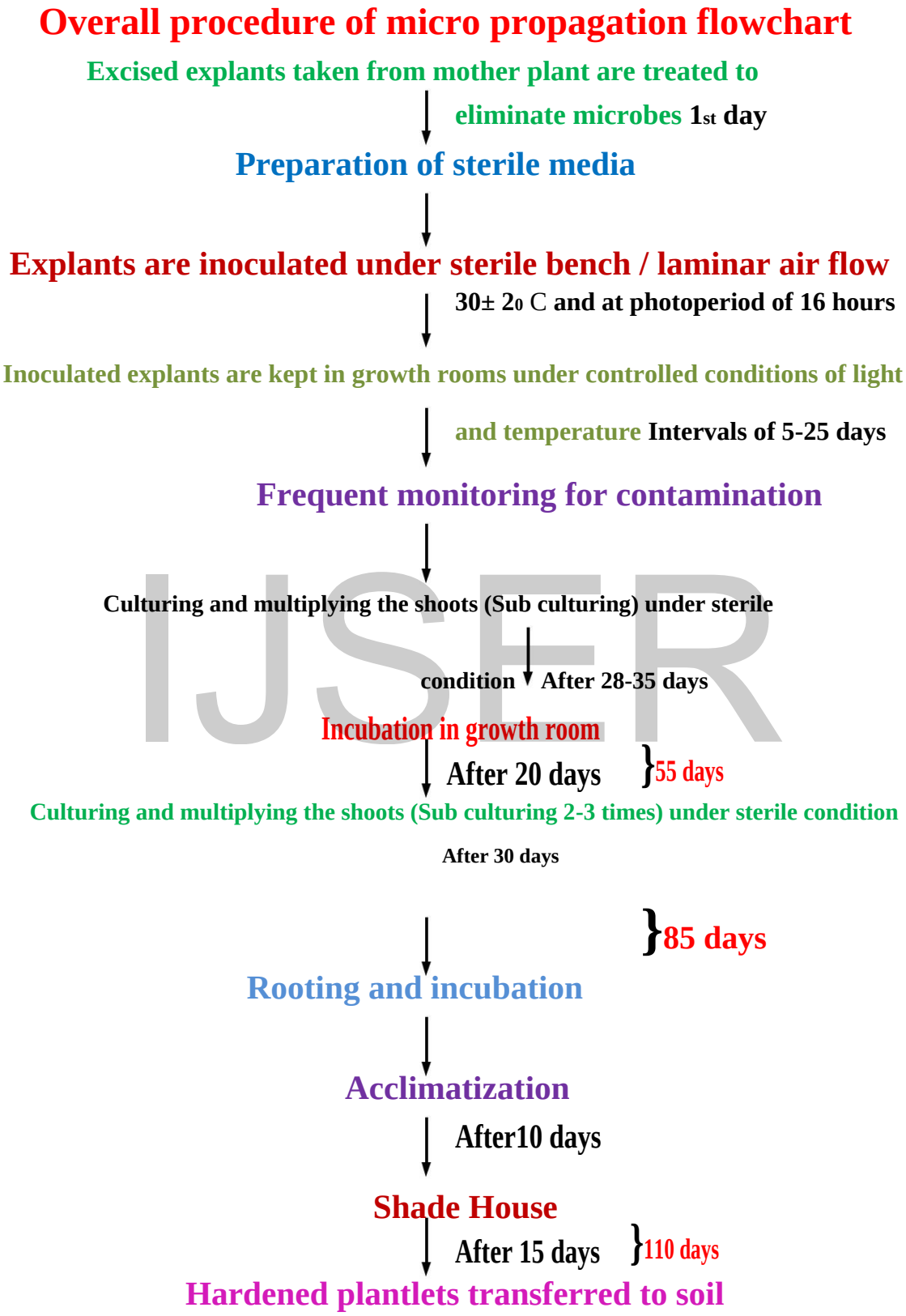

Fig 9: Flow diagram of overall procedure of micro propagation

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2.2 *Aloe ferox*

A. Seeds sterilization:

The seeds were first washed thoroughly in running tap water for 30 minutes. After that they were again washed with liquid detergent Tween 20 (Himedia Laboratories, India) for 10 minutes with vigorous shaking. After washing with detergent seeds were again washed with running tap water to remove any traces of detergent for 30 minutes and kept in 1% w/v solution of Bavistin (BASF India Limited) for 30 minutes.

After washing the seeds were taken inside the laminar flow for further 2-3 repeated sterilization with distilled water. The sterile seeds were taken out and dipped in 70% ethyl alcohol for 30 seconds. They were surface sterilized with freshly prepared 5 % Sodium Hypochloride (Commercial brand Corox 20%) solution for 30 minutes. Followed by 3-4 times with sterile water remove any traces of sodium hypo chloride before germination. All treatments consisted of four replicates with 25 seeds in each. The seeds were placed on two layers of whatman No.1 filter paper in disposable plastic petri dishes (9 cm) (Fig 19). The filter paper was wetted with 4 ml distilled water and kept moist by adding distilled water when required until the germination. Germination was recorded daily and was considered complete once the radical protruded 1cm in length (4 weeks). After 4 weeks, the germinated seedlings were directly kept in MS basal media for 1 month for further growth.

B. Preparation and Sterilization of the media for *Aloe ferox* seeds

The basal medium containing MS (Murashige and Skoog, 1962) salts, vitamins, 3% sucrose and agar. The basal media was supplemented with various concentration and

combination of Growth regulators 2, 4-D (2, 4- dichlorophenoxy acetic acid), BAP (6 benzyl amino purine), NAA (Naphthalene acetic acid), Kinetin (K), IBA (Indole Butyric Acetic acid), and IAA (Indole 3 acetic acid). The medium was adjusted to pH 5.8 with acid or base and dispensed in culture tubes and conical flasks of 100 ml capacity (Fig 20). The media was sterilized by autoclaving at 121^0C and 15 lbs pressure for 20 minutes (cysteine Hi Media Laboratories, AR Grade).

C. *Aloe ferox* shoot root proliferation

Procedure of subculture was similar for both root and shoot culture was similar to *Aloe vera.*

D.Acclimatization: Similar to *Aloe vera*.

3. Phytochemical Investigations

A. Plant material collection: The selected plant germplasm of *Aloe vera* collected and authenticated from National Bureau of plant Genetic Resources (NBPGR), New Delhi was used for phytochemical analysis. The leaves of the plant were made free from dust particles, dried under shade and powdered coarsely. Then the powder was extracted using ethyl acetate and methanol as solvent (increasing order of polarity) by soxhlet apparatus. The resultant extracts were taken for the study.

B. Plant extracts preparation: 500 g of powdered leaves of *Aloe vera*, *Aloe CIM-Sheetal* and *Aloe ferox* extractions were prepared by using different organic solvents.

Aloe CIM-Sheetal was extracted continuously using soxhlet apparatus with methanol and ethyl acetate successively for about 48 hrs at 30°C (Fig 10). The extracts were

concentrated under reduced pressure using rotary vacuum flash evaporator to get a constant volume. The concentrated extract was eluted with ethyl acetate, hexane and methanol in ascending order of polarity by performing column chromatography.

C. Analysis of phytochemical compounds: Analysis was done to find out the presence of phytochemical compounds like flavonoids, flavonols, alkaloids, saponins, total phenols, tannins, alkanes, pyrimidines, proanthocyanidins, fatty acids, indoles, phytosterols, aldehydes, dicarboxylic acids, ketones, organic acids and alcohols by some modified procedures of earlier studies (Shreya et al., 2013; Parmar et al., 2012; Lisa Botes et al., 2008; Loots et al., 2007).

The plant material was subjected to preliminary phytochemical screening for the detection and confirmation of various plant constituents (Table 9).

1. **Tests for Alkaloids: a. Dragendorff's Test:** To the extract, add 1ml of Dragendorff's reagent was added. An orange red colored precipitate indicates the presence of alkaloids.

b. Wagner's Test: 1ml of Wagner's reagent is added to the extract, reddish brown colored precipitate indicates the presence of alkaloids.

c. Mayer's Test: To the extract, add 1ml of Mayer's reagent. A dull white colored precipitate indicates the presence of alkaloids.

d. Hager's Test: To the extract add 3ml of Hager's reagent. Yellow colored precipitate indicates the presence of alkaloids.

2) Tests for Carbohydrates:

a. Molish Test: To the extract, 1ml of α-naphthol solution was added and Conc. sulfuric acid was added along the sides of the test tube. Purple or Reddish violet color at the junction between the two liquids indicates the presence of carbohydrates.

b. Fehling's Test: To the extract, equal quantities of Fehling's solution A and B were added. Upon heating gently, a brick red precipitate indicates the presence of carbohydrates.

c. Benedict's Test: To 5ml of Benedict's reagent, 8 drops of solution under the test was added and mixed well. Then it was boiled vigorously for 2 minutes and cooled. Red precipitate indicates the presence of carbohydrates.

3) Tests for Proteins:

a. Biuret Test: To the extract, 1ml of 40% sodium hydroxide and 2 drops of 1% copper sulphate solution was added. A violet color indicates the presence of proteins.

b. Xanthoprotic Test: To the extract, 1ml of Conc. Nitric acid (HNO_3) was added. When a white precipitate was formed, it is boiled and cooled. Then 20% of sodium hydroxide or ammonia was added. Orange color indicates the presence of aromatic amino acids.

c. Lead Acetate Test: To the extract, 1ml of lead acetate solution was added. A white precipitate indicates the presence of proteins.

4) Test for Amino acids:

a. **Ninhydrin Test:** 2 drops of freshly prepared 0.2% ninhydrine reagent was added to the extract and heated. Development of blue color indicates the presence of proteins, peptides, amino acids.

5) Tests for Steroids:

a. **Libbermann Burchard Test:** The extract was dissolved in 2ml chloroform in dry test tube. 10 drops of acetic anhydride and 2 drops of conc. sulphuric acid were added. The solution becomes red, and then blue and finally bluish green in color indicates the presence of steroids.

b. **Salkowaski Test:** The extract was dissolved in chloroform and equal volume of Sulfuric acid was added to it. Bluish red to cherry red color was observed in chloroform layer; whereas acid layer assumes marked green fluorescence indicates the presence of steroids.

6) Test for Saponnins:

a. **Foam Test:** About 1ml of extract is diluted separately with distilled water to 20ml and shaken in a graduated cylinder for 15 minutes. A 1cm layer of foam indicates the presence of saponnins.

7) Test for Phenolic Compounds and Tannins: Small quantities of alcoholic and aqueous extracts in water were tested for the presence of phenolic compounds and tannins with dilute ferric chloride solution (5%), 1% solution of gelatin containing 10% sodium chloride, 10% lead acetate and bromine solutions.

Fig 10 (a)

Fig 10 (b)

Fig 10: Phytochemical Extractions of Aloe sps by different Organic solvents

4. Chromatographic techniques:

The elution was carried out with solvents of increasing polarity using Methanol and Ethyl acetate in following ratios.

1. 100 % Ethyl acetate
2. 2 % Ethyl acetate in Methanol
3. 5 % Ethyl acetate in Methanol
4. 10 % Ethyl acetate in Methanol
5. 15% Ethyl acetate in Methanol
6. 20 % Ethyl acetate in Methanol
7. 25 % Ethyl acetate in Methanol
8. 40 % Ethyl acetate in Methanol
9. 50 % Ethyl acetate in Methanol
10. 70 % Ethyl acetate in Methanol
11. 80 % Ethyl acetate in Methanol

The elutes were collected in fractions of 200 ml each.

* Fractions were collected and the resolutions of the compounds were checked on commercially available TLC trial plates.
* The fractions showing the same identity of the compounds (i.e., same Rf values) are mixed and then concentrated using rota vapor.
* The dry mass of the compound was dissolved in $CdCl_3$ for determination of compound by 'H -NMR.
* The dry mass of the compound was dissolved in $CdCl_3$ for determination of compound by C^{13} -NMR.
* The remaining compound was used to determine anti-microbial activity done.

5. Spectroscopic studies

A. Isolation of different compounds in the *Aloe vera* leaf extracts

- The crude extract (Powder) was soaked in 100 ml of methanol + 100 ml of ethyl acetate + 100 ml of chloroform for 3-4 days and filtered.
- Filtration of the crude sample was done with whatman filter paper and precipitated. Crude extract was tested in TLC and evaporated by distillation at 48 -49^{0}c and with rotary vacuum flash evaporator with 90 rpm.
- Crude extract was adsorbed by silica and compounds were separated by column chromatography.
- The different compounds of *Aloe vera* and *Aloe ferox* methanolic, ethyle acetate and chloroform leaf extracts were isolated by eluting in the stepwise gradient solvent system determined in TLC, described.
- Column was set with 60 - 70 % silica + compound + cotton (ethyl acetate + methanol).

- Initially the column was run with 100% ethyl acetate and the fractions obtained were checked for compounds by TLC. Chlorophyll is removed by this step completely.
- Similarly the column was run with 70 % ethyl acetate + 30 % methanol a total of 6 fractions were obtained which were tested TLC respectively.
- Finally the Colum was run with 50 % ethyl acetate +50% methanol and followed by TLC.
- Soluble compounds of the three solvents were taken separately in round bottom flask and distillated and separated by rotavapour.
- Unknown compound were collected in the eppendorf separately and kept overnight for evaporation of methanol.
- Finally small pellet was formed bottom at the bottom of eppendorf. 3-4 compounds were observed in70% ethyl acetate + 30% methanol.

Different mobile phases like 10% ethyl acetate: 90% of hexane, 50% methanol: 50% ethyl acetate, 100% ethyl acetate and 100% methanol.

B. High-performance liquid chromatography (HPLC)

- High-performance liquid chromatography (HPLC) was done to determine the individual components in leaf extracts of *Aloe vera* and *Aloe ferox* (Patra et al., 2012). The HPLC analysis revealed the presence of polysaccharide acemannan *Aloe vera g*el. Acemannan (Fig 11) is an anticancer agent derived from *Aloe ferox* leaf gel extract (Nema et al., 2012). Molecular formula of acemannan compound: $C_{64}H_{97}NO_{49}$; molecular weight of acemannan compound: 1664.43.

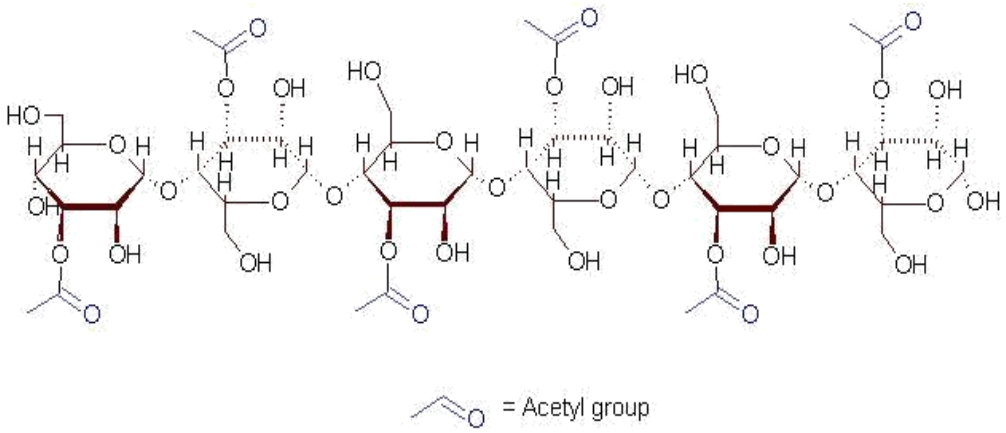


Fig 11: Chemical structure of Acemannan

High performance liquid chromatography (HPLC):

Analysis of methanol extracts of leaf of *Aloe ferox* was performed by HPLC. The HPLC system consists of BioSep- SEC- s2000 prominence liquid chromatography pump (P680), SPD-20A prominence UV-PDA detector, autosampler ASI-100 injector fitted with 50 µl capacity fixed loop, chromatographic oven (TCC-100) and a photodiode array detector (PAD100) all from Agilent company, USA. The column used was Phenomenex luna 5 µ C_{18} (2) 100A (250 mm × 4.6 mm) at 28^0C temperature. The output signals were monitored and processed using chemstation software. The mobile phase optimized for the analysis was ammonium acetate and flow rate was 1 ml/minute and detection wave length was set at 232 nm (Patra et al., 2012). The run time of the method was 4.545 minutes and analytes were separated at the retention time 563.778 mAU. The highest peak was also seen at the retention time.

C. NMR (Nuclear magnetic resonance)

Nuclear magnetic resonance spectroscopy: commonly referred to as NMR, was applied to exploits proton and ^{13}C (Nishant and Anil, 2012).

^{13}C-NMR spectra of methanolic extract of *Aloe ferox* was recorded on a Bruker type AV 300 at 300 MHZ and chemical shifts were recorded as **δ** values. The result graph was compared with the reference chart and possible functional group present in the plant were determined. The solvent used was $CdCl_3$ (Silverstein et al., 2005; Patra et al., 2012).

2. *Aloe ferox*:

A. Plant extracts preparation: 500 g of powdered leaves of *Aloe ferox* was extracted continuously using soxhlet equipment with chloroform, ethyl acetate and methanol for concerning about 48 hrs at 30°C. The extracts were concentrated under reduced pressure using rotary vacuum flash evaporator to get a constant volume. The concentrated extract was eluted with ethyl acetate and methanol in ascending order of polarity by performing column chromatography similar to *Aloe vera.*

B. Separation of compounds by column chromatography:

Elution was carried out with increasing polarity using Chloroform, hexane methanol and ethyl acetate.The elutes were collected around 200 ml from each fraction through column chromatography separation (Fig 12). Fractions were collected and the resolutions of the compounds were checked on commercially available TLC plates. The fractions showing the same identity of the compounds (i.e., same Rf values) are mixed and then concentrated using rota vapor.

Fig 12: Column chromatography extractions

C. Extraction of compounds by Thin Layer Chromatography (TLC):

Resolutions of collected fractions were checked on commercially available TLC plates.

D. **Analysis of phytochemical compounds:** Analysis was done to find out the presence of phytochemical compounds like flavonoids, flavonols, alkaloids, saponins, total phenols, tannins, alkanes, pyrimidines, proanthocyanidins, fatty acids, indoles, phytosterols, aldehydes, dicarboxylic acids, ketones, organic acids and alcohols by some modified procedures of earlier studies (Shreya et al., 2013; Parmar et al., 2012; Lisa Botes et al., 2008; Loots et al., 2007).

D. Antioxidant activity (DPPH assay): The antioxidant activity of the *Aloe ferox* leaf extract was measured in terms of hydrogen-donating or radical scavenging ability by using the DPPH radical scavenging method (Milady and Damak, 2008).

DPPH solution (1.5 ml) was incubated with 1.5 ml of *Aloe ferox* leaf extract fractions at various concentrations (0.01-1 mg). The reaction mixture was shaken well and incubated in the dark for 30 min at room temperature. The control was prepared

without any extract. The absorbance of the solution was measured at 517 nm against a blank (Shreya et al., 2013). The radical scavenging activity (RSA) was measured as a decrease in the absorbance of DPPH (1, 1-diphenyl, 2-picrylhydrazyl) and was calculated using the following equation:

$$\text{Scavenging effect (\%)} = \frac{(1 - \text{Absorbance of the test Sample (517nm)} \times 100}{\text{Absorbance of the Control (517nm)}}$$

E. Anti-inflammatory activity:

Animals: Healthy SD (Sprague Dawley) adult male rats (150-200g) obtained from National Institute of Nutrition (NIN) Hyderabad, India. The animals were housed under standard laboratory conditions maintained at 25 ± 10^{o} C and under 12/12 h light/dark cycle and fed with standard pellet diet, water ad lib. The protocol was approved by the institutional animal ethics committee and by the animal regulatory body of the Indian Government (Registration No. 380/01/a/ CPCSEA) for this experiment.

Drugs: Carrageenan (Sigma Chem.), Carboxy methyl cellulose (SD fine chem.) Ibuprofen (local market), were used during the experimental protocol.

Carrageenan-Induced Paw Edema method:

Experimental design: Rats were divided into 4 groups; each group consists of 6 rats.

Group I: Treated with 0.2 ml of 2% CMC daily for 1 week.

Group II: Treated with Ibuprofen (intraperitoneally) 10 mg/kg body weight (b.w) daily for 1 week.

Group III: Treated with methanolic extract of *Aloe ferox* leaves at the dose of 250 mg/kg b.w. daily for 1 week.

Group IV: Treated with methanolic extract of *Aloe ferox* leaves at the dose of 500 mg/kg b.w. daily for 1 week.

Acute inflammation was induced in all groups by injecting 0.1 ml of 1% (w/v) carrageenan into the sub-plantar region of the right hind paw of the rats (Venkatesa et al., 2001). On the 7th day, paw volume was measured 1h prior to carrageenan injection using a plethysmometer from 0 to 3h and after the carrageenan injection, the increase in the paw volume was measured and percentage inhibition was calculated. Percentage of inhibition =100 (1-Vt/Vc); (Vc = edema volume in control and Vt = edema Volume in test/standard compound) (Mulla et al., 2010; Manjit et al., 2010).

Statistical analysis: The values were analyzed with the analysis of variance (one way ANOVA) to determine the significance of difference within the experimental groups. P-values of 0.05 or less were taken as significant.

4. Antidiabetic and Histopathology

studies: A. Chemicals

Analytical graded chemicals were used for all the experiments. Streptozotocin (sigma chemicals), Glibenclamide (local market) were used in the experimental protocol**.** Streptozotocin was purchased from Sigma chemicals Hyderabad, India and was dissolved in 0.1 M citrate buffer (pH = 4.5) at the dose of 55 mg/kg b.w. This was injected intraperitoneally within 15 min of dissolution in a vehicle volume of 0.4 mL with 1 ml of

liquid syringe fitted with twenty four gauge needle and normal control group was given citrate buffer 0.4 mL only.

(1) Glibenclamide

It is an oral antidiabetic preparation with an efficient hypoglycemic action. Daonil (glibenclamide) manufactured by Aventis Pharma Ltd. Goa, India was collected from local market and preserved at room temperature.

B. Collection and processing of blood for estimation of blood sugar levels:

After 21 days of herbal treatment the experiment was terminated and observations were made. Body weights were taken before and after the experiment, with the help of one pan balance and blood glucose levels were estimated on day 0, 7^{th}, 14^{th} and 21^{st} day of experiment with the help of glucometer using strip method and blood was collected from tip of the tail. Diabetes was confirmed by the determination of fasting glucose concentration on the third day of post administration of STZ (Ogbonnia et al., 2008).

C. Preparation of extract:

500g of powdered leaf extract of *Aloe CIM-Sheetal* was extracted continuously using soxhlet apparatus with methanol and ethyl acetate successively for about 48 hrs at 30°C. The extracts were concentrated under reduced pressure using rotary vacuum flash evaporator to get a constant volume.

D. Animals

Healthy male SD (Sprague Dawley) rats weighing about 150 - 200 g, procured from National Institute of Nutrition (NIN) Hyderabad, India were used in the present investigation. The animals were grouped and housed in polyacrylic cages and maintained under standard laboratory conditions with dark and light cycle (12:12 hrs) and temperature 22±0.5°C. They were fed *ad libitum* everyday with standard chow dry pellet diet procured from NIN and were given free access to water. Animals represented as fasting were deprived of food for at least 18 h but were allowed free access to drinking water. All the rats were given a period of acclimatization for 7 days before starting the experiment and the experimental protocol had been approved by the Institutional animal ethics committee and by the animal regulatory body of the Indian Government (Registration No. 380/01/a / CPCSEA).

Experimental design

Rats were divided into seven groups as follows.

Group I: Consisted of six rats which served as normal control (non - streptozotocin) and were given only citrate buffer (0.4 ml pH 4.5) daily.

Group II: Consisted of six STZ induced diabetic rats and served as diabetic control (STZ-induced@ 55 mg / Kg b.w.) and were given citrate buffer (0.4 ml pH 4.5) daily.

Group III: Consisted of six STZ induced diabetic rats and were treated orally with 100 mg/ kg b.w. methanolic extract of *Aloe CIM-Sheetal* (ALE) once a day for 21 days.

Group IV: Consisted of six STZ induced diabetic rats and were treated orally with a dose of 200 mg/kg b.w. of methanolic extract of *Aloe CIM-Sheetal* leaf extract (ALE) once a day daily for 21 days,

Group V: Consisted of six STZ induced diabetic rats which were treated orally with 300 mg/kg b.w of methanolic extract of *Aloe CIM-Sheetal* leaf extract (ALE) once a day daily for 21 days.

Group VI: Consisted of six STZ induced diabetic rats which were given Glibenclamide (GBC) daily for 21 days, once a day at the dose of 0.60.mg/kg b.w.

Group VII: Consisted of six STZ induced diabetic rats which were treated orally with a dose of 500 mg/kg b.w. of methanolic extract of *Aloe CIM-Sheetal* leaf extract (ALE) once a day daily for 21 days.

E. Toxicity studies

The methanol extract of *Aloe vera* did not produce significant toxic effect in rats during acute and sub-acute treatments. The experimental study was evaluated by the World Health Organization (WHO) guideline and the Organization of Economic Co-operation and Development (OECD) guide line for chemical testing (WHO 2000 : OECD 2001).

(a) Acute toxicity

In this study rats were divided into four groups. The treated groups were given *Aloe CIM - Sheetal* methanol extract orally in single dose of 10, 20, 30 g/kg b.w. while

the control group received only water. The animals were monitored for 14 days for apparent signs of toxicity.

(b) Sub acute toxicity

SD rats were divided into 6 groups and the treated group was orally given the extract at a dose of 1 (dose 1), 5 (dose 2), 10 (dose 3), 15 (dose 4) and 20 (dose 5) g/kg b.w. for 42 days and the control group received the same volume of water. All the rats were observed for apparent signs of toxicity during the experimental period.

After toxicity test the SD rats were sacrified and liver pancreas and kidney sample were collected and given at NIN, Hyderabad for histopathological staining.

Statistical Analysis

Statistically, the values were analyzed with the analysis of variance (one way ANOVA) to determine the significance of difference within the experimental groups. P-values of 0.05 or less were taken as significant.

5. Molecular studies

The present investigation has been undertaken to assess the extent of genetic diversity in 12 elite accessions of *Aloe vera* (*Aloe barbadensis* Miller) i.e. 10 accessions from National Beaureo of Plant Genetic Resources (NBPGR), New Delhi, India and 2 accessions from Central Institute of Medicinal and Aromatic Plants (CIMAP) Hyderabad, India, by using two DNA-based molecular marker techniques, *viz.*, Random Amplified Polymorphism DNA (RAPD) and Inter Simple Sequence Repeat (ISSR),

RAPD technique was used to estimate the genetic diversity in the germplasm collections in *Aloe* spp. All the accessions were maintained at Indian Immunologicals Ltd, Hyderabad, India for genetic diversity. Following procedure was followed.

Molecular techniques:

- DNA isolation
- Agarose Gel electrophoresis
- DNA quantification by Spectrophotometer
- PCR
- Gel documentation

Equipment handled:

- Autoclave (Yorco)
- Centrifuge (Themo Scientific and Tarsons)
- PCR (Eppendorf)
- Agarose Gel Electrophoresis(Genei and Scie-plas)
- Gel Documentation system (Syngene)

A. Collection of Plant Material

Twelve accessions of *Aloe vera* germplasm (NBPGR, New Delhi, India vide voucher No: NBPGR / 2011/ 1771 dated 11.07.2011) representing totally from different geographical regions of India were obtained from Rajasthan (IC111267, IC111269, IC111271, IC111272), Gujarat (IC111279), Haryana (IC111280), Delhi (IC471882, IC471883, IC471884 and IC471885) and Central Institute of Medicinal and Aromatic Plants (CIMAP) Research Center, Boduppal, Hyderabad, Telangana State, India (*Aloe*

CIM-Sheetal and commonly grown wild *Aloe vera* (local)) vide voucher no: CIMAP/ 63/ 6222 dated 20.07.2011. All the plants utilized in the present study were about two years of age. In all cases, the predominantly grown accession was used. In case of some states, where variation has been reported different provenances were collected. Accordingly, the accessions from the different regions of collection from Rajasthan, Gujarat, Haryana, Delhi, Uttarpradesh and Telangana State; the germplasm was maintained at the research farm of Indian Immunologicals Ltd, Hyderabad, India and the details of the accessions are presented in the Table 4 and Fig 13.

B. DNA extraction

Total genomic DNA was extracted from younger leaves of 12 *Aloe vera* accessions by following the standard CTAB method with minor modifications (Doyle and Doyle, 1990). Two grams of leaves were ground in liquid nitrogen, then homogenized in 10 ml of extraction buffer (4 % CTAB, 20 mM EDTA, 2% PVP, 1.4 M NaCl, 100 mM Tris- HCl pH 8.0 and 1% β mercapto ethanol) and incubated at 65^0C for 30 min. The supernatant was twice extracted with chloroform: isoamylalcohol (24:1, v/v) and treated with 2 µl RNase A (100 mg/ml), incubated at 37 ^{o}C for 1 hr. The DNA was precipitated with isopropanol and washed twice with 70% ethanol. The pelleted DNA was air dried and resuspended in 500 µl of sterile millipore water and finally all DNA samples were diluted to get 50 ng/µl and were stored at -20 ^{o}C for use in RAPD and ISSR assay.

DNA isolation:

DNA was isolated using a modified protocol of (Doyle and Doyle, 1990) which is detailed below.

1. Two to three leaf pieces (weighing ~2-3 g) were collected from the plants growing in field in a labeled plastic cover kept in freezer pack. After bringing the plastic cover to lab, they were immediately stored at -20^{0}C.
2. Leaf pieces were cut into small bits in a sterile porcelain mortar and liquid nitrogen was added till the leaf bits were completely immersed. The leaf bits were then ground into fine powder using a sterile porcelain pestle. While grinding, liquid nitrogen was continuously add to ensured that the leaf powder does not thaw.
3. Immediately after the grinding, 10 ml of extraction buffer (4% - CTAB buffer 10 mM Tris HCl pH 8; 20 mM EDTA, 1.4 mM NaCl and 20% PVP and 1% β mercapto ethanol) was added to the mortar, which was then kept in a water bath maintained at 65^{0}C for about 5 min. Once the solution thaws, the contents from the mortar were transferred to 3-5 sterile, labeled 1.5 ml microcentrifuge tubes using a 1000 µl capacity micropipette using micropipette tips cut at their tip portion. The microcentrifuge tubes were incubated for 30 min at 65^{0}C.
4. After the incubation step, approximately equal volume of Chloroform: Iso-amyl alcohol (24:1) mixture (~400 µl) was added to each microcentrifuge tube. The contents were mixed well by inversion for about 10 minutes and centrifuged at 10,000 rpm (~8000 g) for about 10 minutes at room temperature.
5. After centrifugation, the supernatant was aliquated from the micro centrifuge tube into freshly labeled sterile 1.5 ml micro centrifuge tubes. Care was taken that the intermediate layer of insoluble proteins was not disturbed. If the supernatant was

not clear, the Chloroform: Isoamylalcohol 24:1 purification step was repeated once again. To the supernatant 2 µl of RNase (10 mg/ml) was added and incubated for 60 minutes at room temperature (37^0C).

6. To the clear aqueous supernatant, equal volume (500-600 µl) of chilled Isopropyl alcohol was added. The contents were mixed gently and centrifuged at 10,000 rpm (~8000 g) for 10 minutes at room temperature.
7. The supernatant was drained gently and about 200 µl of 70 % ethanol was added to the pellet collected at the bottom of the micro centrifuge tube. The tube was tapped gently to disturb the pellet. Centrifugation is done at 10,000 rpm (~8000 g) for 10 minutes at room temperature. After this the supernatant was drained and the pellet was washed with 70 % ethanol once again as described above.
8. Finally, the pellet was left for air-drying over night at room temperature with the tube cap open. After complete drying of pellet, about 50-100 µl of sterile TE buffer (10mM Tris-HCl, pH 8.0 and 1mM EDTA) was added to the tube depending on the size of the pellet, for dissolving the pellet. The DNA solution was then stored at -20^0C till further analysis.

C. Analysis of quality and quantity of DNA isolated:

The quality and quantity of isolated DNA was checked by Agarose gel (0.8 %) electrophoresis and staining with ethidium bromide as described below 0.8 % Agarose gel was prepared as follows for electrophoresis of DNA samples.

1. 0.3 g of agarose was weighed and added into a 250 ml conical flask.
2. One liter of 50X TAE was prepared as follows: 212 g of Tris base was taken in 500 ml double distilled water, to which 48 ml of Acetic acid (AcOH) was added, mixed well

by stirring. This was followed by the addition of 100ml of 0.5M Na_4EDTA and the volume was finally made up to 1000 ml using double distilled water. After thorough mixing the solution was autoclaved and stored at room temperature. This was used for preparation of 1X TAE, which was prepared by diluting 50X TAE with sterile distilled water.

3. 30 ml of 1X TAE was added to a 250 ml beaker and boiled gently in a microwave oven.
4. After the agarose was properly melted (when the solution was crystal clear with no floating particulate matter), the solution was taken out of the microwave oven and allowed to cool down.
5. Meanwhile, a gel-casting tray was washed thoroughly first with tap water and then with distilled water followed by rinsing with methanol.
6. The gel-casting tray was then placed in a sealing mechanism and a comb (containing 8 lanes) was arranged in its slot on the gel-casting tray.
7. When the boiled agarose had cooled down substantially (to about 45^0C), 2 µl of ethidium bromide solution (10 mg/ml of distilled water) was added and mixed gently. Precautions were taken by wearing gloves while handling ethidium bromide.
8. The melted agarose was then slowly poured in the gel casting tray and allowed to solidify (~20-30 min). Care was taken so that air bubbles are not formed in the gel.
9. After solidification, the gel was removed from its casting tray and put into a gel tank, which was then filled with 1X TAE buffer till the buffer reaches 0.5 cm above the gel surface (~100 ml).

10. Five microlitre of DNA solution was then mixed with~1-2 µl of loading dye (0.0025 % bromophenol blue in 40 % sucrose), loaded into the wells and the electrodes were connected to Power pack. DNA solutions of known concentration (50 ng/µl, 100 ng/µl, 150 ng/µl and 200 ng/µl) were also loaded along with the samples for comparison of the concentration of the DNA samples.
11. The samples were then subjected to electrophoresis at constant voltage (75 V) for 20-30 minutes till the dye front reached 2/3rd of the running length of the gel. The gel was then visualized under UV light in a syngen gel documentation system for checking the quality and quantity of DNA. Based on comparison with intensity of DNA solution of known concentration, the unknown samples were diluted to a final concentration of 50 ng/µl using 1xTE buffer.

D. Spectrophotometer:

1ml of TE buffer was taken in a cuvette which was used as blank to calibrate the OD at 260 nm as well as 280 nm. 10 µl of each DNA sample was added to 990 µl of TE buffer and mixed well. The OD_{260} and OD_{280} values on spectrophotometer were noted.

5.1. Randomly Amplified Polymorphic DNA (RAPD analysis):

A set of 64 decamer primers synthesized from IDT (Integrated and Technologies) were used for DNA amplification with minor modifications (Williams et al., 1990). The PCR amplification was performed in 10 µl reaction volume containing of 5 ng of genomic DNA, 1x PCR buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 100 µM of each the 4 dNTPs, 0.4 µM of RAPD primer and 0.3 U of Taq DNA polymerase (Bangalore Genei). PCR amplifications were performed in Gene Amp 9700 Thermal

Cycler (Perkin Elmer Applied Biosystems) with an initial denaturation at 94^{o}C for 3 min followed by 45 cycles of 94^{o}C for 45 s, 36^{o}C for 30 s, and 72^{o}C for 2 min with a final extension of 72^{o}C for 7 min. The PCR amplified products were separated on 1.5% agarose gel in 1x TAE buffer by electrophoresis at 100 V for 2 h and visualized with ethidium bromide staining and the gels were documented using an Alpha Image Gel Documentation System. We have used 64 primers for RAPD analysis (Table 2).

Agarose Gel Electrophoresis: The amplified products along with λ DNA/ *Eco* R1 and *Hind* III double digest as molecular weight marker were analyzed by electrophoresis in 1.8% agarose gel containing Ethidium bromide and run on 1x TAE buffer at a constant voltage for 3 hrs. The bands were visualized on the gel under ultra-violet light and documented polaroid (667) prints. On the basis of seed yield per plant and plant height the genotypes were classified into seven clusters. The genotypes of medium yielding, semi dwarf type and medium yield, tall groups appeared to have sufficient genetic diversity and could be considered as ideal parents in hybridization programme in order to create wide variation for further improvement.

5.2 ISSR analysis

A total of twenty five ISSR primers (UBC primer set No. 9, University of British Columbia, Canada) were used for the analysis (Table 3). The PCR reaction mixture ($10\mu l$) consisted of 5 ng of genomic DNA, 1x PCR buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM of each the four dNTPs, 0.4 µM of ISSR primer and 0.6 U of Taq DNA polymerase (Bangalore Genei, India). PCR amplifications were performed using a GeneAmp 9700 Thermal Cycler (Perkin Elmer Applied Biosystems) with initial

denaturation at 94^{o}C for 4 min followed by 35 cycles of 30 s at 94^{o}C for 1 min at the annealing temperature (*Ta*), 2 minutes elongation at 72^{o}C and final extension at 72^{o}C for 7 min. The amplified products were electrophoresed at 100 V on a 1.7% agarose gel using *EcoR* I and *Hind* III double digest as the molecular weight standard.

Statistical analysis

For each RAPD and ISSR primer, the presence or absence of bands in each accession was visually scored and set in a binary matrix. The number of polymorphic and monomorphic fragments for each primer pair was scored and the monomorphic markers were excluded from the analysis. The binary matrices were read by NTSYS-pc version 2.02i (Rohlf, 1997) with Jaccard's similarity coefficients and estimates of genetic distances for all pair wise comparisons between accessions were determined using Similarity for Qualitative Data (SIMQUAL). Dendrograms were created independently for both the marker systems and also based on pooled marker data using Unweighted Pair Group Method with Arithmetical Averages (UPGMA). The correlation between matrices was determined using Mantel test. Principal coordinate analysis was also performed and the ordination displayed in two dimensions.

Table 2: List of RAPD Primers

S. No	Primer Code	Primer sequence 5'- 3'	S. No	Primer Code	Primer sequence 5'- 3'
1	OPA-01	CAGGCCCTTC	33	OPD-15	CATCCGTGCT
2	OPA-02	TGCCGAGCTG	34	OPD-17	TTTCCCACGG
3	OPA-03	AGTCAGCCAC	35	OPE-07	AGATGCAGCC
4	OPA-04	AATCGGGCTG	36	OPE-08	TCACCACGGT
5	OPA-05	AGGGGTCTTG	37	OPK-04	CCGCCCAAAC
6	OPA-06	GGTCCCTGAC	38	OPK-08	GAACACTGGG
7	OPA-07	GAAACGGGTG	39	OPL-01	GGCATGACCT
8	OPA-08	GTGACGTAGG	40	OPL-05	ACGCAGGCAC
9	OPA-09	GGGTAACGCC	41	OPL-06	GAGGGAAGAG
10	OPA-10	GTGATCGCAG	42	OPL-10	TGGGAGATGG
11	OPA-13	CAGCACCCAC	43	OPL-11	ACGATGAGCC
12	OPA-14	TCTGTGCTGG	44	OPL-12	GGGCGGTACT
13	OPA-15	TTCCGAACCC	45	OPL-18	ACCACCCACC
14	OPA-16	AGCCAGCGAA	46	OPM-12	GGGACGTTGG
15	OPA-17	GACCGCTTGT	47	OPN-02	ACCAGGGGCA
16	OPA-18	AGGTGACCGT	48	OPN-03	GGTACTCCCC
17	OPA-20	GTTGCGATCC	49	OPN-04	GACCGACCCA
18	OPB-03	CATCCCCCTG	50	OPN-05	ACTGAACGCC
19	OPB-04	GGACTGGAGT	51	OPN-06	GAGACGCACA
20	OPB-16	TTTGCCCGGA	52	OPN-07	CAGCCCAGAC
21	OPB-17	AGGGAACGAG	53	OPN-09	TGCCGGCTTG
22	OPB-18	CCACAGCAGT	54	OPN-10	ACAACTGGGG
23	OPC-01	TTCGAGCCAG	55	OPN-11	TCGCCGCAAA
24	OPC-02	GTGAGGCGTC	56	OPN-12	CACAGACACC
25	OPC-03	GGGGGTCTTT	57	OPN-13	AGCGTCACTC
26	OPC-05	GATGACCGCC	58	OPO-20	ACACACGCTG
27	OPC-06	GAACGGACTC	59	OPP-09	GTGGTCCGCA
28	OPC-07	GTCCCGACGA	60	OPP-10	TCCCGCCTAC
29	OPC-08	TGGACCGGTG	61	OPP-11	AACGCGTCGG
30	OPC-09	CTCACCGTCC	62	OPAJ-02	TCGCACAGTC
31	OPC-11	AAAGCTGCGG	63	OPAJ-04	GAATGCGACC
32	OPC-13	AAGCCTCGTC	64	OPAK-03	GGTCCTACCA

Table 3: List of ISSR Primers

S. No	Primer Code	Primer sequence 5'- 3'
1	UBC-807	AGAGAGAGAGAGAGAGT
2	UBC-808	AGAGAGAGAGAGAGAGC
3	UBC-809	AGAGAGAGAGAGAGAGG
4	UBC-810	GAGAGAGAGAGAGAGAT
5	UBC-811	GAGAGAGAGAGAGAGAC
6	UBC- 813	CTCTCTCTCTCTCTCTT
7	UBC-816	CACACACACACACACAT
8	UBC-817	CACACACACACACACAA
9	UBC-818	CACACACACACACACAG
10	UBC-819	GTGTGTGTGTGTGTGTA
11	UBC-820	GTGTGTGTGTGTGTGTC
12	UBC-825	ACACACACACACACACT
13	UBC-827	ACACACACACACACACG
14	UBC-830	TGTGTGTGTGTGTGTGG
15	UBC-840	GAGAGAGAGAGAGAGAYT
16	UBC-847	CACACACACACACACARC
17	UBC- 848	CACACACACACACACARG
18	UBC-860	TGTGTGTGTGTGTGTGRA
19	UBC-861	ACCACCACCACCACCACC
20	UBC-862	AGCAGCAGCAGCAGCAGC
21	UBC-867	GGCGGCGGCGGCGGCGGC
22	UBC-873	GACAGACAGACAGACA
23	UBC-880	GGAGAGGAGAGGAGA
24	UBC-892	TAGATCTGATATCTGAATTCCC
25	UBC-894	TGGTAGCTCTTGATCANNNNN

CHAPTER-4

RESULTS

4. RESULTS

The germplasm of *Aloe vera* was maintained and characterized by using morphological, *in vitro*, biochemical and molecular traits with the ultimate aim of identifying the bio chemical compounds and also promising genetic diversity among *Aloe vera* accessions.

The details of observations are presented below:

1. Morphological characters

Morphological studies have been carried out on all the collected clones. The data on morphological characteristics have been presented in Table 5 (a) and 5 (b). The morphological details of different accessions are also given below.

In the present study, 12 Aloe accessions were studied for some morphological traits. Some of them were easily separated from other accessions by their distinct phenotypic characteristics of stem, leaves and development.

- Morphological analysis indicated that all the studied characters have a significant difference at $P < 0.01$ among aloe accessions.
- Accession No: IC 111272(4), Accession No: IC 471883(8), Accession No: IC471882 (7), Accession No: IC 111267(1) and Accession. *Aloe CIM-Sheetal* (11), were found to be the tallest (61 - 67 cm range) as it possesses a distinct stem (calescent) with long internodes.
- Minimum leaf thickness was 18.1 mm for Accession No. IC 111271(3) and minimum wideness was 3.9 cm for wild *Aloe vera* (12).

- Lowest aloine concentration was recorded 0.78% from *Aloe CIM- sheetal*, leaf dry weight (12.20 g) was lowest in wild *Aloe vera* and highest leaf dry weight was found 23.70 g in the Accession No: IC 111269 (2) among all accessions.

- Gel fresh weight was recorded 58.52 g as highest in Accession No: IC 111267 (1) and lowest 4.10 g in wild Aloe (12).

- Though the fresh gel weigh was 58.52 g highest in Accession No: IC 111267 (1) but the highest dry gel weight was recorded 14.13g in *Aloe CIM- Sheetal* compared to all other accessions.

- The present results show the occurrence of variation in morphological and biochemical characteristics when we compare the mother plants and their tissue culture generated plants. The extent of variation, however, differs from accession to accession. This could be related to differences in the genotypes of various accessions.

Fig 14: Different accessions of *Aloe vera*

Fig.a. *Aloe barbadensis* Mill IC 111267

Fig.b. *Aloe barbadensis* Mill IC 111269

Fig.c. *Aloe barbadensis* Mill IC 111271

Fig.d. *Aloe barbadensis* Mill IC 111272

Fig.e. *Aloe barbadensis* Mill IC 111279

Fig.f. *Aloe barbadensis* Mill IC 111280

Fig.g. *Aloe barbadensis* Mill IC 471882

Fig.h. *Aloe barbadensis* Mill IC 471883

Fig.i. *Aloe barbadensis* Mill IC 471884

Fig.j. *Aloe barbadensis* Mill IC471885

Fig k. *Aloe barbadensis* Mill *Aloe CIM – Sheetal*

Fig.l. *Aloe barbadensis* Mill *Aloe vera* (Local)

Table 4: Details of *Aloe vera* accessions used in morphological, *in vitro* and diversity studies.

S.No	Species	Accession	Obtained from	Source
1	*Aloe barbadensis* Mill	IC 111267	Rajasthan	NBPGR, New Delhi
2	*Aloe barbadensis* Mill	IC 111269	Rajasthan	NBPGR, New Delhi
3	*Aloe barbadensis* Mill	IC 111271	Rajasthan	NBPGR, New Delhi
4	*Aloe barbadensis* Mill	IC 111272	Rajasthan	NBPGR, New Delhi
5	*Aloe barbadensis* Mill	IC 111279	Gujarat	NBPGR, New Delhi
6	*Aloe barbadensis* Mill	IC 111280	Haryana	NBPGR, New Delhi
7	*Aloe barbadensis* Mill	IC471882	Delhi	NBPGR
8	*Aloe barbadensis* Mill	IC471883	Delhi	NBPGR
9	*Aloe barbadensis* Mill	IC471884	Delhi	NBBPGR
10	*Aloe barbadensis* Mill	IC471885	Delhi	NBPGR
11	*Aloe barbadensis Mill*	*Aloe CIM-Sheetal* (CAL14)	Telangana State	CIMAP, Hyderabad
12	*Aloe barbadensis Mill*	wild *Aloe vera*(local)	Telangana State	CIMAP, Hyderabad

Table 5 (a): Morphological studies of Aloe accessions

S. No	Accession No.	No. of leaves	No. of suckers/plant	Peduncle length	Leaf length (cm)	Leaf width (cm)	Stem length (cm)	Leaf wt (gm)	leaf thickness (cm)
1	IC 111267	13.0 ± 0.3^{b}	3.0 ± 0.2^{c}	88.0 ± 0.5^{h}	51.0 ± 0.4^{b}	4.0 ± 0.06^{e}	13.1 ± 0.3^{a}	353.3 ± 0.25^{j}	22.1 ± 0.04^{c}
2	IC 111269	12.0 ± 0.3^{c}	1.0 ± 0.1^{e}	124.0 ± 0.6^{c}	49.0 ± 0.4^{c}	4.8 ± 0.03^{d}	10.3 ± 0.2^{b}	548.5 ± 0.2^{c}	31.6 ± 0.04^{a}
3	IC 111271	12.0 ± 0.3^{c}	5.0 ± 0.04^{a}	183.0 ± 0.5^{a}	48.0 ± 0.4^{bc}	5.2 ± 0.05^{c}	11.3 ± 0.2^{ab}	483.3 ± 0.07^{d}	18.1 ± 0.06^{e}
4	IC 111272	15.3 ± 0.1^{a}	1.0 ± 0.1 ^{e}	118.0 ± 0.6^{d}	55.0 ± 0.4^{a}	6.1 ± 0.05^{a}	12.3 ± 0.1^{a}	561.6 ± 0.2^{b}	18.8 ± 0.06^{e}
5	IC 111279	12.0 ± 0.3^{c}	1.0 ± 0.1^{e}	83.0 ± 0.4^{hi}	49.0 ± 0.3^{c}	4.5 ± 0.03^{de}	10.6 ± 0.04^{b}	361.7 ± 0.7^{i}	20.3 ± 0.1^{d}
6	IC 111280	12.0 ± 0.3^{c}	4.0 ± 0.04^{b}	85.0 ± 0.4^{i}	51.0 ± 0.3^{b}	4.6 ± 0.05^{de}	8.5 ± 0.06^{d}	370.2 ± 0.2^{h}	19.5 ± 0.04^{de}
7	IC471882	13.3 ± 0.1^{b}	4.0 ± 0.04^{b}	108.0 ± 0.2^{f}	51.0 ± 0.4^{b}	5.9 ± 0.2^{ab}	12.1 ± 0.04^{a}	466.6 ± 0.2^{f}	18.5 ± 0.04^{de}
8	IC471883	13.2 ± 0.2^{b}	2.0 ± 0.05^{d}	115.0 ± 0.4^{e}	52.0 ± 0.4^{ab}	5.8 ± 0.05^{ab}	12.4 ± 0.06^{a}	691.7 ± 0.04^{a}	20.8 ± 0.06^{d}
9	IC471884	12.2 ± 0.1^{c}	2.0 ± 0.05^{d}	103.0 ± 0.6^{g}	48.0 ± 0.2^{bc}	6.1 ± 0.05^{a}	12.7 ± 0.06^{a}	406.7 ± 0.08^{g}	22.3 ± 0.05^{c}
10	IC471885	11.1 ± 0.1^{cd}	1.0 ± 0.1^{e}	88.0 ± 0.4^{h}	48.0 ± 0.2^{bc}	5.2 ± 0.05^{c}	11.3 ± 0.1^{ab}	333.3 ± 0.1^{k}	25.3 ± 0.06^{b}
11	*Aloe CIM-Sheetal* (CAL-14)	14.3 ± 0.1^{ab}	5.0 ± 0.05^{a}	143.0 ± 0.4^{b}	49.0 ± 0.2^{c}	5.6 ± 0.04^{b}	12.2 ± 0.1^{a}	471.6 ± 0.02^{e}	21.6 ± 0.09^{c}
12	Wild *Aloe vera* (Local)	10.2 ± 0.1^{d}	3.0 ± 0.06^{c}	78.0 ± 0.4^{j}	46.0 ± 0.3^{d}	3.9 ± 0.05^{e}	9.8 ± 0.06^{c}	342.6 ± 0.2l	20.3 ± 0.05^{d}

Values are means + SE. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Kuel's multiple comparisons test.

Table 5 (b): Morphological studies of Aloe accessions

S. No.	Accession No.	Leaf dry Wt (g)	Gel fresh Wt (g)	Gel dry Wt (g)	Biomass (g/plant)	Root length (cm)	Aloin Concentration (%)
1	IC 111267	23.5 ± 0.02^{a}	58.52 ± 0.04^{a}	12.4± 0.05^{a}	683.2± 0.37^{c}	14.23± 0.1^{d}	0.86 ± 0.008^{e}
2	IC 111269	23.7 ± 0.03^{a}	56.27 ± 0.08^{b}	12.4± 0.05^{a}	713.2±0.12^{b}	2.32 ± 0.5^{e}	0.9 ± 0.007^{e}
3	IC 111271	20.35±0.05^{b}	49.98±0.03^{c}	12.8±0.03^{a}	720.8±0.4^{a}	23.91±0.1^{b}	0.91±0.007^{e}
4	IC 111272	19.9 ± 0.04^{b}	20.7±0.05^{d}	8.5±0.01^{b}	223.3±0.37^{d}	9.67±0.07^{ef}	1.15±0.005^{c}
5	IC 111279	19.7± 0.04^{b}	15.12±0.06^{e}	6.3±0.04^{c}	199.2±0.32^{e}	14.72±0.07^{d}	1.4±0.007^{b}
6	IC 111280	15.8± 0.02^{c}	8.85±0.03^{f}	3.8±0.05^{d}	123.2±0.38^{f}	15.34±0.1^{d}	1.68±0.001^{a}
7	IC 471882	15.6± 0.03^{c}	6.3±0.05^{g}	3.4±0.02^{d}	86.7±0.38^{g}	26.25±0.2^{a}	1.36±0.009^{b}
8	IC 471883	12.64±0.01^{d}	4.72±0.05^{h}	2.2±0.01^{e}	58.01±0.34^{j}	18.52±0.07^{c}	1.3±0.007^{b}
9	IC 471884	12.81±0.03^{d}	4.79±.01^{h}	2.2±0.05^{e}	58.4±0.19^{j}	14.12±0.1^{d}	1.08±0.003^{c}
10	IC 471885	13.8±0.08^{c}	5.3±0.06^{gh}	3.1±0.04^{d}	74.4±0.17^{h}	7.65±0.07^{f}	0.83±0.002^{d}
11	*Aloe CIM-Sheetal*	14.13±0.06^{c}	5.65±0.02^{gh}	3.2±0.04^{d}	70.28±0.1^{i}	27.34±0.07^{a}	0.78±0.004^{d}
*12	Wild *Aloe vera* (Local)	12.2±0.09^{d}	4.1±0.06^{h}	2.1±0.05^{e}	56.2±0.2^{j}	8.91±0.04^{ef}	1.2±0.007^{c}

Values are means + SE. Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Kuel's multiple comparisons test.

2. *In vitro* Study

Micropropagation in *Aloe vera* and *Aloe ferox*

A. Shoot Initiation

For *in vitro* clonal propagation of *Aloe vera* plants shoot tips were used as explants. Explants were obtained from healthy parent plants of twelve accessions for micro propagation. After sterilization, shoot apices were directly inoculated into various media. These explants were inoculated in wide mouth culture bottles containing MS medium. The media was supplemented with different concentrations of BA alone or in combination with IBA or IAA. The explants cultured in combination of MS basal medium with BA, NAA, KIN and IBA started showing signs of proliferation after 15 days of culturing. Best growth was observed on MS medium supplemented with 1.5 mg/l BAP, 0.2 mg/l IBA, 0.1 mg/l IAA, 0.5 mg/l KIN and 0.5 mg/l GA_3. New buds appeared from the axils of leaves of shoot explants and bud developed.

B. Shoot Multiplication

After initiation of the growth on the explants (30 days culturing) the newly formed shoots were excised individually with the help of sterilized blade and re-cultured on fresh bottles containing same medium (MS medium with different supplements) to increase the number of shoots. It was observed that BAP at concentration of 1.5 mg/l gave better shoot multiplication. All the cultures showed shoot proliferation on MS medium with different concentrations (Table 6.) On an average each explants gave rise to 5-8 shoots (Fig 14).

Table 6: Shoot proliferation response from shoot tips cultured on medium with different concentrations of growth regulators

S.No	Growth Regulator (mg/l)	% response	Average No. of shoots /explant	Average length of shoots (cm)
1	IAA+ BAP 0.3 + 3.00	18.79 ± 0.01^{e}	3.80 ± 0.05^{e}	2.60 ± 0.05^{e}
2	IBA+IAA+NAA+BAP 0.1+0.1+0.1 + 4.5	27.84 ± 0.04^{d}	4.58 ± 0.03^{c}	3.18 ± 0.05^{c}
3	KIN+IBA+IAA+NAA+BAP 0.5+0.2+0.1+0.2+1.5	87.89 ± 0.02^{a}	8.84 ± 0.03^{a}	4.89 ± 0.03^{a}
4	KIN+IBA+IAA+NAA+BAP+ GA_3 0.5+0.2+0.1+0.2+1.5+0.5	77.34 ± 0.08^{b}	6.71 ± 0.07^{b}	3.56 ± 0.02^{b}
5	KIN+IBA+IAA+NAA+BAP+GA_3 0.5+0.2+0.1+0.2+1.5+1.0	65.68 ± 0.05^{c}	4.23 ± 0.03^{d}	2.88 ± 0.01^{d}

Values are means $\pm$ SE. Means followed by the same letter in a column are not significantly different ($p \leq 0.05$) by Newman-Kuel's multiple comparisons test.

C. Rooting

The *in vitro* raised 3-4 cm long shoots were excised individually from the proliferated shoot clumps and cultured on rooting media where MS medium was supplemented with different concentrations of NAA (Table 7). All the combinations showed induction of roots. Maximum number of roots (2-7) per plant was obtained in plantlets cultured on MS + 0.2 mg/l NAA. The plantlets cultured on MS medium supplemented with 2 mg/l to 10 mg/l NAA showed induction of only one root per shoot. The roots obtained were creamish yellow in colour and with/without branching. Newly formed micro roots measuring 2-3cm in length (Fig 16) were excised individually from the parent explant and transferred to rooting media. Two types of rooting medias were used one is MS basal media with 3 types of hormones NAA, IAA, IBA and other half strength MS media (Fig 15). Data were recorded after 30 days of culture.

D. Hardening of Plantlets

After 30 days of culture on rooting media, the plantlets were successfully acclimatized. Pots (8×6 cm) were kept readily filled with garden soil, compost and sand in the proportion of 2:1:1 respectively. The plants were then transplanted into the pots then thoroughly watered and kept under plastic house having 80% humidity and 31°C temperature for ten days. Then the plants were shifted to shade house with less humidity and indirect sunlight. After 15 days, the hardened plantlets were transferred to the soil. The plants were watered periodically and upper layer of the soil mulched occasionally whenever necessary. The result of acclimatization showed that 96% of plantlets survived to grow under greenhouse conditions and were morphologically similar to mother plants.

Table 7: Effect of auxins on root induction from *in vitro* regenerated shoots of *Aloe*

Conc. of auxins in the medium (mg/l)	Root induction percentage	Average number of roots	Average length of roots (cm)
0.1 NAA	65.57 ± 0.05^{d}	4.98 ± 0.005^{d}	2.09 ± 0.03^{c}
0.2 NAA	91.12 ± 0.05^{a}	6.89 ± 0.007^{a}	3.08 ± 0.04^{a}
0.5 NAA	79.87 ± 0.3^{b}	6.15 ± 0.005^{b}	2.97 ± 0.005^{b}
0.1 IBA	49.83 ± 0.2^{e}	2.98 ± 0.003^{f}	1.88 ± 0.005^{d}
0.2 IBA	74.93 ± 0.03^{c}	5.51 ± 0.003^{c}	2.91 ± 0.005^{b}
0.5 IBA	40.64 ± 0.07^{f}	2.75 ± 0.008^{g}	2.04 ± 0.05^{c}
0.1 IAA	33.65 ± 0.05^{g}	3.65 ± 0.005^{e}	0.97 ± 0.003^{e}
0.2 IAA	26.72 ± 0.08^{h}	1.31 ± 0.007^{h}	0.84 ± 0.005^{f}
0.5 IAA	-	-	-

Values are means $\pm$ SE. Means followed by the same letter in a column are not significantly different ($p \leq 0.05$) by Newman-Kuel's multiple comparisons test.

Fig 15: Regeneration of Shoot tip in *Aloe vera*

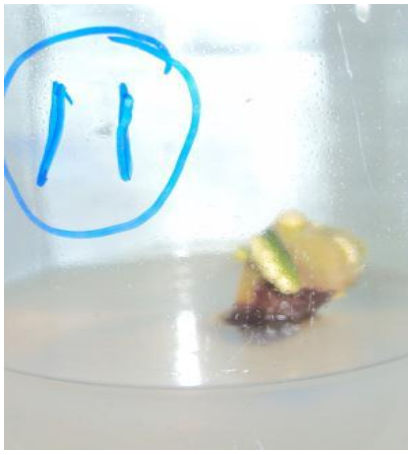


Fig a: Shoot tip regeneration in 1 week *Aloe CIM Sheetal* (11)

Fig b: Shoot tip regeneration in 1 week –Accession IC 111271(3)

Fig c: 30 days regenerated Shoot tip of Accession IC471882 (7)

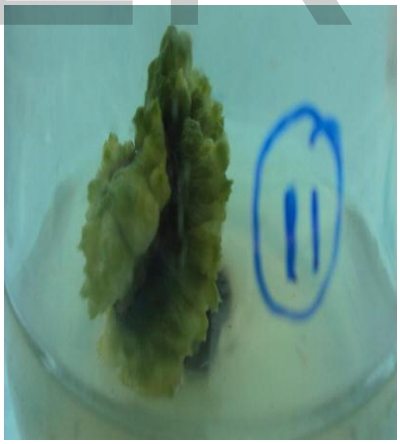


Fig d: Regeneration of Multiple shoots in *Aloe CIM Sheetal* (11)

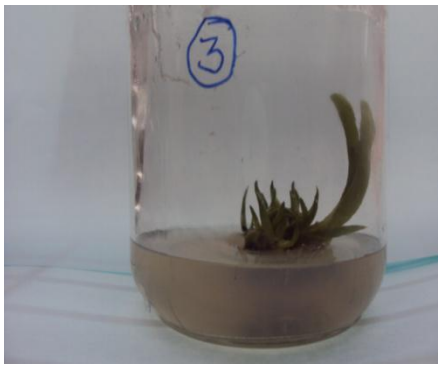


Fig e. Multiple shoots in early stage before subculture in IC 111271(3).

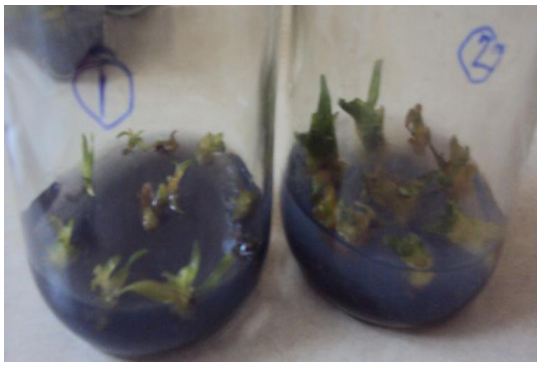


Fig f. Subcultured shoot tips in IC 111267(1) and IC 111269(2).

Fig g. Shoot multiplication of wild *Aloe vera* (12)

Fig h. Multipleshoot regeneration after Subculture of IC 111271(3)

Fig 16: Root regeneration of *Aloe CIM- Sheetal*

Fig 17: Measurement of root *Aloe* length

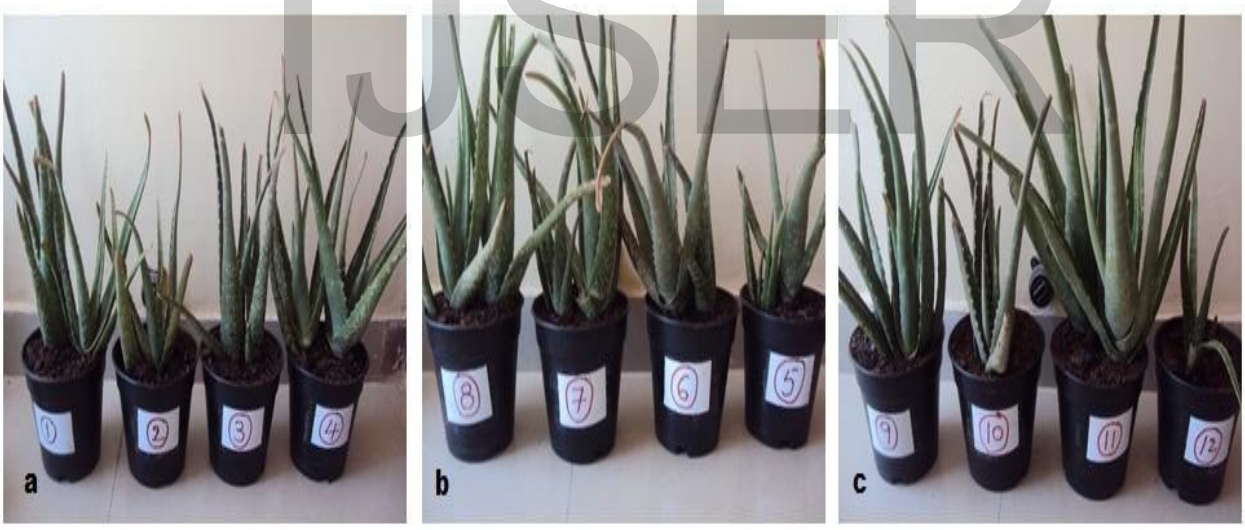


Fig 18: Hardened plantlets of different Aloe accessions ready to transfer to soil from pots.

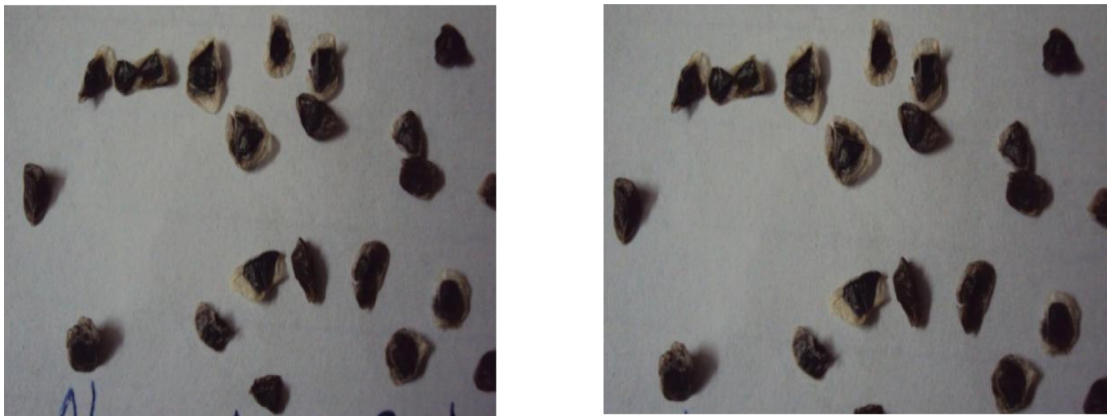


Fig 19: *Aloe ferox* Seeds

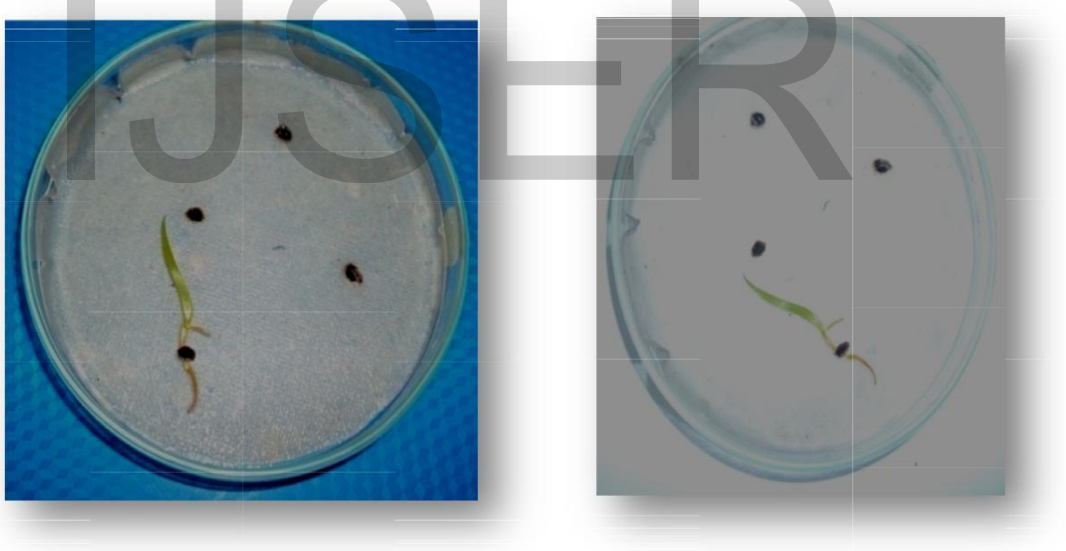


Fig 20: Seed germ ination of *Aloe ferox* in Petri dish es

A **B**

C

Fig 2 1: Seed germination o f *Aloe ferox* in bottles a nd dishes

3. Biochemical analysis

We have taken three varieties i.e 1. *Aloe CIM- Sheetal* (11), wild *Aloe vera* (12) and *Aloe ferox* for Phytochemical, antioxidant, anti-inflammatory, HPLC and NMR studies.

A. Preliminary phytochemical screening of *Aloe ferox* and *Aloe CIM-Sheetal* leaf extracts: The phytochemical analysis of *Aloe ferox* leaf extract revealed the presence of flavonoids, flavonols, alkanes, alkaloids, aldehydes, ketones, alcohols, total phenols, saponins, tannins, phytosterols, proanthocyanidins, fatty acids, indoles, pyrimidines, organic acids and dicarboxylic acids (Table 8).

Table 8: Phytochemical constituents of ethyl acetate and methanol extract of *Aloe ferox*.

S.No	Phytochemical compounds	Ethyl acetate extract	Methanol extract
1.	Total phenols	+	+
2.	Flavonoids	+	+
3.	Flavonols	+	-
4.	Proanthocyanidins	+	-
5.	Tannins	+	-
6.	Alkaloids	+	+
7.	Saponins	+	+
8.	Phytosterols	-	+
9.	Fatty acids	-	+
10.	Indoles	-	+
11.	Alkanes	-	+
12.	Pyrimidines	-	+
13.	Organic acids	-	+
14.	Aldehydes	-	+
15.	Dicarboxylic acids	-	+
16.	Ketones	+	+
17.	Alcohols	-	+

Medicinal plants are used for the treatment of various diseases due to the presence of different phytochemicals. The concentrations of compounds extracted in the different solvent extracts (Fig 21) were significantly different from each other.

Fig 21: Concentrated solvent extracts with different concentrations

Phenols, flavonoids**,** alkaloids, saponins and ketones contents were found in both ethyl acetate and methanol extracts of *Aloe ferox*. These compounds aloin, aloe-emodin and aloesin may be a contributing factor towards its antioxidant activity. Absence of flavonols, proanthocyanidins and tannins were observed in the methanol extracts when compared with the ethyl acetate extract. Phytosterols, fatty acids, indoles, alkanes, pyrimidines, organic acids, aldehydes, dicarboxylicacids and alcohols were present in methanol extract but absence in ethyl acetate extract.

Table 9: Phytochemical constituents of different extracts of *Aloe CIM- Sheetal* leaves

TESTS	ALE- EA	Gel Extract - EA	ALE- MET	ALE -AC	ALE -CH	ALE- H_20
Alkaloids	+	-	-	-	-	-
Sterols	-	+	-	-	-	-
Glycosides	+	-	+	+	-	+
Steroids	-	-	-	-	+	-
Saponins	+	-	-	-	+	-
Phenolics	+	-	+	-	-	+
Tanins	+	-	+	-	+	+
Terpenoids	+	-	-	-	-	-
Acid compounds	+	-	-	-	-	-
Reducing sugars	-	+	+	-	+	-
Carbohydrates	-	+	+	+	+	+
Flavonoids	+	-	+	-	-	-
Anthraquinones	+	-	-	-	-	-
Resins	+	-	-	-	-	-
Phlobatanins	-	-	+	-	+	-

+ **Present, - absent**, ALE-EA (*Aloe CIM - Sheetal* leaf extract with Ethyl Acetate), Gel extract with Ethyl acetate, MET: Methanol, AC: Acetone, CH: Chloroform and H_2O: Water

B. Antioxidant activity:

DPPH radical scavenging activity: The DPPH scavenging potential of the whole leaf extracts of *Aloe ferox* was depends on the concentration of the extracts. The percentage inhibition of DPPH by the various solvent extracts and the standard drugs was recorded in decreasing order: BHT > methanol >ethyl acetate. The percentage inhibition of BHT was significantly different from other extracts. The ethyl acetate extract showed the least inhibition.

A freshly prepared DPPH solution exhibited a deep purple color with a maximum absorption at 517 nm. This purple color disappears once an antioxidant is present in the medium and antioxidants molecules will quench DPPH free radicals and convert them to a colorless product, resulting to a decrease in absorbance at 517 nm. The radical scavenging activity of values of methanol and ethyl acetate fractions are presented in the Table 10; results are expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH solution in the absence of an extract at 517 nm. Radical scavenging activity percentage was proportional to the concentration of the extract.

Table 10: *Aloe ferox* leaf ethyl acetate and methanol extracts fractions.

Concentration (µg/ml)	Absorbance	
	***Aloe ferox* ethyl acetate extract**	***Aloe ferox* methanol extract**
10	0.02	0.06
100	0.14	0.23
200	0.25	0.41
500	0.38	0.73

The activity was carried out in triplicate and the results were expressed as mean values ± standard deviation. The concentration of extract providing 50% inhibition (EC50%) was calculated from the graph of scavenging effect percentage against the extract concentration. α-tocopherol and butylated hydroxyl toluene (BHT) were used as standards. Because, the presence of the polyphenols, alkaloids and indoles of the *A. ferox* leaf extract shows antioxidant capacity as confirmed by DPPH assay.

The strong antioxidant activity of plant extract exhibited by the presence of phenolic compounds, which have direct antioxidant properties due to the presence of hydroxyl groups, which act as hydrogen donor. Also, they are found to be effective in scavenging free radicals as a result of their redox properties that allow them to act as reducing agents. The potent water soluble antioxidants, flavonoids are hydroxylated phenolics which help in radical scavenging and prevention of oxidative cell damage and they have reported to possess strong antioxidant activities.The concentration was low in the whole leaf extracts of *Aloe ferox* .But, methanol extracts has more flavonoids than the other solvent extracts during this study.

A. ferox whole leaf extracts showed the reductive capabilities when compared with BHT. The reducing power of the extracts was concentration dependent and the antioxidant activities in all the solvents used were comparable with BHT. DPPH is a relatively stable free radical scavenger that converts the unpaired electrons to paired ones by hydrogen proton donation. Scavenging of DPPH radical during this study indicates the potency of the plant extracts in donating hydrogen proton to the lone pair electron of the radicals.

C. Anti - Inflammatory activity:

Anti-inflammatory activity of various solvent extracts of *Aloe ferox* leaf was presented in the tables given below **(**Table 11, Table 12, Table 13 and Table 14).

Table 11: Paw volume in control group animals

S.No.	Paw volume (ml)			
	0 hr	**0 hr**	**0 hr**	**0 hr**
1	0.21 ml	0.41 ml	0.73 ml	1.05 ml
2	0.22 ml	0.37 ml	0.67 ml	0.99 ml
3	0.19 ml	0.36 ml	0.69 ml	1.01 ml
4	0.25 ml	0.39 ml	0.71 ml	0.96 ml
5	0.21 ml	0.38 ml	0.74 ml	1.02 ml
6	0.23 ml	0.39 ml	0.70 ml	1.01 ml

Table 12: Paw volume in standard group animals.

S.No.	Paw volume (ml)			
	0 hr	**1 hr**	**2 hr**	**3 hr**
1	0.17 ml	0.20 ml	0.25 ml	0.29 ml
2	0.14 ml	0.18 ml	0.21 ml	0.21 ml
3	0.15 ml	0.22 ml	0.19 ml	0.24 ml
4	0.16 ml	0.19 ml	0.25 ml	0.27 ml
5	0.15 ml	0.21 ml	0.23 ml	0.28 ml
6	0.14 ml	0.22 ml	0.20 ml	0.26 ml

Table 13: Paw volume in *Aloe ferox* methanol extract 250mg/kg treated animals.

S.No.	Paw volume (ml)			
	0 hr	**1 hr**	**2 hr**	**3 hr**
1	0.20 ml	0.35 ml	0.42 ml	0.66 ml
2	0.21 ml	0.36 ml	0.39 ml	0.63 ml
3	0.19 ml	0.29 ml	0.44 ml	0.58 ml
4	0.22 ml	0.28 ml	0.45 ml	0.65 ml
5	0.21 ml	0.34 ml	0.42 ml	0.62 ml
6	0.19 ml	0.32 ml	0.44 ml	0.64 ml

Table 14: Paw volume in *Aloe ferox* methanol extract 500 mg/Kg treated animals

S.No.	Paw volume (ml)			
	0 hr	**1 hr**	**2 hr**	**3 hr**
1	0.17 ml	0.32 ml	0.44 ml	0.52 ml
2	0.18 ml	0.27 ml	0.40 ml	0.51 ml
3	0.15 ml	0.31 ml	0.39 ml	0.49 ml
4	0.18 ml	0.30 ml	0.43 ml	0.53 ml
5	0.19 ml	0.28 ml	0.42 ml	0.52 ml
6	0.18 ml	0.29 ml	0.41 ml	0.51 ml

The present study shows the scavenging activity of the leaf extracts of *A. ferox* in methanol and ethyl acetate. Among the ethyl acetate and methanol extractions of *Aloe ferox,* the methanol extract showed the highest antioxidant activity when evaluated by the DPPH and reducing power method. The observed results suggest further analyses to confirm its prophylactic effect in the treatment of free radical mediated diseases. Most antioxidant activities rely upon the amount of the phytochemicals present in the plants. In Indian system of medicine, certain herbs are claimed to produce relief of pain and inflammation. The claimed therapeutic reputation needs to be verified in an exceedingly scientific manner. In the current study one such drug *Aloe ferox* leaf extract was taken (ALE).

In this study *A. ferox* traditionally used for different health disorders was studied for their *in-vitro* antioxidant and anti-inflammatory activities. The anti-inflammatory activity of this plant material has not been reported till now in the literature. In this study *in vitro* anti-denaturation of bovine serum albumin and reducing antioxidant activity was evaluated. The results have clearly showed that, *Aloe ferox* plant had moderate to significant antioxidant and anti-denaturation activity. It was observed that *Aloe ferox* methanol extract (AFME) showed significant inhibition against carrageenan-induced paw edema in the dose dependent manner (Table 15).

Table 15: *Aloe ferox* leaf extract with Carrageenan Induced Paw Edema in Rats anti-inflammatory activity of *Aloe ferox*, leave values expressed as Mean ± SD; Number of animals in each group: 6

Group	Treatment	Mean paw volume (ml)				edema inhibition (%)
		0 hr	1 hr	2 hr	3 hr	
I	2% CMC	0.23 ± 0.02	0.37 ± 0.02	0.71± 0.02	1.01 ± 0.03	-
II	Ibuprofen 10 mg/kg	0.14 ± 0.01	0.18 ± 0.01	0.23 ± 0.02	0.28 ± 0.02	75%
III	AFME 250 mg/kg	0.18 ± 0.02	0.32 ± 0.02	0.40 ± 0.02	0.61 ± 0.02	38%
IV	AFME 500 mg/kg	0.17 ± 0.02^{b}	0.29 ± 0.02^{b}	0.41 ± 0.01^{b}	0.52 ± 0.01^{b}	50%

P< 0.01*: a, P< 0.001**: b and P< 0.0001***:

Among them methanolic extract was more potent than the ethyl acetate extract. **Chemical analysis in *Aloe ferox***

Analytical HPLC and ^{13}C- NMR spectroscopy was undertaken to assess the various components present in the *Aloe ferox* leaf extract.

3 (D). HPLC analysis of methanol extract of leaf of *Aloe ferox* was carried out with the mobile phase methanol: acetonitrile: water in the ratio 25:35:40 gave total of 6 peaks at retention time 4.545Min. The highest peak was seen at the retention time 563.778mAU (Fig 22).

3 (E). ^{13}C-NMR analysis of the same methanol extract of leaf of *Aloe ferox* showed a number of peaks in between δ 16.832 to 71.196 and δ 174.6 to 176.777 (Indicating presence of aromatic rings). The highest peak was seen between 48.996 - 49.007 (Fig 30).

These signals confirmed the presence of aromatic ring indicating towards the occurrence of possible chemical compounds – respectively.

Chemical analysis in *Aloe vera*

3 (D). HPLC Profile

Column: BioSep-SEC-S 2000, 300X 7.80mm; **Mobile Phase**: 50% ACN IN 20mm Ammonium Acetate; **Detection:** 210 nm; **Flow Rate:** 2.0ml/min.

HPLC results:

- High-Performance Liquid Chromatography. For the HPLC methods, the analyses were carried out on a Dionex system (Dionex, Sunnyvale, CA, USA), consisting of an autosampler (ASI 100), pump (P680), chromatographic oven (TCC-100), and a photodiode array detector (PAD100). UV absorbance was monitored from 200 to 400 nm (Fig 23).
- The extract of *Aloe CIM- Sheetal* was injected for HPLC and the compound peaks were identified at 4.508 Min Ret Time - Area 293.135 mAU (Fig 24).
- *Aloe ferox* - peak was identified at 4.545Min Ret Time - Area 563.778 mAU (Fig 22).
- Wild *Aloe vera* - Small peak was identified at 4.510Min Ret Time - Area 22.18 mAU (Fig 25) Compared with Standard compound - 4.615Min Ret Time - Area 1363. 15 mAU (milliabsorbance units) (Fig 26).

Fig 23: HPLC column: (Bio Sep - SEC- s2000)

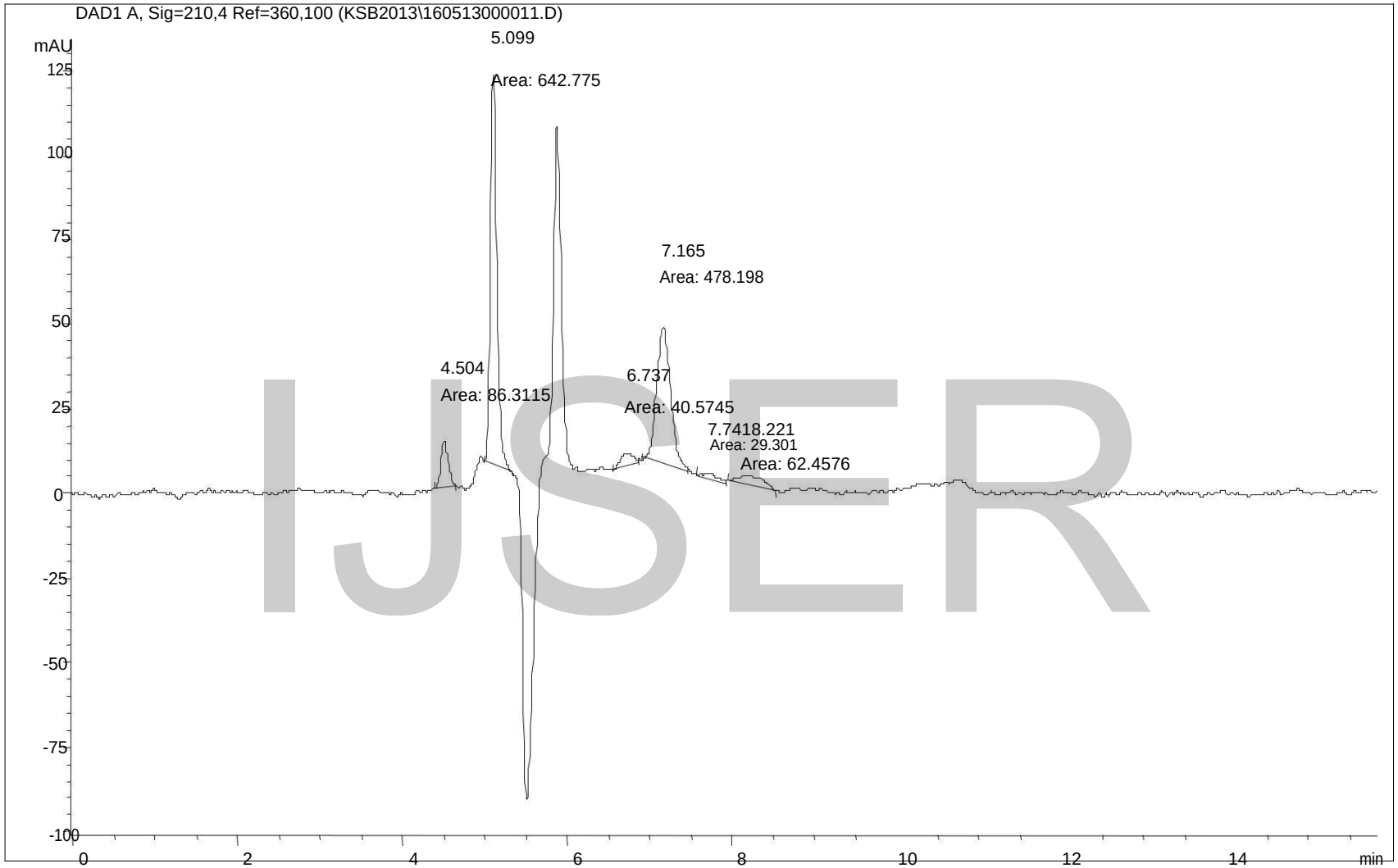


Fig 23: Compound extracted by HPLC in *Aloe ferox*

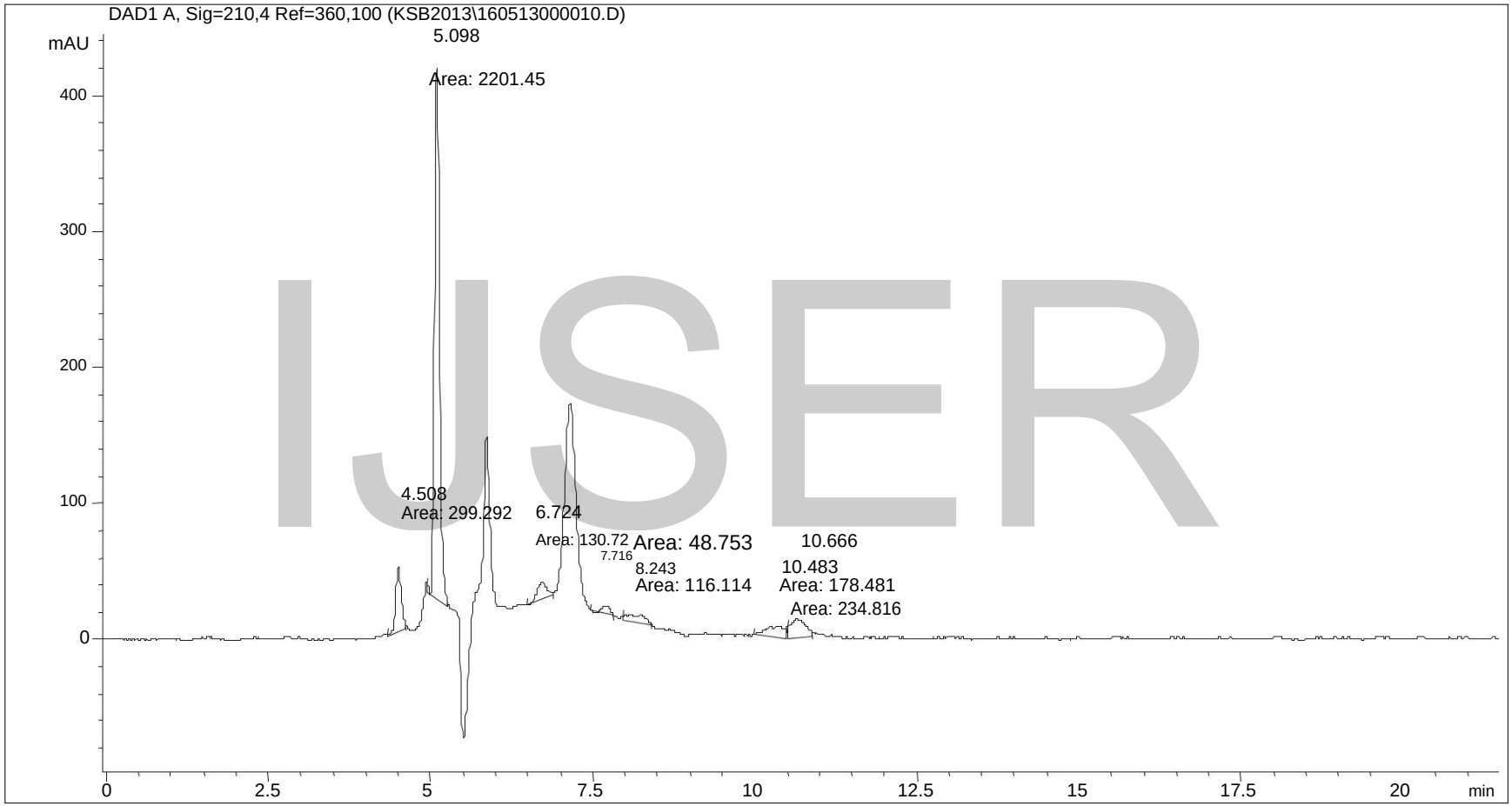


Fig 26: *Aloe CIM-Sheetal* extracted compound by HPLC

Fig 27: Compound extracted by HPLC in *Aloe vera* (wild)

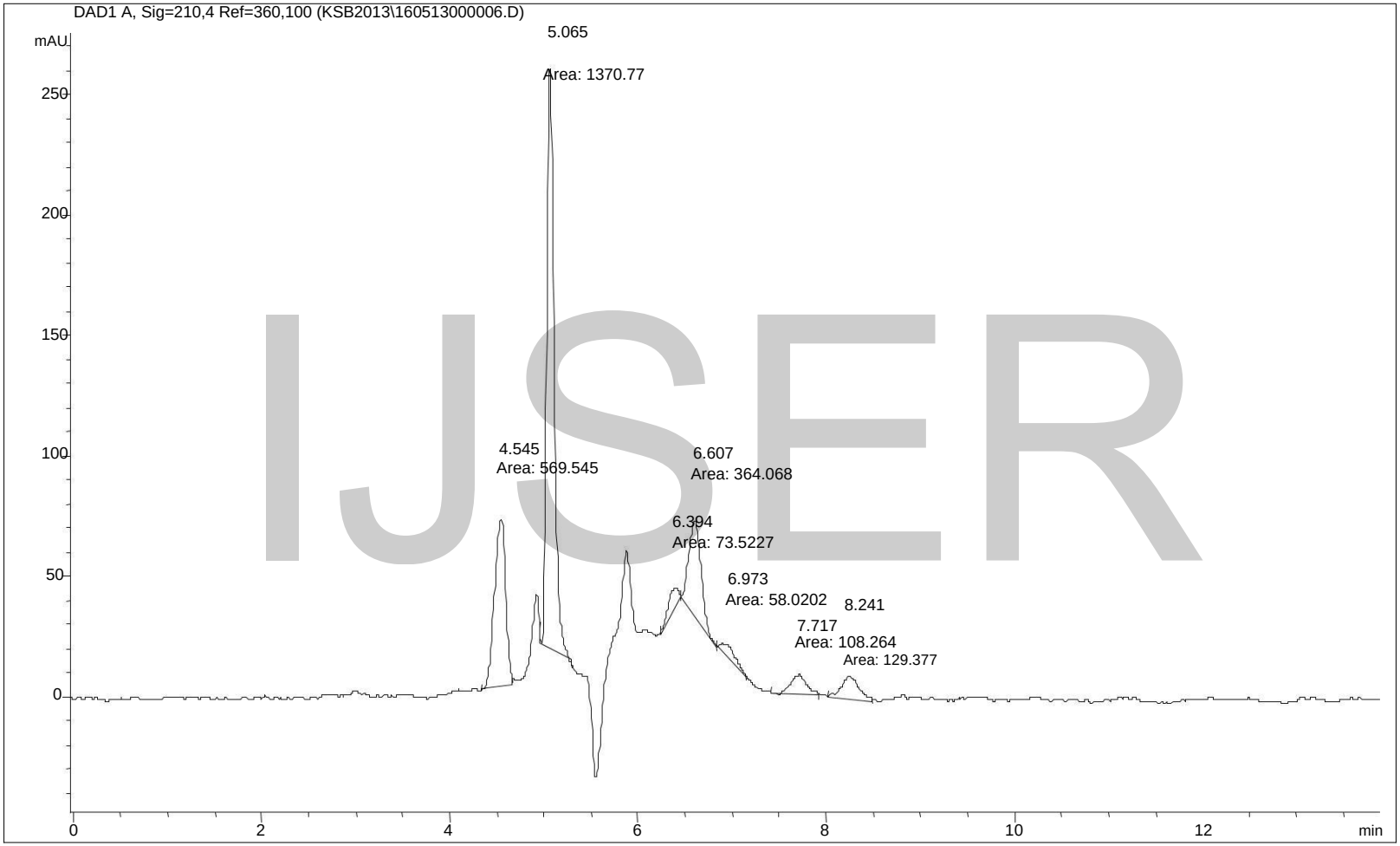


Fig 28: Standard Compound (Acemannan) extracted by HPLC

^{18}C- HPLC Profile

Column: WATERS HR C18 300X3.9MM 6U; **Mobile Phase:** 50% Methanol in water;

Detection: 210nm; **Flow rate:** 1.0ml/min

Desirable compounds were not identified in ^{18}C- HPLC (Fig 29 and Fig 30).

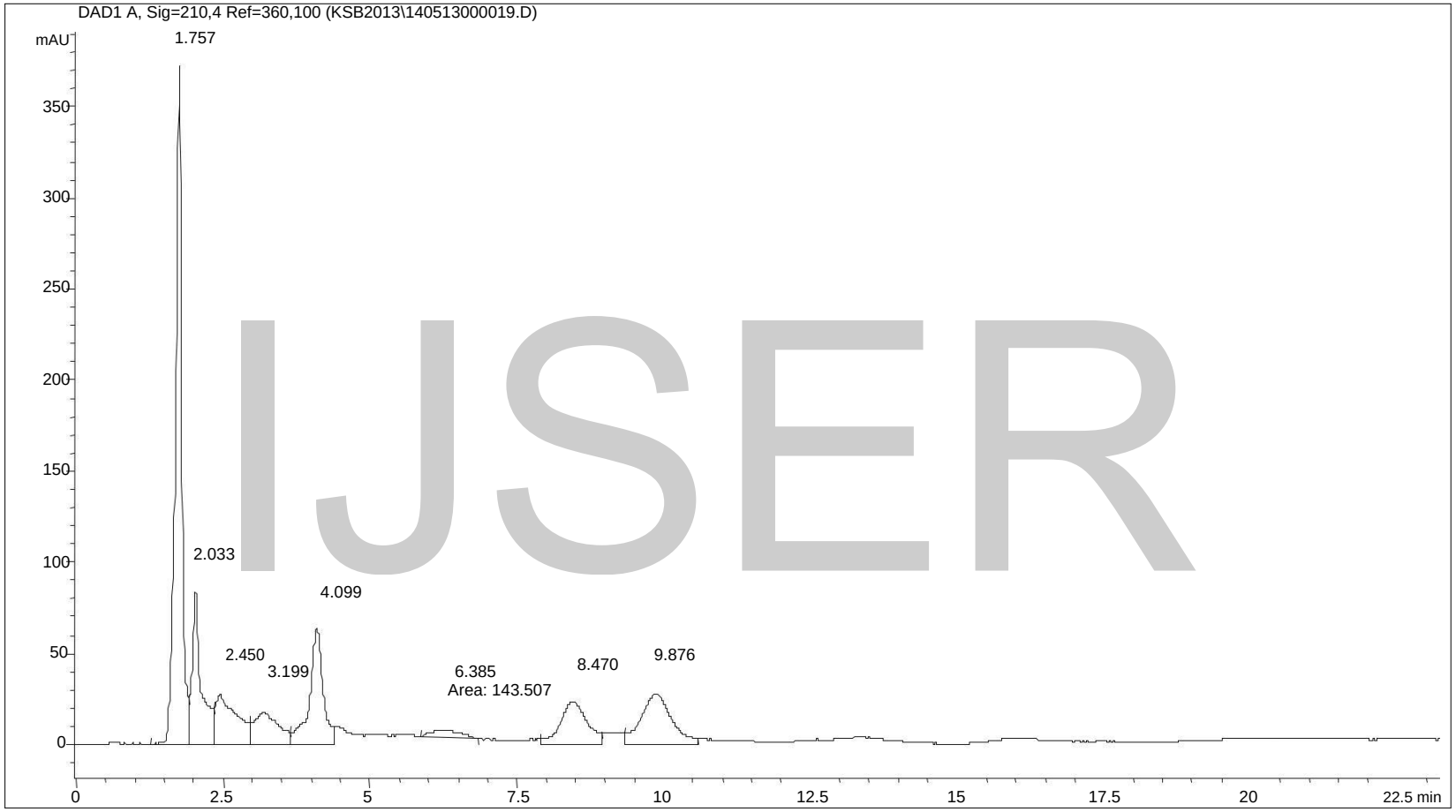


Fig 29: Compound extracted by HPLC – ^{18}C in *Aloe CIM-Sheetal*

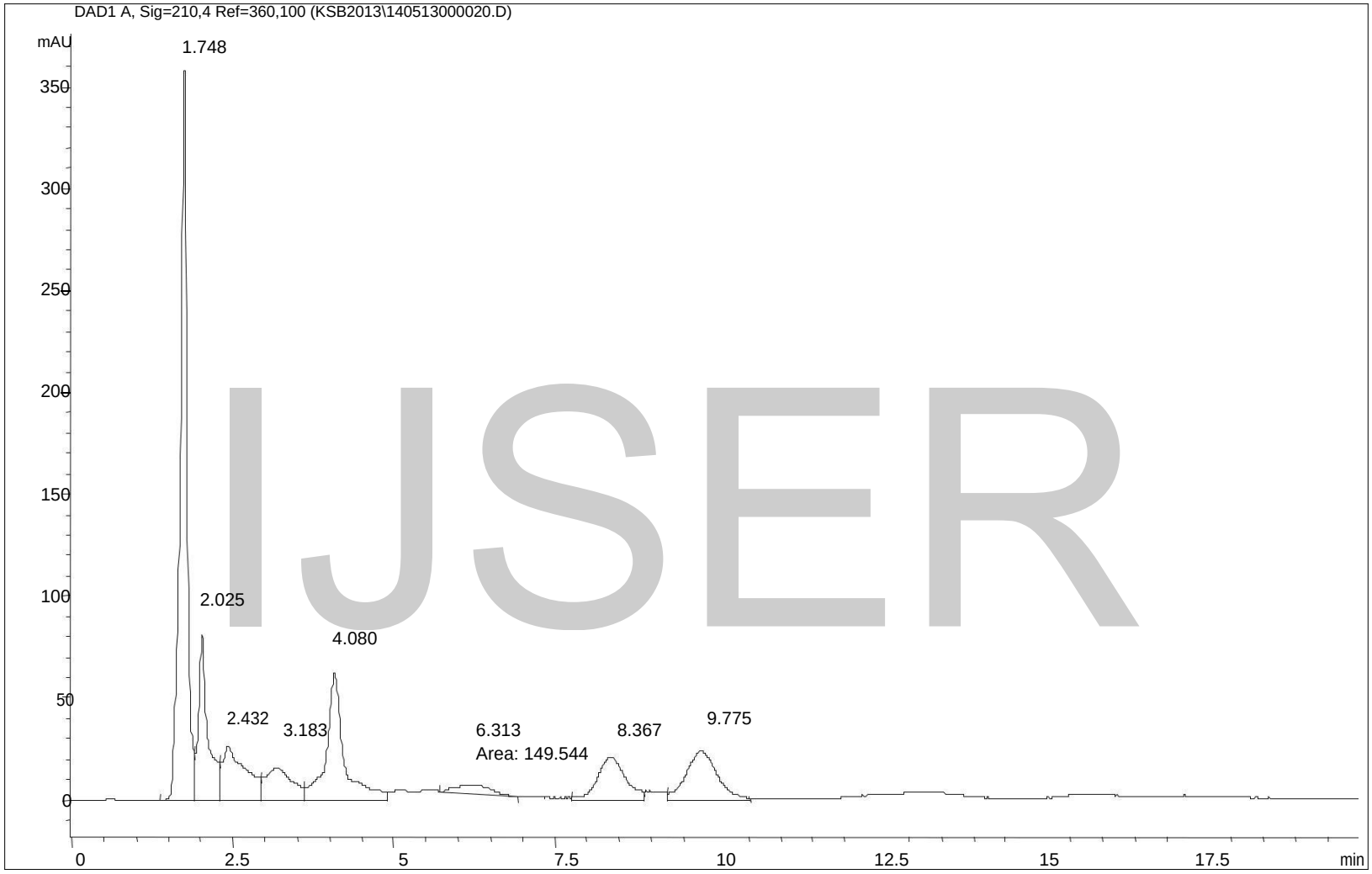


Fig 30: Compound extracted by HPLC - ^{18}C in wild *Aloe vera*

3. (E). NMR Spectroscopy

Nuclear magnetic resonance spectroscopy: commonly referred to as NMR, is a technique which exploits the magnetic properties of certain nuclei to study physical, chemical and biological properties of matter.

^{13}C-NMR Spectroscopy

Organic compounds contain carbon. Unfortunately, the C-12 nucleus does not have a nuclear spin, but the ^{13}C nucleus does due to the presence of an unpaired neucarbon-1tron. ^{13}C nuclei make up approximately 1% of the carbon nuclei on earth. Therefore, ^{13}C NMR will be much less sensitive than ^{1}HNMR.

The presence of spin-spin coupling between a ^{13}C nucleus and the nuclei of ^{1}H atoms bonded to the ^{13}C, splits the carbon-13 peaks and causes an even poorer signal-to-noise ratio.

Each nonequivalent ^{13}C gives a different signal. A ^{13}C signal is split by the ^{1}H bonded to it according to the (n + 1) rule. Coupling constants of 100-250 Hz are common, which means that there is often significant overlap between signals, and splitting patterns can be very difficult to determine. The most common mode of operation of a ^{13}C-NMR spectrometer is a proton-decoupled mode.

NMR Results:

Identified the compound peaks and eluted from the injected compound by ^{13}C NMR

a) *Aloe CIM- Sheetal* (11) - between 48.979 - 49.031 at 50 ppm (Fig 31).

b) *Aloe ferox* - between - 48.996 - 49.001 at 50 ppm (Fig 32).

c) Wild *Aloe vera* - No compounds were identified at 50 ppm (Fig 33).

d) Compared with Standard compound - between 49.003 at 50 ppm **(**Fig 34).

Identified the compound peaks and eluted from the injected compound by ^{1}H NMR

a) *Aloe CIM- Sheetal* - between 2.598 at 2.5 ppm (Fig 35).

b) *Aloe ferox* between 2.793 - 2.822 at 2.9 ppm (Fig 36).

c) Wild *Aloe vera* - No compounds were identified at 2.070 - 2.912 ppm (Fig 37).

d) Compared with Standard compound - between 2.627 at 2.0 ppm (Fig 38).

NMR Spectras

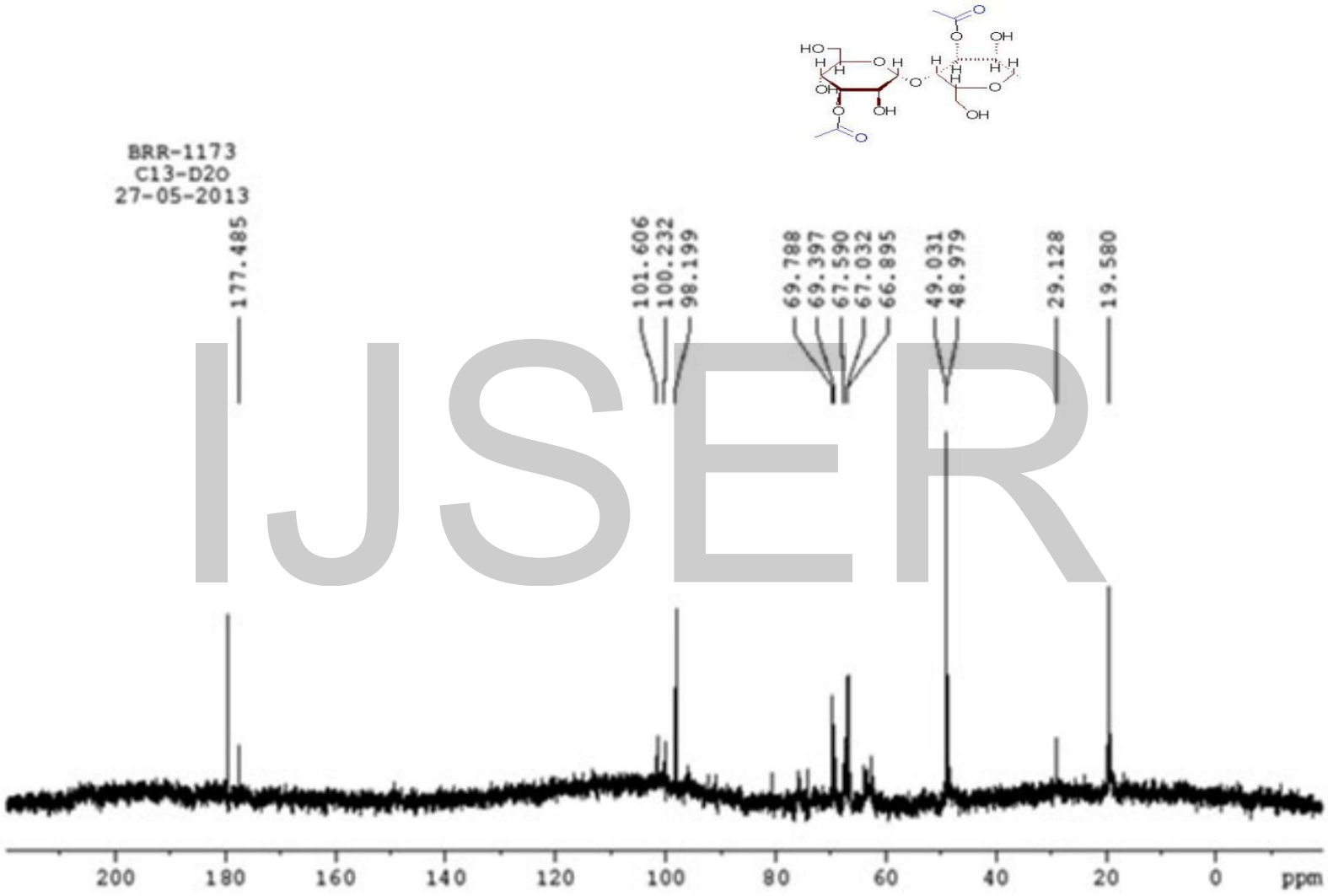


Fig 31: Eluted compound in *Aloe CIM-Sheetal* by NMR ^{13}C

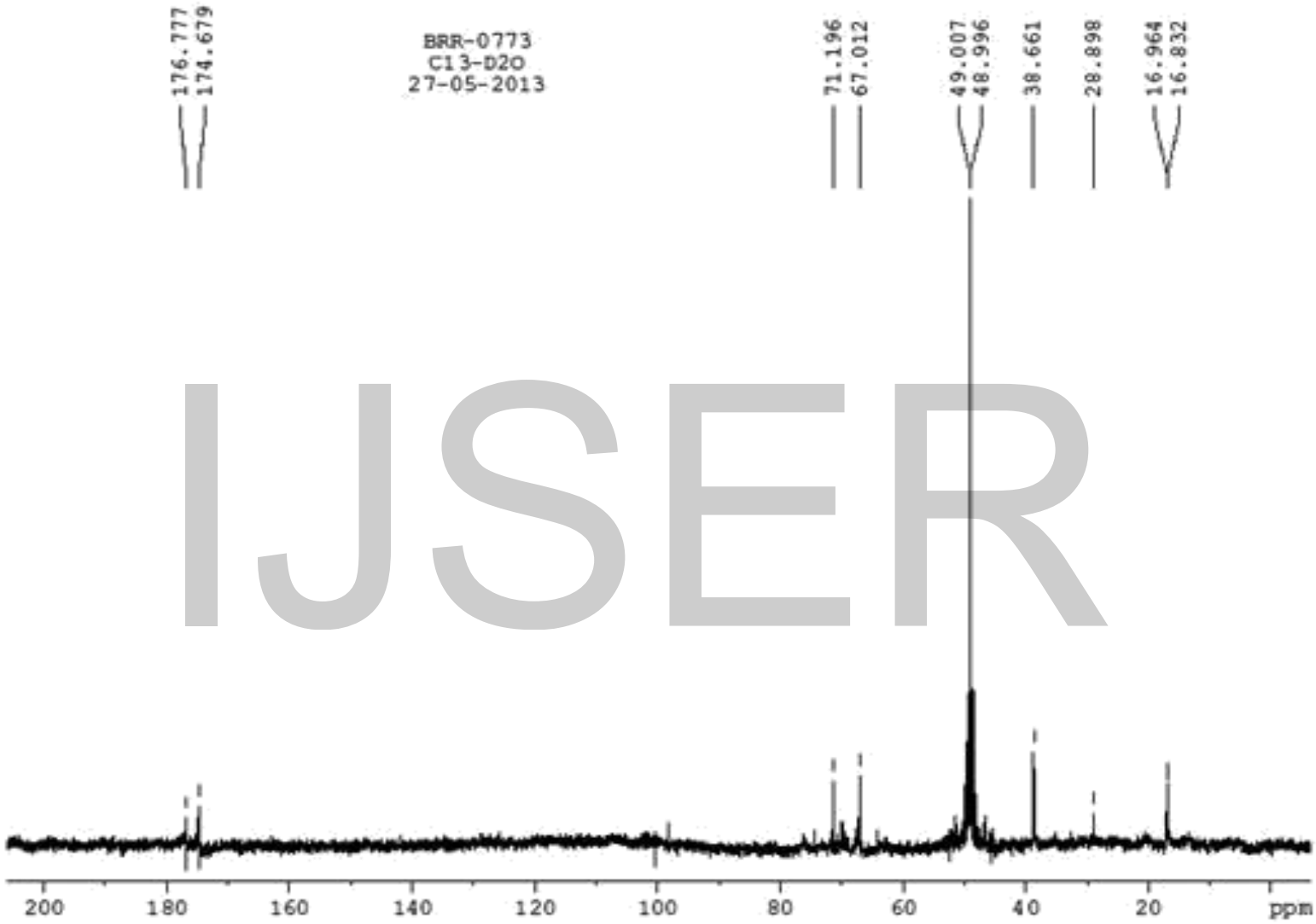


Fig 32: Eluted compound in *Aloe ferox* by NMR ^{13}C

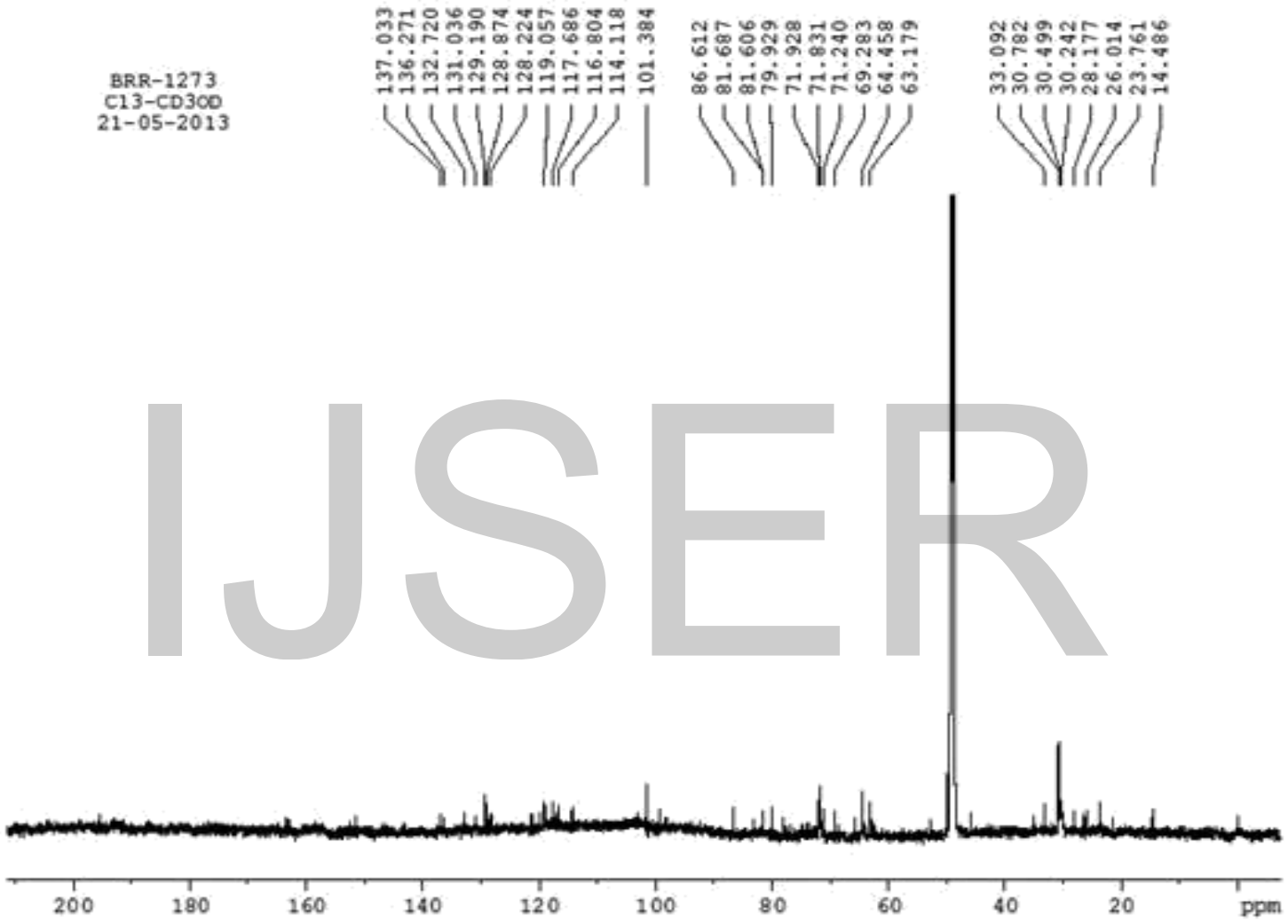


Fig 33: No compounds eluted in wild *Aloe vera* by NMR ^{13}C

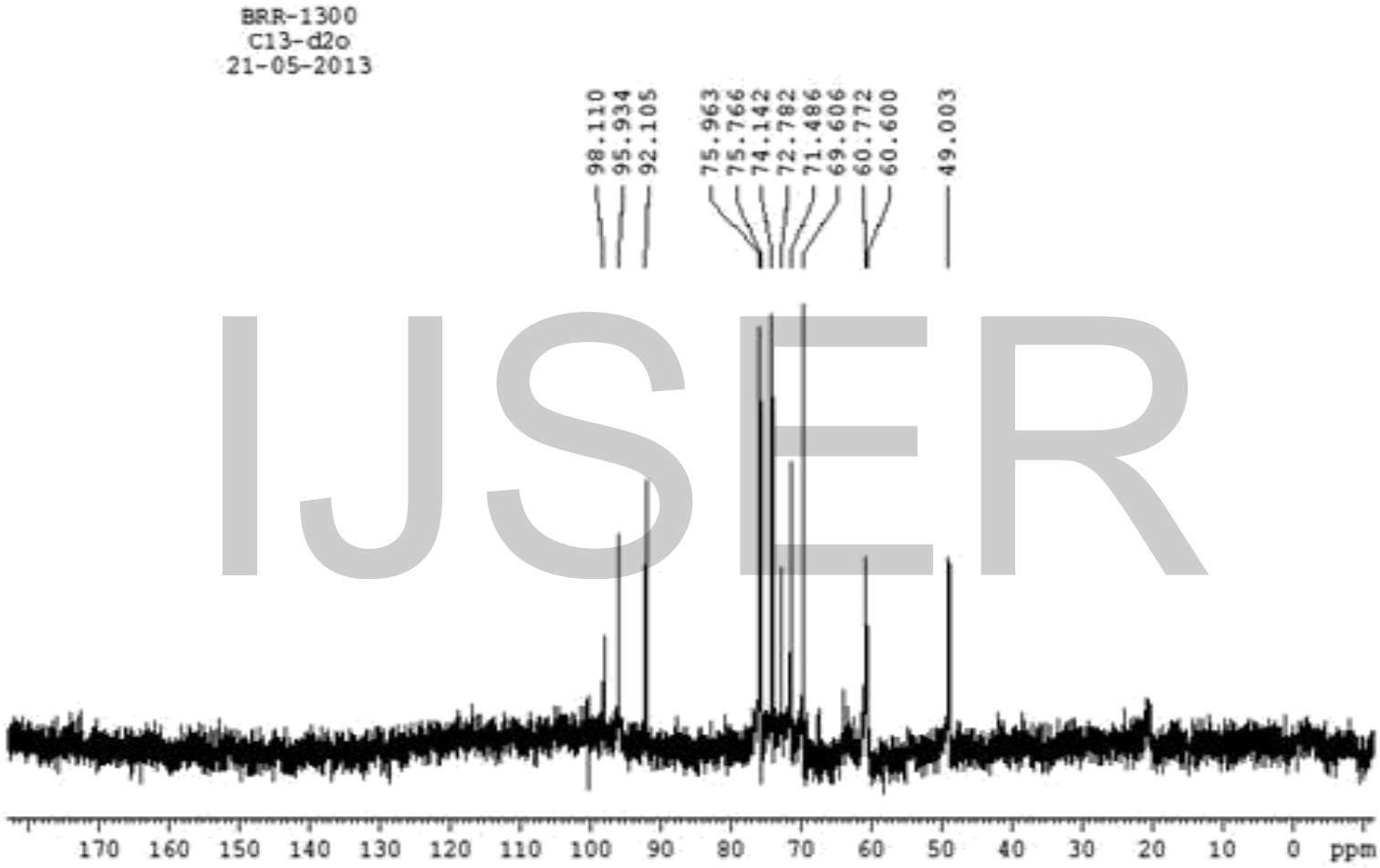


Fig 34: Standard compound of NMR^{13}C

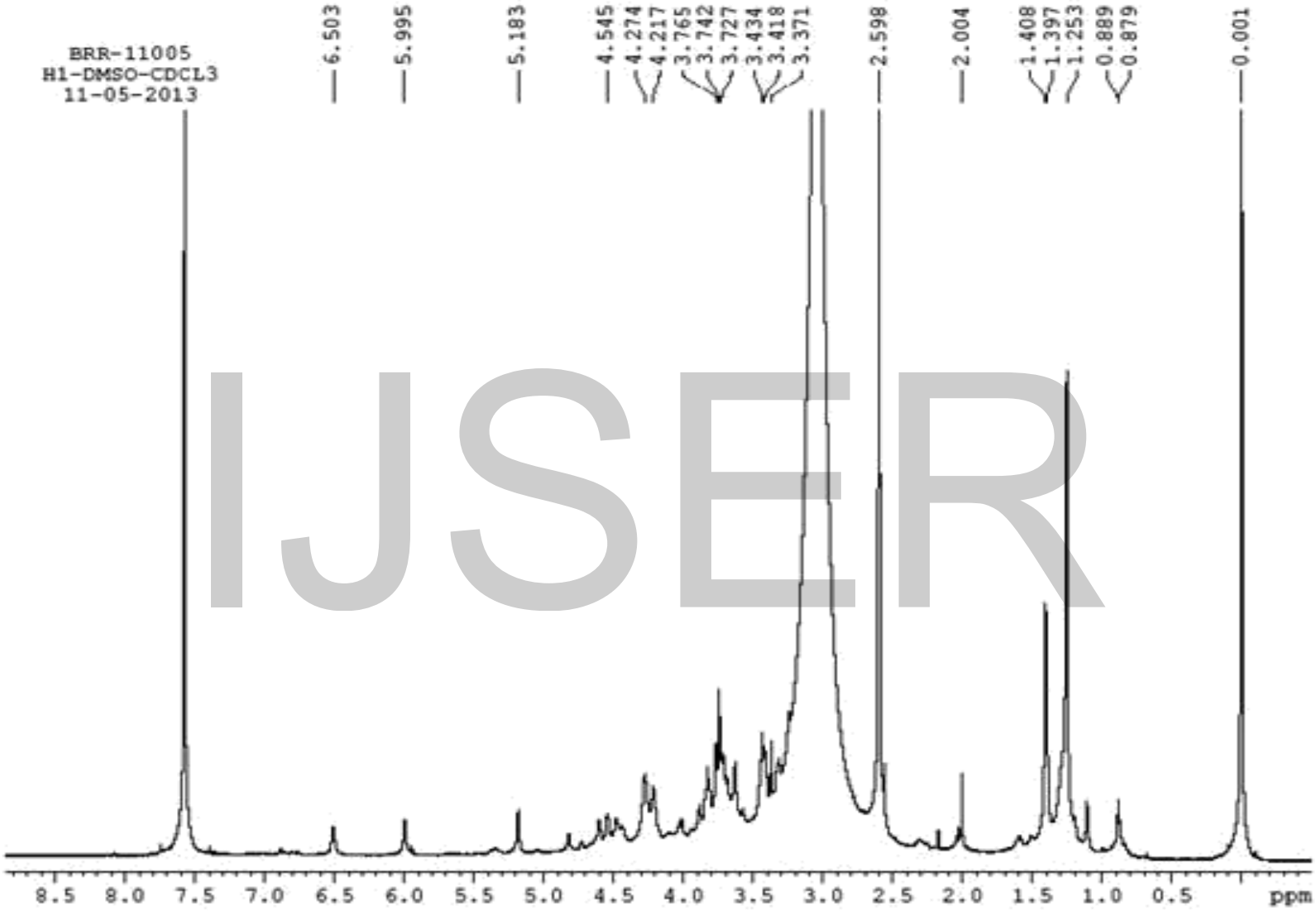


Fig 35: Eluted compound in *Aloe CIM-Sheetal* by ^{1}H NMR

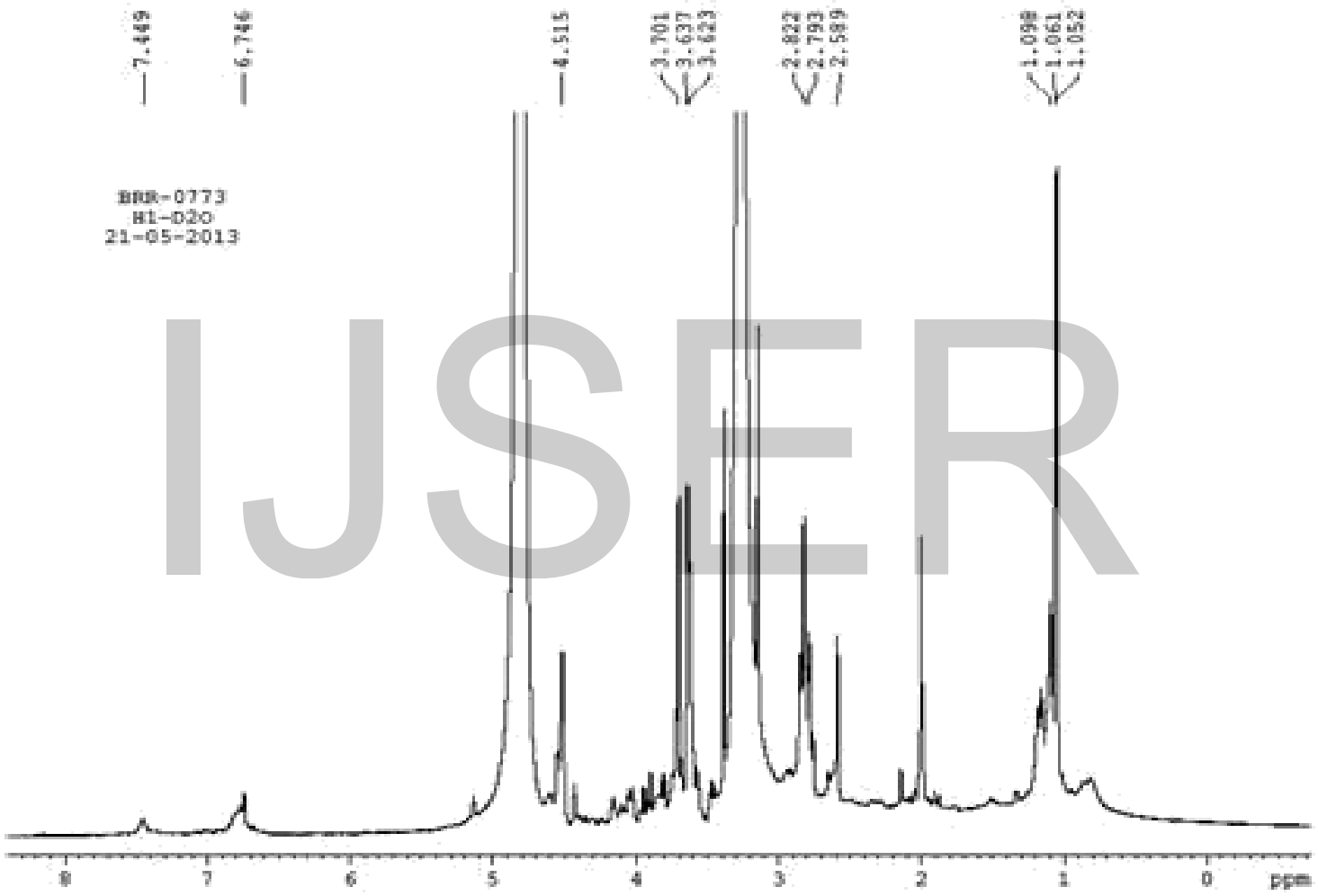


Fig 36: Eluted compound in *Aloe ferox* by ^{1}H NMR

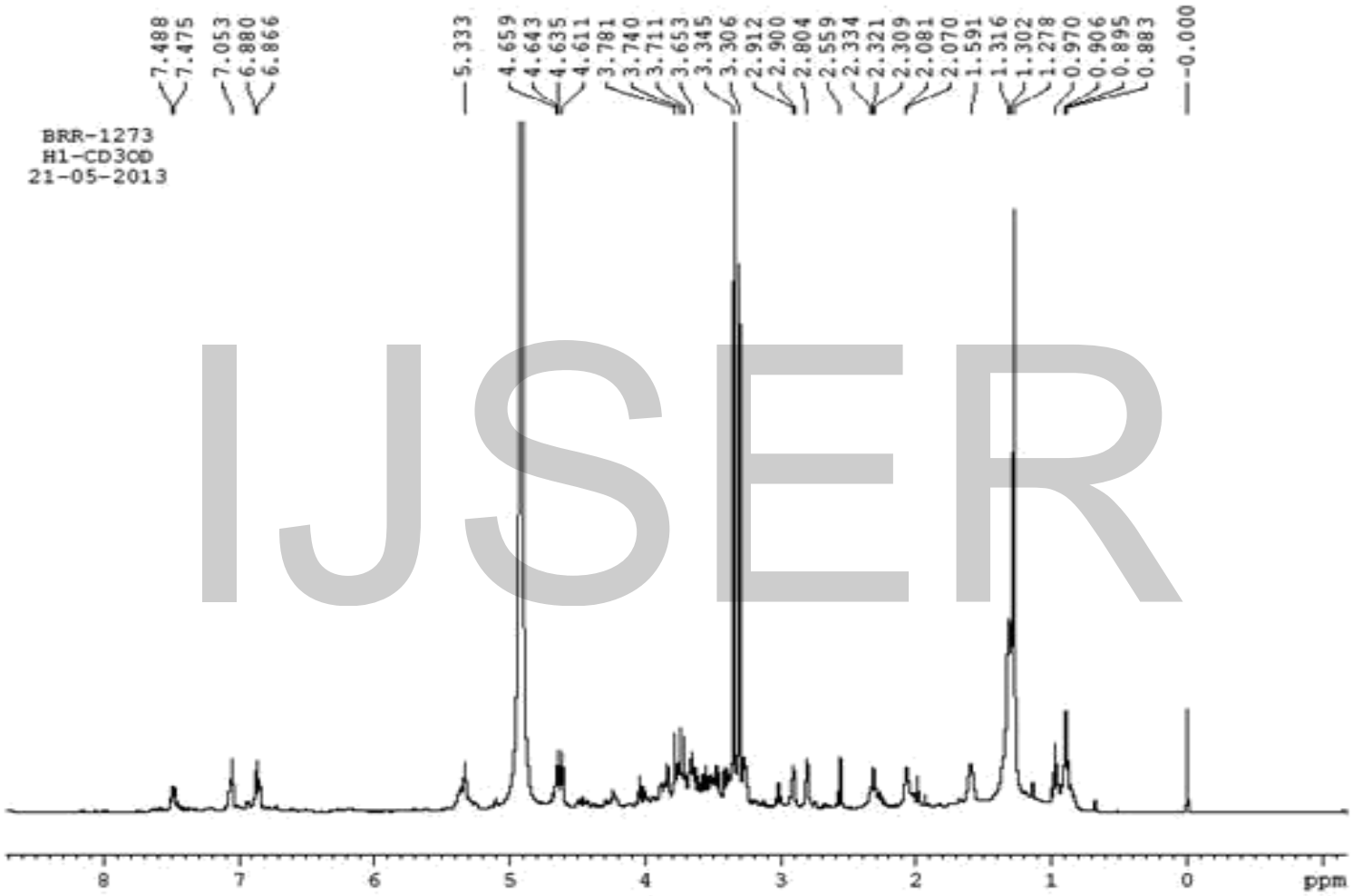


Fig 37: No compounds eluted in Wild *Aloe vera* by ^{1}H NMR

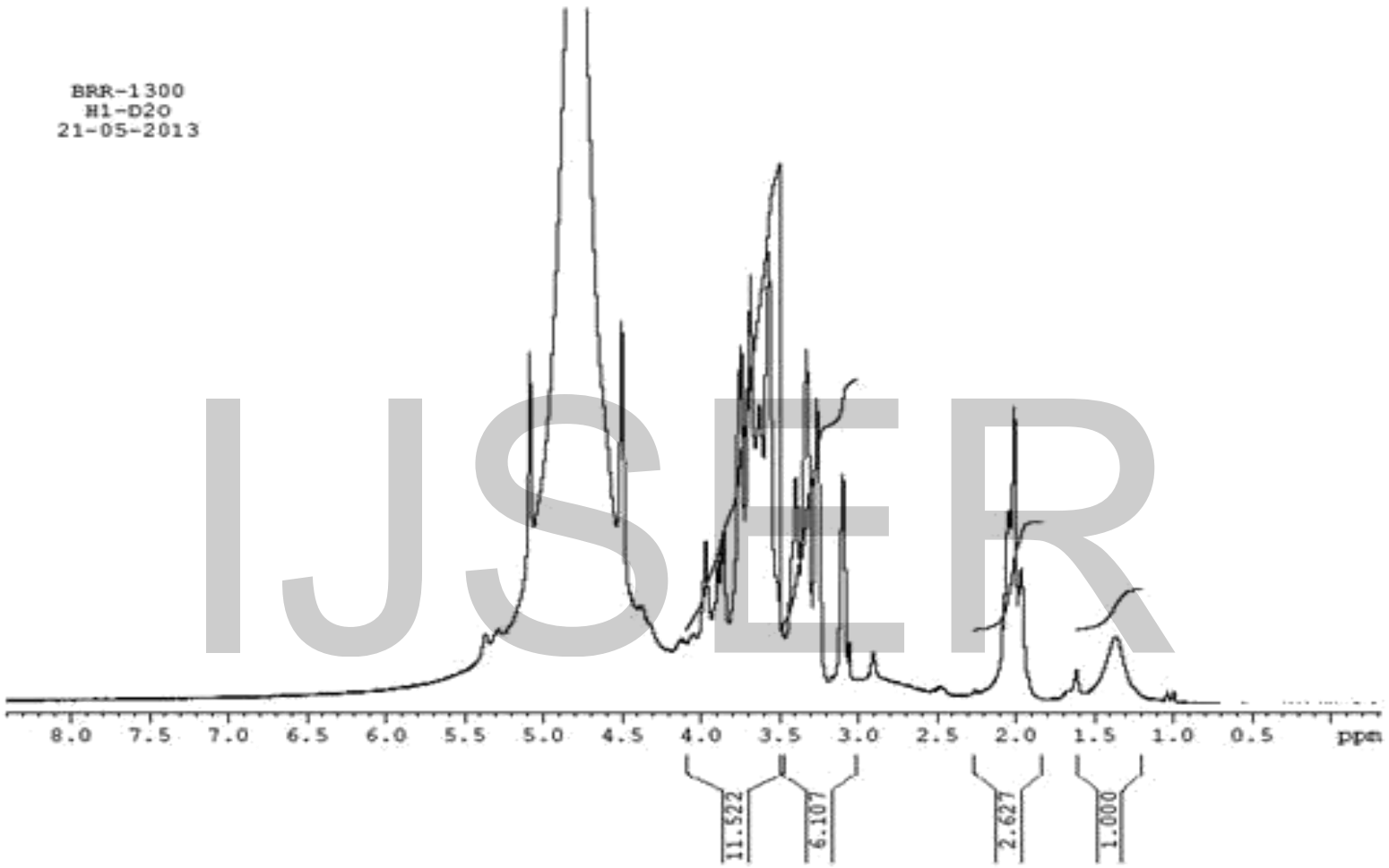


Fig 38: Standard compound of ^{1}H NMR

4. Anti diabetic studies

In the present study, the methanolic extract of *Aloe CIM- Sheetal* produced a significant decrease in the blood glucose level at a dose of 300mg/kg and 500 mg/kg in diabetic rats. The animals which are treated with 300 mg/kg of aloe leaf extract showed a significant decrease in the blood glucose levels when compared to the100, 200 and 500 mg/kg. Streptozotocin (STZ) selectively destroyed the pancreatic insulin β cells, leaving less active cell resulting in a diabetic state. STZ action in β cells is accompanied by characteristic alterations in blood insulin and glucose concentrations. The significant decrease in the blood glucose levels in diabetic rats treated with methanolic extract has substantially prevented the body weight loss. Daily treatment of animals with extract for three weeks led to a dose dependent fall in blood glucose levels. Maximum effect was seen in animals which are treated with 300 mg/kg of ALE.

4 (A). Anti diabetic activity

The results revealed that, the body weight was significantly diminished by the treatment with the extracts (300 mg/kg) after 21 days (Table 16). It was observed that there was a significant effect of *Aloe CIM-Sheetal* (300 mg/kg) on plasma glucose level of STZ treated SD rats. Glibenclamide (0.60 mg/kg) and *Aloe CIM-Sheetal* (300mg/kg) groups showed significant decrease in the blood glucose level when compared to diabetic groups (Fig 39). Further, a similar trend like that of the 7^{th} day was observed on 14thday $P<0.05$ and 21st day $P<0.05$ also. It was observed that the healthy control group rats showed a stable body weight in comparison to diabetic rats which showed reduction in body weight. The acute and sub- acute toxicity studies of *Aloe CIM-Sheetal* shows that the maximum dose of 20g/kg b.w. of rats.

The rats were orally given a multiple dose of the methanol extract from the leaf of *Aloe CIM - Sheetal* at 5, 10, 15 and 20 g/kg and neither signs of toxicity nor death of rats were observed during the 14 days of the acute toxicity study and body weights were recorded as shown in the Table 17 and significant difference was noticed as compared to control group. The sub-acute toxicity study of methanol extract of *Aloe CIM–Sheetal* with the same above doses did not reveal any toxicity symptoms revealed in body weights (Fig 40). The experimental animal body weights and body weights of control rats were high throughout the duration of oral feeding.

Table 16: Antidiabetic study for various groups of SD rats using *Aloe CIM-Sheetal*.

Group	Treatment	Mean Fasting blood glucose level (mg/dl)			
		Basal value	1st week	2nd week	3rd week
Group I	Normal Control	93.66 ± 3.5^{c}	93.33 ± 4.08^{f}	91.83 ± 3.97^{e}	91.33 ± 4.08^{d}
GroupII	DiabeticControl	299.00 ± 1.78^{a}	296.16 ± 1.6^{a}	295.33 ± 3.72^{a}	297.16 ± 1.47^{a}
GroupIII	ALE 100 mg/kg	298.83 ± 3.31^{a}	290.50 ± 3.27^{b}	280.66 ± 6.77^{b}	266.33 ± 5.68^{b}
GroupIV	ALE 200mg/kg	296.83 ± 2.22^{b}	282.83 ± 5.52^{d}	273.50 ± 5.30^{c}	262.83 ± 4.44^{b}
GroupV	ALE300 mg/kg	299.16 ± 1.94^{a}	276.16 ± 10.18^{e}	227.50 ± 9.52^{d}	190.00 ± 7.61^{c}
GroupVI	Standard + Glibenclamide 0.60 mg/kg	298.66 ± 2.16^{a}	274.50 ± 11.04^{e}	226.00 ± 16.02^{d}	192.00 ± 4.14^{c}
GroupVII	ALE500 mg/kg	299.16 ± 2.92^{a}	288.16 ± 5.49^{c}	293.00 ± 5.72^{a}	298.50 ± 2.16^{a}

Values are rendered as Mean ± SD (n=6) ($p<0.05$).

Statistically significant compared with Group - I (normal control) and diabetic rats treated with Glibenclamide. Statistically significant decrease compared with diabetic

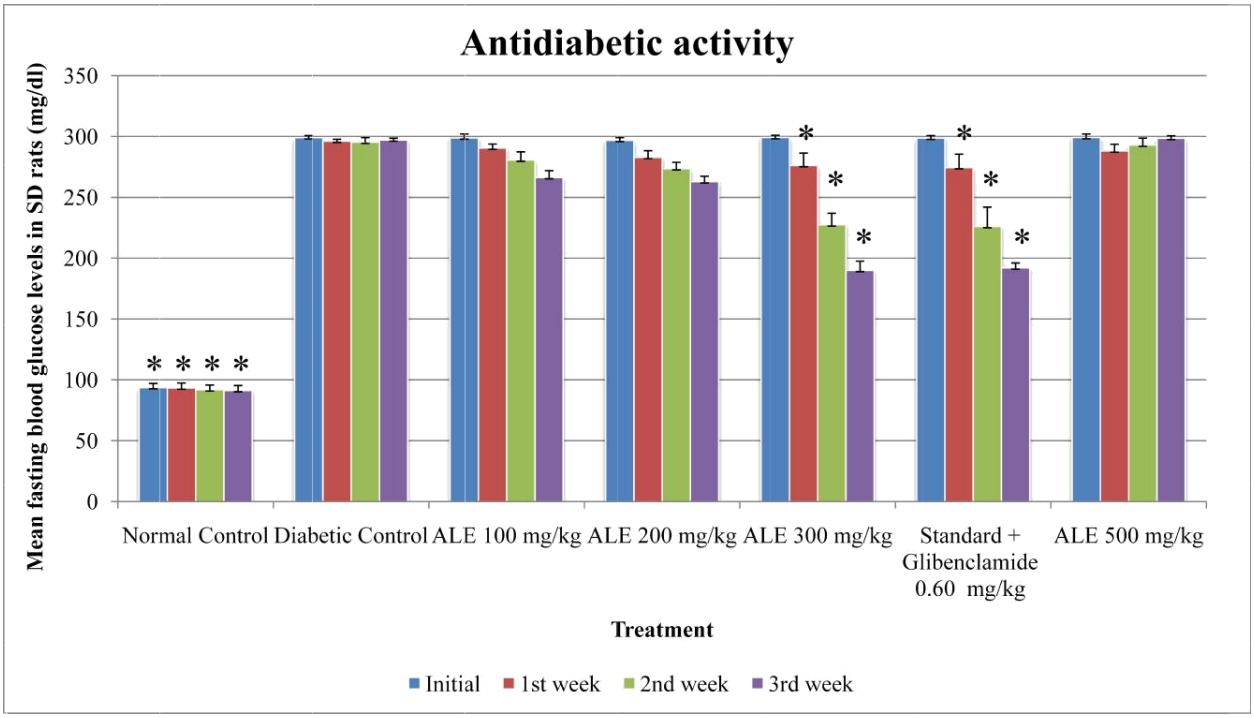
control (Group - II) and diabetic rats fed with *Aloe CIM-Sheetal* (Group V, IV, III and VII): $p<0.001$

Day 0, corresponds to the start of *Aloe CIM-Sheetal* feeding with confirmation of diabetes in diabetic- induced rats.

Table 17: Effect of *Aloe CIM–Sheetal* leaf *e*xtracts on body weights (in grams) in control and experimental group rats.

Group	Treatment	Initial	1st week	2nd week	3rd week
Gr - I	Normal control	171.50 ± 10.33^{d}	177.33 ± 6.77^{a}	187.5 ± 5.46^{a}	196.83 ± 2.48^{a}
Gr - II	Diabetic control (STZ 55 mg /Kg)	170.83 ± 7.22^{d}	161.50 ± 3.45^{c}	155.16 ± 3.06^{e}	152.33 ± 2.42^{e}
Gr - III	ALE 100 mg/kg	171.33 ± 5.75^{d}	174.66 ± 5.20^{ab}	171.16 ± 4.79^{d}	177.33 ± 5.98^{d}
Gr - IV	ALE 200 mg/kg	172.83 ± 9.04^{c}	176.33 ± 9.02^{a}	182.66 ± 4.92^{b}	190.33 ± 3.83^{b}
Gr -V	ALE 300 mg/kg	173.33 ± 5.24^{c}	178.50 ± 3.56^{a}	186.16 ± 2.92^{a}	197.50 ± 2.07^{a}
Gr -VI	Standard+ Glibenclamide 0.60 mg/kg	175.16 ± 2.92^{b}	178.66 ± 3.01^{a}	184.83 ± 2.63^{ab}	192.33 ± 2.65^{ab}
Gr -VII	ALE 500 mg / kg	180.33 ± 15.66^{a}	173.16 ± 8.70^{b}	177.83 ± 7.96^{c}	182.83 ± 7.65^{c}

Values are shown as means $\pm$ SD. Means followed by the same letter in a column are not significantly different ($p \leq 0.05$) by Newman-Kuel's multiple comparisons test.

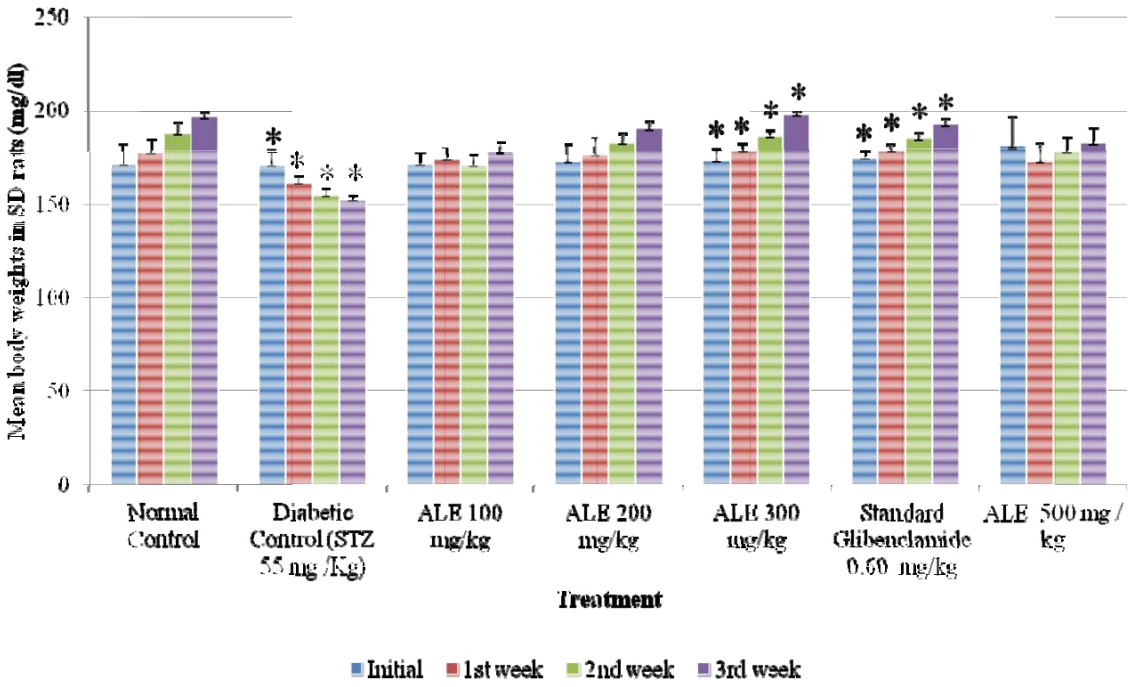


Error bars indicate standard devi ation. Signifi cance of di ference was analysed b one-way ANOVA and N wman-Kuel's multiple comparison test and is indicated by one asterisk ($p \leq 0.05$).

Fig 39: Graph representing the effect of *Aloe CIM-S heetal* extr act on blood glucose in oral glucose toler ance test.

Mean (± SD) of blood glucose after 21 days treatment in treated, diabetic and control r ats.

R epresents significant d ifference between diabetic rats a nd control / treated groups ($p<0.05$), n= .



Error bars indicate standard deviation. Significance of difference was analysed by one-way ANOVA and Newman-Kuel's multiple comparisons test and is indicated by one asterisk ($p \leq 0.05$).

Fig 40: Effect of methanol extracts *Aloe CIM-Sheetal* on the relative on body weights. Values are rendered as mean ± SD.

4 (B). Histopathology

At the end of 21st day, all the rats were euthanized by pentobarbitone sodium (60 mg/kg) and sacrificed by cervical dislocation. The pancreas, liver and kidneys were dissected out, washed immediately in ice-cold saline. These organs were fixed overnight in 10% neutral buffered saline and sent to the histopathology department of NIN. In the department these organs were sliced, processed and histology sections were done on paraffin embedded blocks and 5 micron thick sections were stained with hemotoxylin and eosin stains and were mounted with DPX. The H and E slides were then visualized under light microscope by a pathologist who gave a report.

(1) Liver

In diabetic rats fed with *Aloe CIM-Sheetal* no significant changes were observed in liver. A few insignificant changes in liver have autolysed cells were seen in the Fig 41.

(2) Kidneys

Histological sections from kidneys of diabetes induced rats (Positive control) showed increased PAS (Periodic Schiff) positive mesangial matrix in the glomeruli with dilated tubules. Histological sections from the kidneys of the experimental animals showed lesser changes consisting of mild decrease in PAS positive mesangial matrix which was however less than the positive control thus indicating improvement in histology with the extract. The results were shown in the Fig 42.

(3) Pancreas

Presence of sections from the positive control and animals fed with *Aloe CIM Sheetal* leaf extract 100 mg/kg body weight showed decrease in the number of islets along with presence of increased vacuolation. Sections from the rest of the experimental animals however showed similar positive changes of improvement consisting of increase in the islet cell number with decrease in the vacuolation. The results were shown in Fig 43.

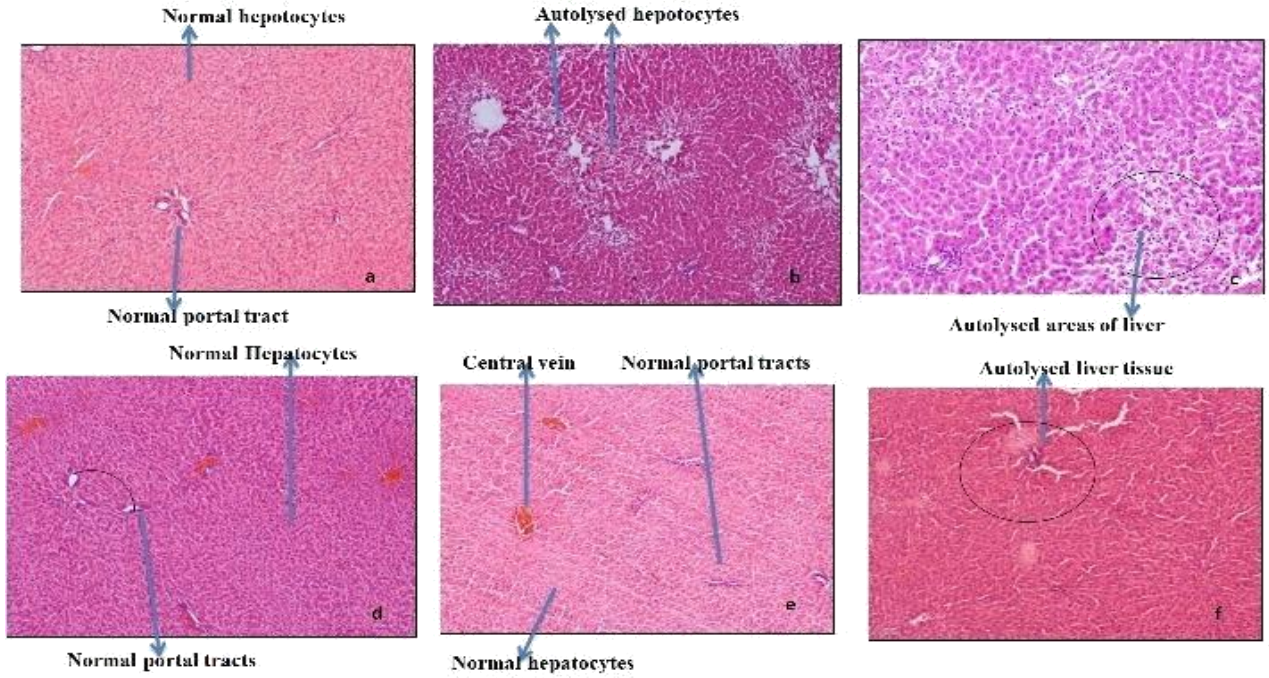


Fig 41: Histopathological results of various groups of SD-Rats of liver : (a) Gr I–Liver Normal Control, H&E Stained section from control shows normal liver histology with normal portal tracts& hepatocytes,H&E,10X; (b) Gr-II Liver (Diabetic Control, STZ induced and untreated) H&E Stained section, shows few autolysed hepatocytes in addition to normal hepatocytes & portal tracts H&E, 10X; (c) Gr III- Liver (Diabetic + Plant extract treated with 100 mg/kg conc.) H&E Stained section shows areas of autolysis, H&E, 10X (d) Gr V- Liver (Diabetic +Plant extract treated with 300 mg/kg) Section shows normal portal tracts & hepatocytes H&E,10X; (e) Gr VI –Liver (Diabetic + standard drug Glibenclamide 0.60 mg/kg) Section Shows normal hepatocytes, Portal tracts & Central vein, H&E,10X (f) Gr VII- Liver (Diabetic + Plant extract treated with 500 mg/kg) Section shows focal autolysed areas H&E,10X.

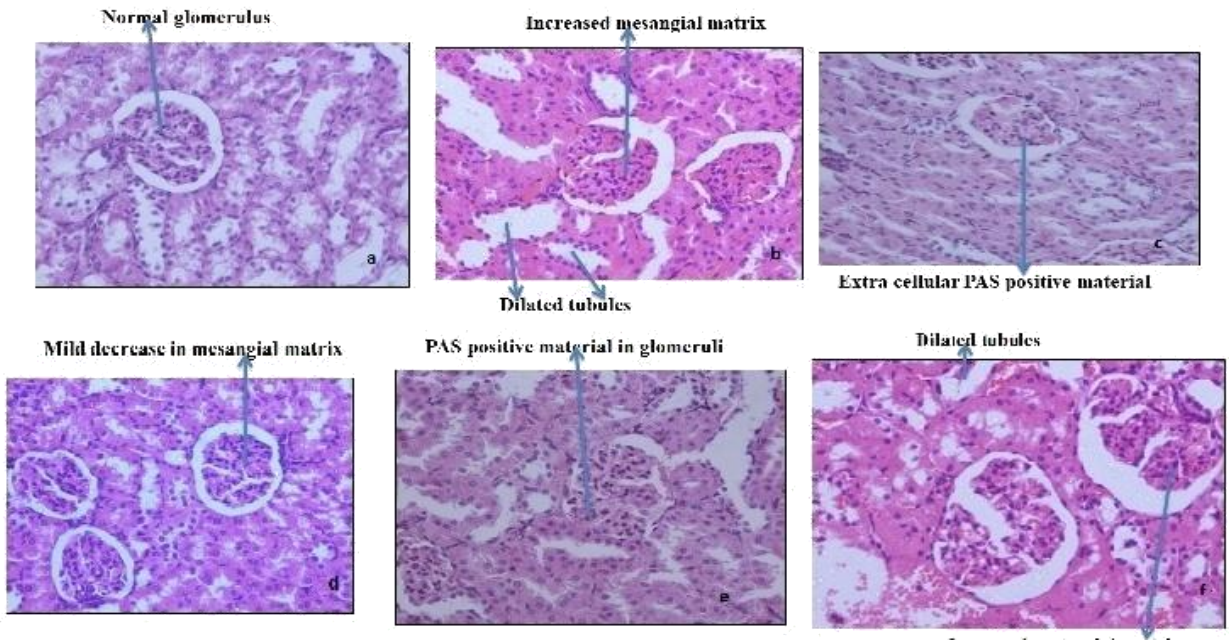


Fig 42: Histopathological results of various groups of SD-Rats of Kidney:

(a) Gr 1- Kidney normal control, PAS stained section shows normal renal glomeruli and normal tubules with no deposits PAS, 40X: (b) Gr II - Kidney (Diabetic Control STZ induced and untreated), PAS stained section shows glomeruli with increase in mesangial matrix which is PAS positive and normal tubules PAS 40X: (c) Gr III - Kidney (Diabetic + *Aloe CIM Sheetal* leaf extract treated 100 mg / kg with conc.) PAS stained section shows mesangial matrix which is mildly increased and is PAS^{+}ve, PAS 40X: (d) Gr V - Kidney (Diabetic + *Aloe CIM- Sheetal* leaf extract treated with 300 mg / kg conc.) PAS stained section shows decreased mesangial matrix in glomeruli with normal surrounding tubules, PAS 40X: (e) Gr VI- Kidney (Diabetic + standard drug treated with Glibenclamide 0.60 mg/kg conc.) PAS stained section shows PAS^{+}ve material indicating mildly decreased mesangial matrix in the glomeruli PAS 40X: (f) Gr VII - kidney (Diabetic + *Aloe CIM- Sheetal* leaf extract treated with 500mg / kg conc.) PAS stained section showed dilated tubules around glomeruli with decreased mesangial matrix PAS 40X.

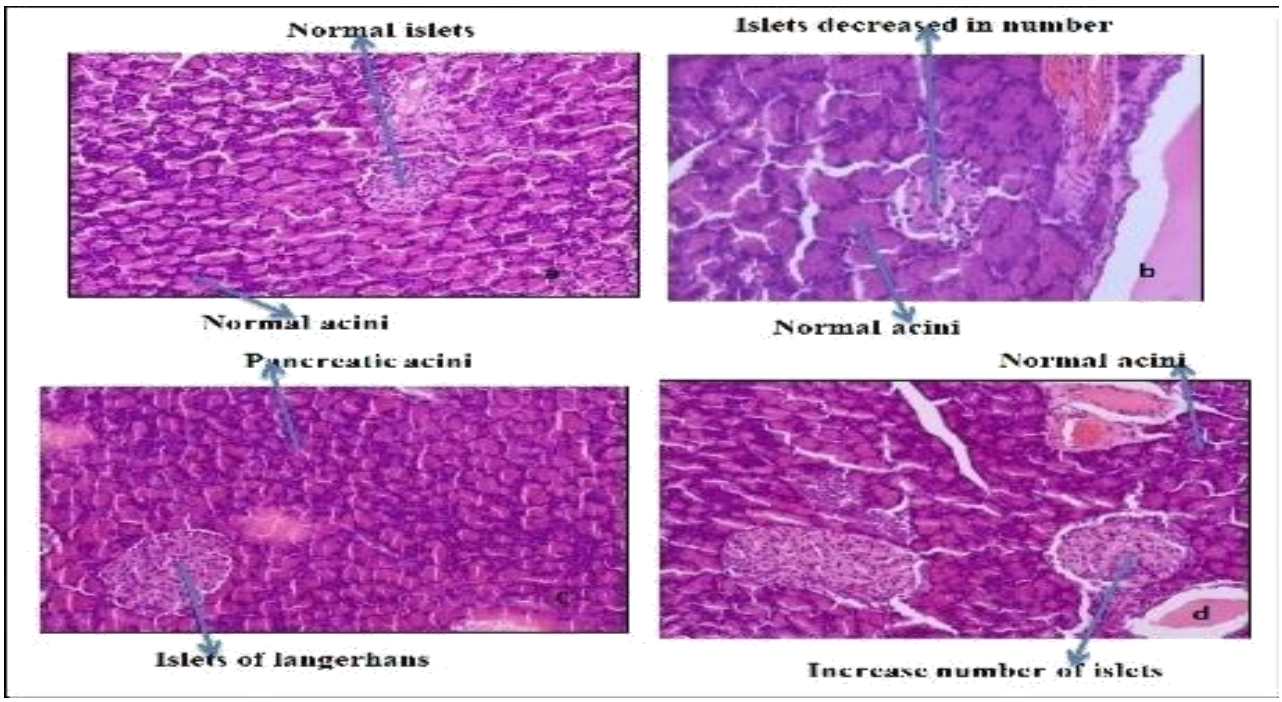


Fig 43: Histopathological results of various groups of SD-Rats of Pancrease**:** (a) Gr1- Pancreas Normal Control, H&E stained section shows normal sized islets of langerhans surrounded by normal pancreatic acini H&E, 20X: (b) Gr II- Pancreas (Diabetic Control STZ induced and untreated), H&E stained section shows decrease in size & number of pancreatic islets with normal surrounding acini, H&E, 40X: (c) Gr IV- Pancreas (Diabetic +Plant extract treated with 200mg/kg conc.) H&E stained section shows islets with moderate increase in number and normal acini H&E, 40X: (d) Gr V- Pancreas (Diabetic +Plant extract treated with 300mg / kg conc.) H&E stained section shows normal islets in number with normal acini H&E, 20 X.

Statistical Analysis: Statistically, the values were analyzed with the analysis of variance (one way ANOVA) to determine the significance of difference within the experimental groups. P-values of 0.05 or less were taken as significant.

5. Genetic diversity

Both the marker systems being employed to assess the genetic diversity in *Aloe vera* accessions were quite informative and were able to generate adequate polymorphism (Fig 44 and Fig 45).

5 (A). RAPD analysis

Out of 64 RAPD primers tested, 61 primers produced amplification products, of which 58 revealed polymorphic fingerprint patterns. The number of bands amplified per primer varied from 4 (OPA-5) to 17 (OPA-3). A total of 351 bands were amplified, of which 252 were observed to be polymorphic resulting in a polymorphism frequency of 71.8% and average of 4.34 polymorphic bands / primer. The molecular size of the bands varied between 100-2600 bp and several accession specific bands have been identified (Fig 44).

The extent of polymorphism per primer ranged from ten alleles per primer OPA-9 (0.98%) to OPA-17 (0.87%) with three alleles per primer. The similarity matrix values using Jaccard's coefficient based on RAPD markers ranged from 0.082 between IC111272 and IC111269 to 0.890 between accessions IC111280 and IC111279. At 17% similarity, the accessions separated out into two major clusters and clustering of accessions based on dendrogram and PCO analysis.

5 (B). ISSR analysis

Of the 25 ISSR primers screened, 24 primers produced amplification products, of which 19 primers revealed polymorphic loci across the *Aloe vera* accessions tested. A total of 105 bands were amplified of which 85 amplicons were observed to be polymorphic resulting in polymorphism of 80.9%. The average number of polymorphic amplicons per primer was 4.47%. The number of bands amplified per primer varied between 2 (UBC primer # 860) and 14 (UBC primer # 867). The size of the amplicons varied between 100 bp to 2800 bp and several accession specific bands have been identified as in the Fig 45.

The extent of polymorphism varied with ten alleles between UBC 867 (0.98%) to UBC 861 (0.82%) with two alleles per primer. Similarity matrix values based on ISSR markers ranged from 0.171 between IC111280 and IC111267 to 0.745 between accessions IC471883 and IC471882. Dendrogram analysis separated the accessions into two clusters at 29% similarity. Clustering of accessions was based on dendrogram and PCO analysis was similar for most accessions except for the accessions IC111269, which is separately sub-grouped in cluster II.

5 (C).Combined RAPD and ISSR analysis

The genetic similarity matrix data generated using RAPD and ISSR systems were compared. Mantel test for congruence of RAPD and ISSR data matrices indicated a goodness of fit (r = 0.87158) indicating good correlation between the two molecular marker systems. Although the two marker systems sampled different segments of the genome, the clustering pattern of the genotypes was almost similar with both the marker

systems and most of the accessions were placed in their respective clusters with minor changes.

The genetic relatedness of the accessions was determined using Jaccard's similarity coefficients. Cluster analysis (UPGMA) performed from combining polymorphic data of both markers (RAPD and ISSR) generated a dendrogram that separated the accessions into two major clusters at 48% of variation (Fig 46).

The first cluster (I) comprised IC111267, IC111271 and IC111272. The second cluster (II) further divided into 2 sub-clusters. Among them the first sub-cluster (IIa) comprised of IC111269, IC111279, IC111280, IC 471885, wild *Aloe vera* and *Aloe CIM-Sheetal* (CAL14) and the second sub-cluster (IIb) comprised of IC471282, IC471883 and IC471884. The accession IC111280 and IC111279 appeared to be closer to each other with a similarity coefficient of 0.847 (Table 18), while the divergent accessions were IC111279 and IC111272 (similarity coefficient 0.143). Principal coordinate analysis (PCO) based on genetic similarity showed the relationship among accessions in two dimensional spaces. The PCO analysis based on pooled data of RAPD and ISSR primers grouped the accessions into 3 groups as given in (Fig 47**)**, which is similar to the UPGMA clustering pattern.

T able 18: Jaccar d's similarity coefficient values of 12 *Aloe vera* accessions based on pooled data of RAPD and ISSR primers.

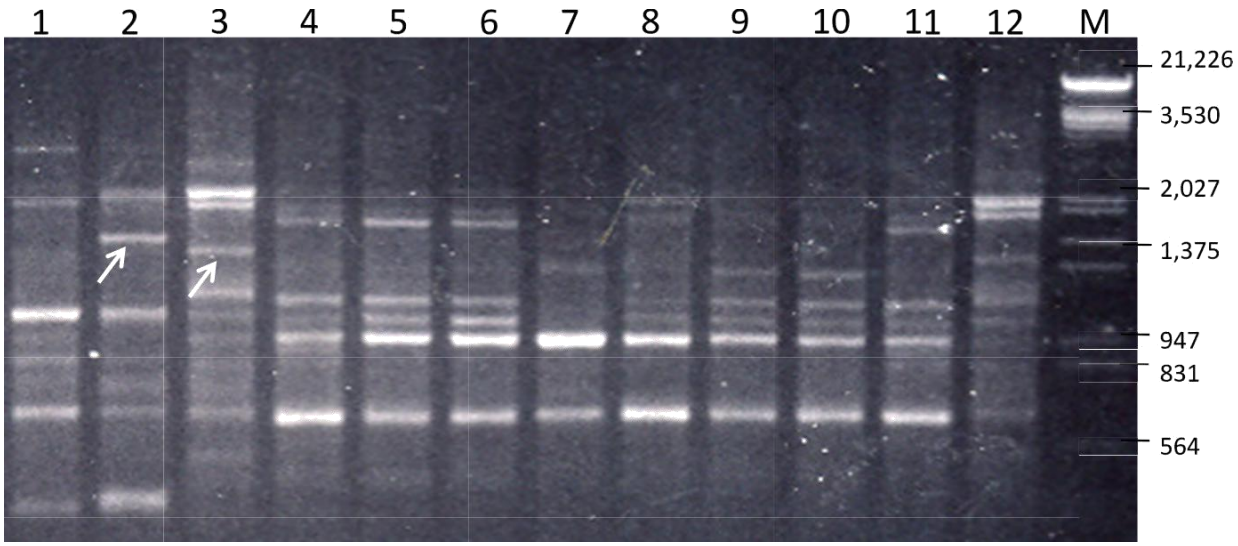


Fig 44: RA PD profile f 12 *Aloe vera* accessi on amplifie d with OPA 9 Primer. Lanes 1-2 the sam ples used in the study as listed i Table 18 and lane M represents λ DNA ouble dig est with *Eco*RI and *Hind*III restriction e nzymes. A rrows indi cates the ccession sp ecific bands.

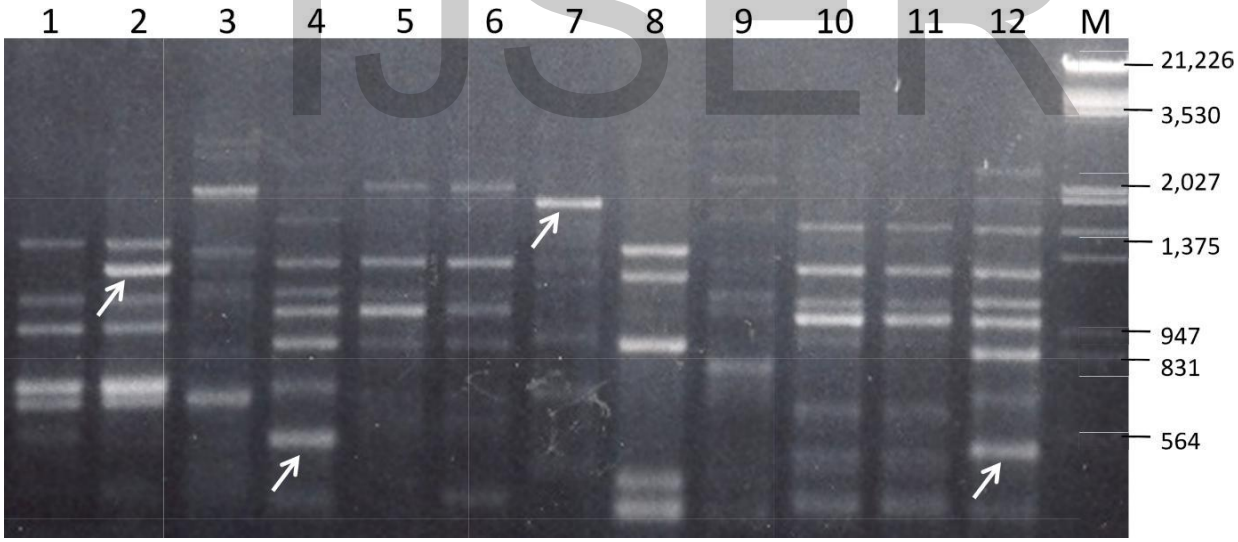


Fig 45: ISS R profile of 12 *Aloe vera* accession amplified with UBC-8 61 primer. Lanes 1-2 the sam ples used in the study as listed i Table 18 and lane M represents λ DNA ouble dig est with *Eco*RI and *Hind*III restriction e nzymes. A rrows indi cates the ccession sp ecific bands.

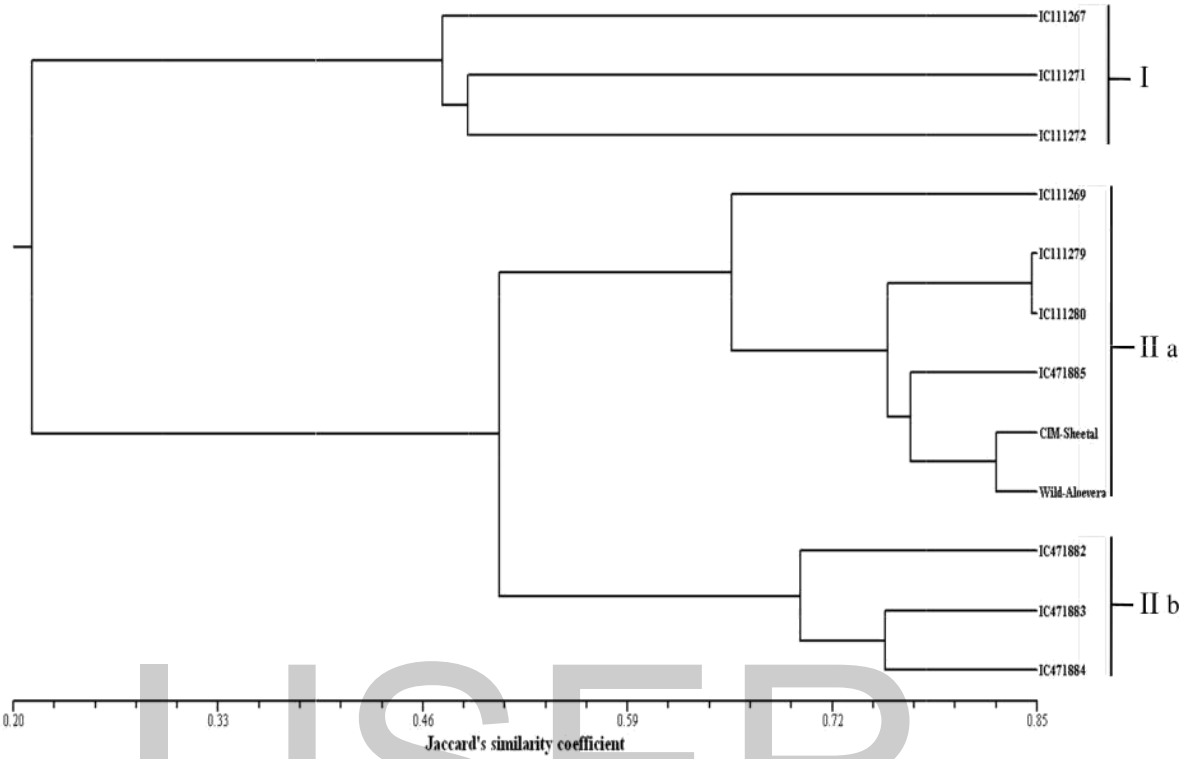


Fig 46: Dendogram (UPGMA) representing genetic relations ips amon g 12 accessions o *Aloe vera* based on J accard's si milarity coefficients obtained using the pooled alleli c profile of RAPD + ISSR primers .

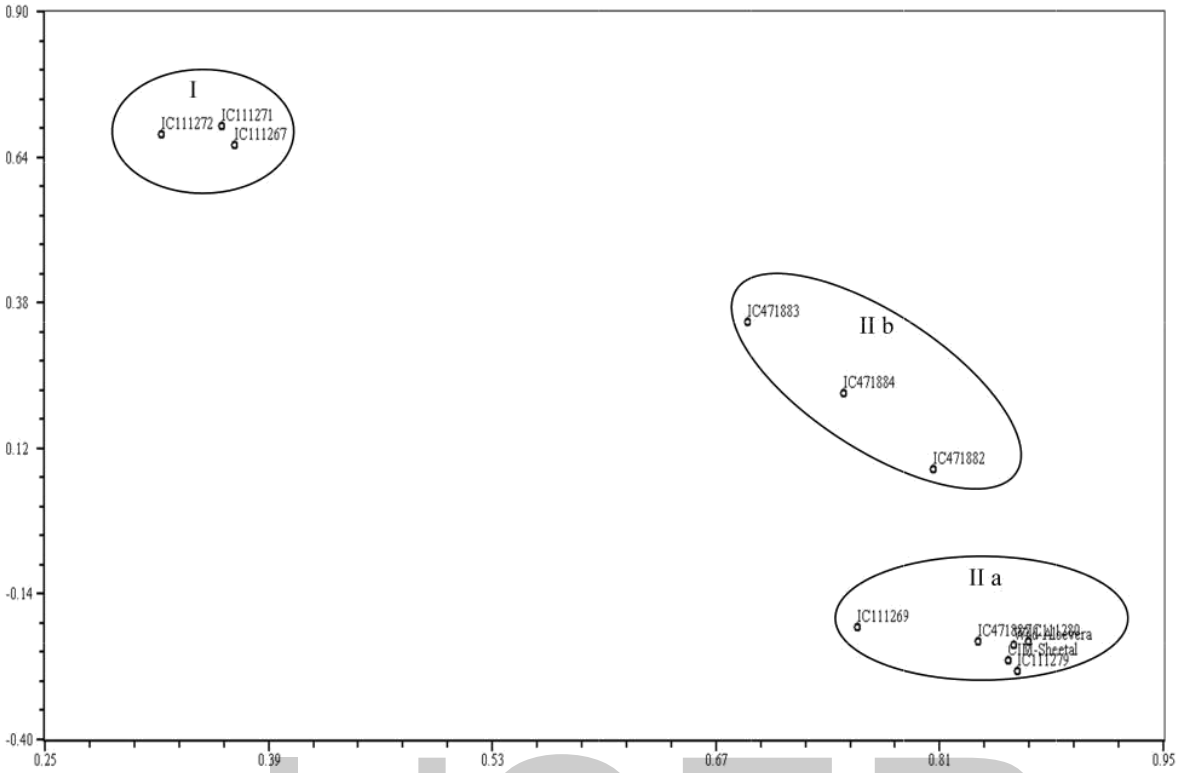


Fig 47: Two-dimen sional scaling of *Aloe vera* accessions by p rincipal component analysis using poole d data of R APD and ISSR prime rs.

CHAPTER - 5

DISCUSSION

5. DISCUSSION

Plant products have been a source of curative and therapeutic agents since time immemorial. The application of plants as medicine dates back to prehistoric period. The early civilizations reveal that a considerable number of drugs that are used in the modern medicine have figured in the ancient manuscripts such as The Rigved, The Bible, The Quran, The Iliad, The Odyssy, History of Herodotus etc. Over 6000 years ago, the ancient Chinese were probably the first to use the natural vegetation as a medicine. About 20,000 species of plants in the world are being used as medicinal plants and their products constitute about 25% of the prescribed medicines in world. The most commonly used drugs of modern medicines such as aspirin, quinine, diosgenin, cortisone, etc. have been originated from the medicinal plant sources (Jafri et al., 2000).

India has been a traditionally rich in various types of medicinal plants. Since ancient times, people have been using herbal medicines to cure diseases. The 'Rig Veda', perhaps the oldest repository of human knowledge written between 4500 and 1600 B.C., contains the earliest mention of the medicinal use of plants (67 plants). Records are available in the ancient manuscripts. Demand for medicinal plants is increasing in both developed and developing countries due to growing recognition of natural products being equally effective, safe, non-narcotic and affordable having no side effects. The acceptance of herbal medicines at global level is increasing day by day, which reflects the generalized adoption of medicinal plant products in the world.

Aloe vera is a unique plant with a rich source of many chemical compounds known as 'pharmacy in a pot'*Aloe vera* has a long history as herbal remedy and is most

popular herbal plant and it has been used worldwide due to its medicinal properties. Because of its demand, it is cultivated in large quantities in many parts of the world (Newall et al., 1996). *Aloe vera* is a xerophytic perennial plant with a short, cylindrical, simple stem of 30-60 cm. The leaves are 40-60 cm long, erect, broad, thick and fleshy which are crowded in a basal rosette. The leaves are succulent, glaucous-green in colour, narrow lanceolate in shape with long acuminate tip with small thorns on both edges. Flowers are yellow to red in colour and are borne in dense racemes terminating in the cylindrical spike.

The demand of Indian medicinal plants has increased over the years in the international market. The area under *Aloe vera* cultivation worldwide is about 23,000 hectare (IASC). American countries possess 19189 ha of Aloe plantation while Australia and Africa cultivate 4170 ha and 300 ha each India possess only negligible area under this crop (Yogeeswaran et al., 2005). The cultivation of *Aloe vera* has acquired great commercial importance for medicinal products and cosmetic preparations but information is scarce about agronomic management of this crop (Hernandez Cruz et al., 2002).

1. Morphological studies

A. Morphological evaluation of germplasm

The evaluation and characterization of germplasm is prerequisite for any breeding programme aimed at improvement of yield. The qualitative as well as quantitative evaluation of germplasm is not only conducive in core collection but also for its utilization in cultivation and breeding. The assessment of variability existing in the germplasm of accessions is of great interest for conservation of genetic resources and

also for broadening of genetic base of species to be exploited by plant breeders. It helps in systematic cultivation of the plant for commercial purposes.

Presently out of 12 accessions of *Aloe vera* (*Aloe barbadensis* Mill.) ten accessions were obtained from National Bureau of Plant Genetic Resources (NBPGR), New Delhi i.e IC 111267, IC111269, IC111271, IC111272, IC111279, IC111280, IC471882, IC471883, IC471884 and IC471885 (each 10-12 samples) and other 2 accessions i.e *Aloe CIM-Sheetal* and wild *Aloe vera* (local) accessions were collected from Central Institute of Medicinal and Aromatic Plants (CIMAP), Boduppal, Hyderabad, India were collected respectively for the evaluation of morphological characters. All accessions were maintained and multiplied at Indian Immunologicals Ltd, Gachibowli, Hyderabad. Investigations were carried out to characterize and evaluate the collected germplasm for the following morphological characters. Number of leaves, number of suckers, leaf length, leaf width/breadth, stem length, peduncle length, leaf thickness, gel fresh weight, leaf dry weight, biomass, root length and aloin concentration were the basic characters selected for evaluation.

The leaf characters of the *Aloe vera* are an important factor which can be attributed in determining the yield of the crop (Saeid Hazrati et al., 2012). There is a lack of expression of reproductive characters in some of the species due to which it is impossible to distinguish morphological variations in some economically important *Aloe species*. It is essential to characterize the medicinally and economically important genus to regularize the commercial and economically important characters. Based on the morphology, the present studies has revealed that the accessions of *Aloe vera* germplasm can be divided into two morphotypes viz. Small *Aloe vera* (SAV): plant size up to 0- 40

cm and large *Aloe vera* (LAV): plants with leaves more than 40 cm in size. The LAV type of accessions show highest incidence.

Type Accessions

1). Small *Aloe vera* (up to 40cm)

IC 111269, IC 111271, IC111279, IC111280, IC 471884, IC471885 and *Aloe vera* (wild / local).

2). Large *Aloe vera* (Above 40 cm)

IC111272, IC471883, IC471882, IC111267 and *Aloe CIM- Sheetal.*

Among the collected *Aloe vera* accessions, the fresh gel content varies between 4.10 ml/100gm to 58.52 ml/100gm with overall mean value of 20.025.

The medicinal and cosmetic value of the Aloe depends upon the quantity of its biochemical constituents. Presently, an attempt has been made to evaluate the collected *Aloe vera* germplasm for its quality and yield components. The quality of the germplasm depends upon the relative composition of leaves/leaflet. The plants producing higher amount of fresh gel, dry gel, biomass, presence of chemical compounds and producing lower amount of aloin are consider being of good quality. Data on the presently studied accessions of Aloe germplasm are given in Table 4. It shows that IC 111271, IC 111269, IC111267 and *Aloe CIM- Sheetal s*how better performance i*n vitro* and *in vivo* than the local *Aloe vera* (wild) obtained from Hyderabad region. Among the present studies of Aloe accessions, the leaf yield per plant ranged between 0.652 kg to 23.2486 kg/plant and dry leaf weight 12.20g/kg to 23.70 gm/ kg. The accessions with high yield are IC 111271, IC 111269, IC111267 and *Aloe CIM- Sheetal* .The amount of gel (dry) obtained from one

plant in one year is estimated to 2.10 gm/plant to 12.80 gm/plant. High yield of gel can be obtained from accessions IC 111271, IC 111269 and *Aloe CIM-Sheetal*.

Morphochemical evaluations can provide insights into the genetic structure and diversity within and among varieties from different geographical origins, producers and distributors. Without out this information there is no means of selecting appropriate plant material for the participation in screening programs with a view to the introduction of the novel varieties for the industrial purpose (Radhamadhavi et al., 2012). In India, 2 or 3 easily recognizable varieties are found, but their exact delimitations are not clear. In *Aloe vera*, variety chinensis Baker is common all over the Deccan, the leaves have a distinct purple colour towards the base and the spines are not sharp. The leaves of *Aloe vera* var. littoralis Koenig ex Baker available on the beach shingles in Madras right up to Rameswaram are smaller in size and have a rough margin. Another species *Aloe variegata* Linn, a close to kin of *Aloe vera*, is found in parts of Maharashtra. It has giant, fleshy, green leaves with sharp spines and white specks at the base of the leaves. A variety which thrives on the Kathiawar coast is called *Aloe abyssinica* is the source of the Jaffarabad aloes (Farooqi and Sreeramu, 2004).

In the International Cosmetic Ingredient Dictionary and Handbook, different kinds of aloe species are marketed for different Aloe products (Gottschalk and McEwen, 2004). The important species contributing to the great market of Aloe products throughout the world are *Aloe andongensis, Aloe arborescens* (Kidachi Aloe), *Aloe vera* (*Aloe barbadensis* Miller), *Aloe ferox* (Cape Aloe). Out of these species, *Aloe vera* with fleshy leaves near about 60 cm long, 10 cm broad and 1.5 to 2 cm thick, are densely crowded, strongly cuticularized scores high in quantity and quality over the others.

2. *In vitro* Studies

The ability of plant cell, tissue and organs to differentiate into plants in culture has resulted in widespread applications in propagation and plant breeding. The technique of tissue and organ culture is used for rapid multiplication of plants for genetic improvement of crops and for preserving valuable germplasm (Bhojwani and Razdan, 1992). Conventional method of propagation in *Aloe vera* is through vegetative means (side buds) which is slow. There is no viable seed setting in the plants. Aloe is exclusively a vegetatively propagated crop where young side branches are used as planting material. Single plant may produce 2-3 side shoots per year making availability of planting material in good quantity and quality, a problem.

Keeping the above things in mind tissue culture studies were undertaken in the *Aloe vera* plants. The present study deals with direct shoot multiplication from shoot tips (micro propagation) of twelve accessions.

Tissue culture research has begun nearly four decades ago. A number of protocols have been developed for micropropagation of Aloe plants using a variety of explants like shoot tip, axillary bud, stem cuttings etc. by various researchers (Richwine et al. 1995; Abrie and Staden, 2001; Aggarwal and Barna, 2004; Campestrini et al., 2006; Hosseini and Parsa, 2007; Hashemebadi and Kaviani, 2008).

The method used for clonal propagation is also an important tool for raising pathogen free plants in culture (Walkey, 1980). Tissue cultures of *Aloe barbadensis* were established by Sanchez et al., (1988) using vegetative meristems and leaf explants. Shoot tips and auxillary buds are popular explants for micropropagation of Aloe (Natali et al.,

1990; Liao et al., 2004; Aggarwal and Barna, 2004). In the present investigation shoot tip was used as an explant.

2.1 Shoot proliferation

Establishment of aseptic cultures was difficult, but once a healthy culture was established, there was no further contamination. Under given conditions and over a culture of 60 days explants from all the treatments produced multiple explants and roots simultaneously. In the earlier studies *Aloe vera* has been sterilized using the mercuric chloride which is harmful for environment. Our method is easy and new for explants sterilization for *in vitro* culture of *Aloe vera*.

In the present study, we have optimized the surface sterilization procedure to avoid any type of endogenous and exogenous contaminants and also standardized media composition for the mass multiplication and fast growth of *Aloe vera*. Shoot tip explants were cultured on MS supplemented medium with various concentrations and combinations. Most researchers proposed the use of shoot tip and apical meristem for micropropagation of aloe (Debiasi et al., 2007; Liao et al., 2004; Aggarwal and Barna, 2004; Campestrini et al., 2006).

Cytokinin level produced a significant response upon the number of explants formed per plant and also showed influence on production of leaf numbers and rooting (Dwivedi et al., 2014). The shoot tip of explants initially produces two - three shoots within two weeks after inoculation. But in our method 15-35 shoots/culture were produced from single explant by subsequent 2-3 subcultures with same medium which indicate the high efficiency of this protocol (Fig 14). The average length of shoots per

culture was 4.89 ± 0.03 cm. Formation of the roots was best observed in *Aloe CIM-Sheetal* and IC111271 in the media containing MS basal media with 3 types of hormones NAA 0.2mg/l, IAA 0.1mg/l and IBA 0.2 mg/l within four weeks after inoculation for rooting. Proliferating shoots obtained from shoot tip explants of *Aloe* took maximum 6-7 weeks from the time of establishment to attain the size (2-3 cm) suitable for rooting (Fig 15). The highest percentage of shoots that induced roots (91.12%) was observed in MS medium supplemented with NAA (0.2 mg/l), IAA (0.1 mg/l) followed by IBA (0.2 mg/l) (Table 7).

Based on our studies, accession *Aloe CIM-Sheetal* (11), IC 111271 (3), IC 111279 (5), IC 111269 (2) are high potential accessions, IC 471882 (7), IC111267 (1), IC471885 (10) have shown moderate proliferation and IC 111280 (6), IC 111280 (8), IC471884 (9), wild *Aloe vera* (12) and *Aloe ferox* were shown poor shoot proliferation and rooting among all accessions for their multiplication ratio of axillary buds, multiple shoots/ clumps and roots were regenerated after sub culturing.

Cytkinins are one of the most important growth regulators for affecting the shoot proliferation (Lane, 1979; Garland and Stolz, 1981). A range of cytokinins BA, kinetin and 2-ip have been used in micropropagation (Bhojwani and Razdan, 1992). BA variations affecting shoot proliferation were also reported by Bhandari et al., (2010); Gantait et al., (2010); Mangal Singh, (2009). Abrie and Staden, (2001); Chaudhuri and Mukundan, (2001) had also reported the use of BA in shoot proliferation of *A. polyphylla* and *Aloe vera* respectively. Some researchers have shown that presence of both of auxin

and cytokinin is necessary for shoot proliferation (Rout et al., 2001; Velcheva et al., 2005).

In our method of explants preparation, sizing and a single mother plant can be multiplied 30- 35 explants per mother plant and 8.84 ± 0.03 shoots per explants were obtained on MS medium supplemented with BAP (1.5 mg/l), KIN (0.5 mg/l), NAA (0.2 mg/l), IAA (0.1mg/l) and IBA (0.2mg/l) in 7 weeks, in comparison to 30 shoots from 18 explants obtained from 18 mother plants in 8 weeks and 20 shoots per plant in 8 weeks was reported (Balraj Singh and Neelu Sood, 2009; Baksha et al ., 2005) also reported that the enhancement of shoots was observed by using BA and NAA. Cluster of shoots were separated into pieces and each was sub-cultured individually on the same medium periodically. Cytokinin level produced a significant response upon the number of explants formed per plant and also showed influence on production of leaf number and rooting (Dwivedi, 2014). After third subculture the shoot multiplication rate remained constant. On the other hand, regeneration of shoot buds was moderate (65-77 %) on a medium containing: 1.5 mg /l BAP, 0.5 mg/l kinetin, 0.2 mg/l of IBA, 0.1 mg/l of IAA, 0.2 mg/l of NAA and 1.5 mg /l BAP, 0.5 mg/l kinetin, 0.2 mg/l of IBA, 0.1 mg/l of IAA, 0.2 mg/l of NAA and 1.0 mg/l of GA_3 respectively. Comparatively a lowest number of (18-28%) adventitious shoots were observed in the medium containing 0.3 mg/l of IAA, 3.0 mg/l of BAP (IM) and 0.1 mg/l NAA, 0.1 mg/l of IBA, 0.1 mg/l of IAA and 4.5 mg/l BAP. The highest concentration 4.5 mg/l of BAP did not increase shoot proliferation. It was also reported that highest shoot proliferation in *A. vera* was found in MS medium containing BA and IBA (Aggarwal and Barna 2004; Mukesh Kumar et al., 2011), where better proliferation occurred on medium containing Kinetin instead of BA (Dwivedi,

2014). NAA and IBA are most commonly used for root induction (Bhojwani and Razdan 1992). Effect of 0.1 mg/l of NAA, 0.5 mg /l of IAA and 0.5 mg /l of IBA of MS medium in rooting was shown poor response. Similar result was also obtained by (Liao et al., 2004).

For shoot proliferation, growth regulators especially cytokinins (Bhojwani, 1980) are one of the most important factors affecting the response. A range of cytokinin (Kinetin, BA, 2-ip and Zeatin) has been used in micro propagation of work. Studies conducted by different workers clearly indicated that BA was more effective, reliable and useful cytokinin for shoot proliferation in *Aloe vera* (Velcheva et al., 2005; Debiasi et al., 2007). IBA (Chaudhuri and Mukundan, 2001) and acetic acid (Mukherjee and Roy Chowdhury, 2008) also reported to be helpful in shoot proliferation in Aloe. Meyer and Staden, (1991) reported auxillary shoot formation using IBA whereas Roy and Sarkar, (1991) obtained shoot on medium containing 2, 4-D with Kinetin. Richwine et al., (1995) reported induction of shoots using Zeatin. Liao et al., (2004) reported that the best medium for micro propagation of *Aloe vera* was MS + 2 mg/l BA + 0.3 mg/l NAA. Budhiani, (2001) demonstrated that the best initiation and multiplication of shoot on MS medium supplemented with 0.2 mg/l BAP + 0.002 mg/l NAA and 2 mg/l BAP +0.002 mg/l NAA, respectively. Hashemabadi and Kaviani, (2008) reported that MS with 0.5 mg/l BA and 0.5 mg/l NAA produced highest number of shoots. According to Liu Fen 2001 best medium for shoot proliferation is MS with BA (1.0 mg/l) and IBA (0.3 mg/l). Best medium for bud initiation according to Liao et al., (2004) is MS with 2.0 mg/l BA + 0.3 mg/l NAA with 30 g/l sucrose + 0.6 g/l PVP (pH- 5.8). Hirimburegama and Gamage, (1995) cultured the plant on MS medium supplemented with 0.18 mg/l IAA and 2.25

mg/l BA. Zhou et al., (1999) suggested MS + 6 BAP (3 mg/l) as the best medium for the induction of buds.

In the present study also, shoot proliferation occurred in the presence of cytokinin. Among the different phytohormonal combinations BAP proved to be more effective. MS medium supplemented with 1.5 mg/l BAP + 0.2 mg/l IBA+0.5 mg/l KIN +0.1mg/l IAA+ 0.2 mg/l NAA+0.5 mg /l GA_3, gave (77-88 %) best shoot proliferation/multiplication. This is in contrast to earlier work by Meyer and Staden, (1991) who reported better proliferation in *Aloe vera* on medium containing kinetin instead of BA. This difference may be due to difference in the genotype of plant used (Abrie and Staden, 2001).

2.2 Rooting

Highest root response in *Aloe vera* was reported in hormone free medium (Bhandari et al., 2010; Aggarwal and Barna, 2004). In the present study, healthy rooting was observed in NAA (0.2 mg/l) and IBA (0.2 mg /l) medium. Healthy roots (number > 7 and length > 3 cm) were obtained in 8 weeks of time. Hardening is important step in tissue culture. Rooting response of micro shoots is reported to be controlled by growth regulators in the medium (Bhojwani and Razdan 1992; Abrie and Staden, 2001), basal salt composition (Garland and Stoltz, 1981), genotype (Rines and McCoy, 1981) as well as cultural conditions. NAA and IBA are most commonly used auxins for root induction (Bhojwani and Razdani, 1992). By use of IBA many plants such as *Lycoperscicon esculentum* (Sibi, 1982); *Hedychium roxburgii* (Tripathi and Bitaillion, 1995) and Carnation (Werker and Leshem, 1987) gave *in vitro* rooting. Rooting was achieved on MS medium + 0.18 mg/l NAA + 0.226 mg/l BA (Hirimburegama and Gamage, 1995).

Zhou et al., (1999) used supplements NAA (0.3 mg/l) and IBA (0.3 mg/l) for rooting and found that NAA was better than IBA in the average number of roots produced and rooting rate. Best rooting was observed by Liao et al., (2004) by using ½ MS + 0.2 mg NAA.

In the present study, shoot tips were subjected to MS medium supplemented with different concentration of NAA. Maximum rooting was observed in MS + 0.2 mg/l NAA+0.2 mg/l IBA+ 0.1mg/l IAA. This study supports the observation by Zhou et al., (1999). However, Natali et al., 1990; Meyer and Staden 1991; Richwine et al., 1995; Aggarwal and Barna, 2004 reported rooting in hormone free medium. In the present study no rooting was obtained in hormone free medium even on prolonged waiting.

2.3 Regeneration from callus

Regeneration of plants from callus may help to induce variability in the Aloe germplasm for future improvement. Over the last years a number of micropropagation protocols have been developed using a variety of explants like shoot tips (Hashemabadi and Kaviani, 2008), auxillary buds (Hirimburegama and Gamage, 1995), stem cutting and leaf explants (Sanchez et al., 1988). Plant regeneration via callus formation in *Aloe barbadensis* occurs at low frequency (Sanchez et al., 1988). Successful establishment of calli and subsequent plantlet regeneration is reported in *Aloe pretoriensis* (Groenewald et al., 1975), *Aloe ferox* (Racchi, 1987), *Aloe saponaria* (Yagi et al., 1983) and *Aloe vera* (Gui et al., 1990; Roy and Sarkar, 1991). Racchi, (1987) used MS medium supplemented with 0.5 mg/l 2,4-D and 1 mg/l kinetin for root explants and 0.2 mg/l 2,4-D and 1 mg/l kinetin for leaf meristems. In A. saponaria best results were obtained using root tissue

with a combination of 1 ppm indoleacetic acid (IAA) and 0.5 ppm 2,4 - D and 2 ppm kinetin (Yagi et al., 1983). However, the occurrence of plant regeneration from these calli was not reported. Gui et al., (1990) used stem segments of *Aloe vera* on MS medium with different hormones and successfully regenerated a large number of plantlets via callus. The best results were obtained on the medium with zeatin 2 ppm + 0.5 ppm NAA. Roy and Sarkar, (1991) reported that MS basal medium supplemented with 1 mg/l 2,4- D and 0.2 mg/l kinetin gave the best callus induction.

In the present study, shoot tips were used as explants and cultured on different combinations of auxins and cytokinins. Among all the combinations no response was observed in MS basal medium with 2, 4 - D (0.5 mg/l) and kinetin (0.2 mg/l).

2.4 Hardening

Plantlets with actively growing roots were transferred to pots containing three different types of soil mixture. The highest 96% of survivability was recorded in mixture of garden soil, compost, and sand in proportion 2:1:1 where rapid shoot length was also observed (Fig 17). It was also revealed that regenerated plants were morphologically similar to the mother (control) plant and that the method of micropropagation used in this investigation (axillary bud method) does not usually produce some clones. The result of acclimatization showed that 96% of plantlets survived to grow under greenhouse conditions and were morphologically similar to mother plants. The leaves also started thicken in shade house.

The tissue culture plants were hardened and these plants are could face ambient environmental conditions (Bhojwani and Razdan, 1992). Rooted plantlets were

transferred from culture bottles to plastic pots containing 1:1 ratio of soil: rice husk. Natali et al., (1990) suggested mixture of soil, sand and perlite. While Aggarwal and Barna (2004) suggested mixture of soil and farmyard manure, cocopeat and perlite (Hashemabadi and Kaviani, 2008). There is decrease in the glycoprotein, verectin (Yagi et al., 2000) and barbaloin content in the clonally regenerated plants of *Aloe vera*. According to Yagi et al., (2006) the clonally induced mutations are associated with the phenotypic variation observed in *Aloe vera*. The present results show the occurrence of variation in morphological and biochemical characteristics when we compare the mother plants and their tissue culture generated plants. The extent of variation, however, differs from accession to accession. This could be related to differences in the genotypes of various accessions.

The aim of tissue culture is to rapidly produce a large number of true type plants. Somaclonal variations are therefore, undesirable. However, in the present case, the somaclonal variants can be a valuable source of genetic variation in the germplasm. The somaclonal variants thus obtained need detailed genetic characterization before being put to use. Genetic variation is an essential component of any conventional breeding programme. Aloe germplasm that lacks natural genetic recombination mechanism due to absence of sexual reproduction the tissue culture technology has great potential for induction of genetic variability.

3. Biochemical analysis

The chemical composition of the *Aloe vera* plants has been investigated by many workers. Vogler and Ernst, (1999) reviewed the phytochemical investigations and listed

75 potentially active constituents including vitamins, enzymes, minerals, sugars, lignin, saponins, salicylic acid and amino acids. This list was modified from a large list of ingredients reported by Antherton 1998; Shelton 1991; Dagne et al., 2000; Chauhan et al., 2007 have further enlarged this list. The *Aloe vera* plant contains about 99 to 99.5% water with pH in the range of 4.4 to 4.7. The remaining solid material contains over 45 different ingredients including vitamins, minerals, enzymes, sugars, anthraquinones, lignins, saponins, sterols, amino acids and salicylic acid. According to Lawrence, (1997) Aloe plant gel or juice contains low amount of components such as protein, carbohydrate, lipid, minerals such as calcium, magnesium, sodium, potassium and some of the vitamins.

Aloe gel usually contains 0.5 - 0.7% of total solids. Inter accession phytochemical variations within and between populations of a species are not uncommon. Diversity in morphological and biochemical parameters has been reported among populations, land races, accessions, etc. in a number of crops (Wilson et al., 1990; Zeinali et al., 2004). Such variations have been shown to have genetic as well as environmental basis. Variation in medicinal plants is often noticed at chemical level which is due to the synthesis and accumulation of various bio-chemicals. This diversity can be continuous or discrete and perhaps coded by many or few genes together with some inputs from the environment (Mallet, 1996). The phytochemical variation has been detected among various accessions of *Aloe vera* maintained at Indian Immunologicals Ltd, Hyderabad. Since these accessions are growing in the same environmental and edaphic conditions the presently observed variability may be related to their genetic makeup. Femenia et al., (1999) reported variation between cultivars of Aloe in terms of quantity of

polysaccharides in parenchyma cells. Singh et al., 1995 evaluated 44 genetic stock of *Aloe vera* collected from Rajasthan and other areas and observed considerable variation in growth, yield and quality parameters.

Carbohydrates: Sugars are derived from mucilage layer of the plant under the rind, surrounding the inner gel. They constitute 25% of the solid fraction and comprise both mono and polysaccharides. The most important are the long chain polysaccharides known as glucomannans b (1, 4)-linked acetylated mannan (Chauhan et al., 2007). Quantitative variation has been found in polysaccharides estimated in different aloe types.

Quantitative analysis of the *Aloe vera* extract done by Cock and Ruebhart, 2008 for the carbohydrate content using a modified anthrone assay revealed that the extract has 201 mg total carbohydrate at a concentration of 167.5 mg/ml and the polyphenol content of the *Aloe vera* extract was 46.8 mg. In WHO monograph the aloe gel containing 0.3% carbohydrates are considered best medicinal plants (WHO, 1999). A recent study by Joubert et al., (2005) reported the polyphenolics compounds in *A. linearis* to account for about 15% of the mass of extracted solids.

The phytochemical analysis of *Aloe vera* leaf extract revealed the presence of flavonoids, flavonols, alkanes, alkaloids, aldehydes, ketones, alcohols, total phenols, saponins, tannins, phytosterols, proanthocyanidins, fatty acids, indoles, pyrimidines, organic acids and dicarboxylic acids (Table.1). The plants are used for the treatment of various diseases due to the presence of phytochemicals. The concentrations of compounds extracted in different solvent extracts were significantly different from each

other. It gives an indication that solvents possess different extracting capacity for each compound. Phenols, flavonoids**,** alkaloids, saponins and ketones contents were found in both ethyl acetate and methanol extracts of *Aloe ferox*. These compounds aloin, aloe-emodin and aloesin may be a contributing factor toward its antioxidant activity **(**Fig 5-8) Extraction of components from plant material depends on the polarity (Wintola et al., 2011). Absence of flavonols, proanthocyanidins and tannins were observed in the methanol extracts against ethyl acetate. Phytosterols, fatty acids, indoles, alkanes, pyrimidines, organic acids, aldehydes, dicarboxylicacids and alcohols were absent in ethyl acetate where as they are present in methanol extract.

Aloin: Aloin or barbaloin is known as the main laxative component of Aloe preparations and it has generally been used as a key component for the quality control of pharmaceuticals containing Aloe. The barbaloin content of latex from different *Aloe species* was accessed by a number of methods and found to be between 10 - 25% on a dry weight basis of the latex and about 1% on a leaf dry weight basis. Aloin is reported to be present to the extent of 18.2% in *Aloe littoralis* Baker (Zonta et al., 1995). 2.9 - 3.7 ppm in *Aloe arborescens* and up to 70% of total dry weight of *Aloe ferox* leaf exudates (VanWyk et al., 1995).

The Aloin content in the leaves has been reported to vary in Aloe collected from different geographic locations. Jansz et al., 1981 reported that aloin content varied between 12.5-16.5% in fresh juice on Mannar plants (Sri Lanka). They observed that Mannar aloes are outstanding in its mean aloin content of 57% (dry basis). The range of aloin content in plants from different localities has been reported as 4.5 - 25.0% (Farooqi and Sreeramu 2004; Rajendran et al., 2007), 22-29% (Viljoen, 1999). Study conducted on

Aloe ecotypes named Tiruvannamalai (TVM) and Trichy (TCY) showed good quality of gel of 36.62 t/ha. The highest aloin yield of 49.22 kg/ha on wet basis and 39.63 kg/ha on dry basis was obtained (Alagukannan et al., 2006). The barbaloin content of Indian Aloe was found to be as low as 4.24% (Ujjwala, 2013) while aloin content of *Aloe barbadensis* is 35% according to Singh and Soma Dey, (2005).

Present study has revealed the variation in aloin content in the exudates of leaves of various accessions. Aloin content ranged between 0.78 to 1.68 % with overall mean value of 1.11 % (Table 5 a, b). Earlier TLC and HPLC studies conducted on two cultivars revealed that aloin content varied between 0.03- 0.06% and 10.0- 15.4% in meetha and khara cultivars respectively (Anonymous, 2004). As per above findings all the presently collected and studied accessions of Aloe fall in line with meetha aloes.

The leaf weight increases during winter and decrease in summer. Seasonal fluctuations have been attributed to water availability (Waller et al., 2004). The increased hydration of Aloe plants was considered responsible for increase in leaf thickness and gel content and drought conditions resulted in low gel content (Genet and Van Schooten, 1992). In addition to total gel content the composition of active ingredients are subtly affected by seasonal, climatic and soil variations (Ramachandra and Srinivasa Rao, 2008). The anthraquinone content is subjected to seasonal variation in Aloe. Janik, (1973) estimated aloin content in *Aloe arborescens* and found with Aloin and Aloe-emodin anthrone content increased with age of plant. Most significant increase was found in plants 2-3 years old. Season variation in aloin content was also revealed by Jansz et al., (1981).

The present study has shown that there exists variability in various phytochemical constituents not only among various accessions of Aloe but also within an accession. Every accession has been evaluated quantitatively twice for various biochemicals and significant variation has been observed in many cases i.e phytochemical analysis, NMR and HPLC studies. Our present estimation has shown that four accessions of aloe germplasm (IC111271, IC111269, *Aloe CIM- Sheetal* and IC111267) have gel content greater than other accessions particularly wild *Aloe vera*.

3. A. Anti Oxidant activity in *Aloe ferox*

The strong antioxidant activity of plant extract exhibited by the presence of phenolic compounds, which have direct antioxidant properties due to the presence of hydroxyl groups acts as hydrogen donor. Also, they are found to be effective in scavenging free radicals as a result of their redox properties that allow them to act as reducing agents. The potent water soluble antioxidants and flavonoids are hydroxylated phenolics which help in radical scavenging and prevention of oxidative cell damage and they have reported to possess strong antioxidant activities. The concentration was low in the whole leaf extracts of *Aloe ferox*. But, methanol extracts has more flavonoids than the other solvent extracts during this study. *A. ferox* whole leaf extracts showed the reductive capabilities when compared with BHT. The reducing power of the extracts was concentration dependent and the antioxidant activities in all the solvents used were comparable with BHT. DPPH is a relatively stable free radical scavenger that converts the unpaired electrons to paired ones by hydrogen proton donation. Scavenging of DPPH radical during this study indicates the potency of the plant extracts in donating hydrogen proton to the lone pair electron of the radicals. As a result of the inhibition was more at a

higher concentration in all the solvent extracts, it can be suggested that the plant extracts contains compounds capable of donating protons to the free radicals and it has proven the effectiveness of the extracts in an exceedingly concentration-dependent manner (Wintola et al., 2011).

The present study shows the scavenging activity of the leaf extracts of *A. ferox* in methanol and ethyl acetate. Among the ethyl acetate and methanol extractions of *Aloe ferox,* the methanol extract showed the highest antioxidant activity when evaluated by DPPH and reducing power method. The observed results suggest further analyses to confirm its prophylactic effect in the treatment of free radical mediated diseases. Most antioxidant activities rely upon the amount of the phytochemicals present in the plants. In Indian system of medicine, certain herbs are claimed to produce relief of pain and inflammation. The claimed therapeutic reputation needs to be verified in an exceedingly scientific manner.

In the current study one *Aloe ferox* leaf extract was taken (ALE). In this study *A. ferox* traditionally used for different health disorders was studied for their *in-vitro* antioxidant and anti-inflammatory activities. The anti-inflammatory activity of this plant material has not been reported till now in the literature. Reactive Oxygen Species (ROS) generated endogenously or exogenously are associated with the pathogenesis of various diseases such as diabetes, cancer, arthritis, atherosclerosis and aging process (Hajimahmoodi et al., 2008). The Inflammation process is a complex and ROS play an important role in the pathogenesis of inflammatory diseases reported by Scandalis et al., (1997). Thus antioxidants which can scavenge ROS are expected to improve these

disorders. Many literatures have correlated the protein denaturation activity and autoimmune diseases.

Many studies on antioxidants have proved that oxidative stress has great importance in generation of autoimmune bodies responsible for autoimmune diseases. Since most of plants have poly phenolic compounds that has a sensible reducing and singlet oxygen quenching effect on free radicals. Literature suggest that, the anti-denaturation property of BSA was due to the presence of two interesting binding sites in the aromatic tyrosine rich and aliphatic threonine and lysine residue regions of the BSA (Williams et al., 2008). They have also reported that therapeutic molecules could be activating the tyrosine motif rich receptor dually with many studies on antioxidants have proved that oxidative stress has great importance in generation of autoimmune bodies responsible for autoimmune diseases. Most of the plants have poly phenolic compounds which have a good reducing, singlet oxygen quenching effect on free radicals.

Thus in this study *in vitro* anti-denaturation of Bovine serum albumin and reducing antioxidant activity was evaluated. The results have clearly demonstrated that all the plant species have moderate to significant antioxidant and anti-denaturation activity. It is well known that, phenolics constitute one of the major groups of compounds antioxidants (Cakiri et al., 2003). The abundance of plant extracts in polyphenol content should also explain the antioxidant activity results perhaps it could be the possible reason for antidenaturation and reducing antioxidant activity. It was observed that *Aloe ferox* methanol extract (AFME) showed significant inhibition against carrageenan-induced paw edema in the dose dependent manner (Table 15).

3. B. Anti-inflammatory activity in *Aloe ferox*

This response tendency of the extract in carrageenan-induced paw edema revealed good peripheral anti-inflammatory properties of the methanolic extract. This anti-inflammatory effect of AFME leaves may be because of flavonoids and also reported that number of flavonoids possessed anti-inflammatory activity (Mascolo et al., 1987). The presence of flavonoids might be responsible for the anti-inflammatory activity in methanolic extract. From the *in vitro* findings, it was clear that both methanolic and ethyl acetate extracts of *A. ferox* leaves showed good antioxidant and anti-denaturation activities. Among them methanolic extract was more potent than the ethyl acetate extract. Many chemical compounds have been identified and separated from different medicinal plants with the help of several biophysical techniques like chromatography, NMR **(**Nishant and Anil, 2012) and mass spectroscopy. Analytical HPLC and ^{13}C- NMR spectroscopy was undertaken to assess the various components present in the *Aloe ferox* leaf extract.

3. C Chemical analysis of *Aloe ferox*:

HPLC analysis of methanol extract of leaf of *Aloe ferox* was carried out with the mobile phase methanol: acetonitrile: water in the ratio 25:35:40 gave total of 6 peaks at retention time 4.545Min (Fig. 23). The highest peak was seen at the retention time 563.778mAU.

^{13}C-NMR analysis of the methanol extract of leaf of *Aloe ferox* showed a number of peaks in between δ 16.832 to 71.196 and δ 174.6 to 176.777 (Indicating presence of aromatic rings). The highest peak was seen between 48.996 - 49.007 (**Fig. 32**). These

signals confirmed the presence of aromatic ring indicating towards the occurrence of possible chemical compounds respectively.

Chemical analysis in *Aloe vera*:

We took two samples i.e 1. *Aloe CIM- Sheetal* (11) and wild *Aloe vera* (12) for chemical analysis through HPLC and NMR among twelve accessions.

HPLC analysis of methanol extract of leaf of *Aloe vera* was carried out with the mobile phase methanol: acetonitrile: water in the ratio 25:35:40. Identified the compound highest peaks and eluted from the injected compound by HPLC in *Aloe CIM- Sheetal* - peaks were identified at 4.508 Min Ret Time - Area 293.135 mAU. Wild *Aloe vera* - Small peak was identified at 4.510Min Ret Time - Area 22.18 mAU Compared with Standard compound- 4.615Min Ret Time- Area 1386. 26 mAU.

NMR analysis

^{13}C-NMR analysis of the same methanol extract of leaf of *Aloe vera* showed a number of peaks. These signals confirmed the presence of aromatic ring indicating towards the occurrence of possible chemical compounds respectively.

Identified the compound peaks and eluted from the injected compound by ^{13}C NMR

- a) *Aloe CIM- Sheetal* (11) - between 48.979 - 49.031, at 50 ppm
- b) Wild *Aloe vera* - No compounds were identified at 50 ppm.
- c) Compared with Standard compound - between 49.003 at 50 ppm.

Identified the compound peaks and eluted from the injected compound by ^{1}H NMR in

- a) *Aloe CIM- Sheetal* - between 2.598 at 2.5 ppm.
- b) Wild *Aloe vera* - No compounds were identified at 2.070 -2.912 ppm.
- c) Compared with Standard compound - between 2.627, at 2.0 ppm.

The present study stated that the methanol and ethyl acetate leaf extracts of *Aloe ferox* contain a variety of phytochemical compounds having potential antioxidant and anti-inflammatory activities. Aloin, aloe-emodin and aloesin are extracted from the results of methanol and ethyl acetate mixture. Aloesin was isolated in a large proportion from the methanolic extract. The review of literature revealed that aloesin has much biological activities including anti-inflammatory activity as well as potential anti-cancer agent. From *in vitro* studies, the antioxidant and anti-inflammatory activities of *Aloe ferox* are attributed to its free radical scavenging activity due to the presence of flavonoids and phenolic compounds. For chemical screening, HPLC coupled with NMR provides a great deal of preliminary information about the content and nature of constituents found in the active extracts. With all these results, we can conclude that *Aloe ferox* leaf extract can be used as a source of safe and natural antioxidant as well as anti-inflammation compound.

This study proposing its potential application as a lead compounds for designing potent anti-inflammatory activity and they can be used for treatment of various diseases such as diabetes, antiviral, antifungal, anti-inflammatory and immuno-modulator. Further, pharmacological and biochemical investigations will clearly elucidate the mechanism of action of compound and will be helpful in projecting this plant as a therapeutic target in diabetes and inflammation research.

Correlation analysis

The morphological and biochemical characteristics of all the accessions of Aloe have been studied. The correlation coefficients among the major characteristics are given in Table-5 (a, b). A character by character examination showed that different characters are positively associated with each other. The plant (*Aloe CIM-Sheetal*) height was found positively and significantly correlated (0.96) with leaf size. The Aloe plants are valued for the presence of high quantity gel in their leaves. The present correlation study revealed that both amount of gel and biomass in the leaves is positively and significantly correlated with morphological characters i.e. plant height and leaf size. The results clearly indicate that selection for yield component of Aloe depend upon big sized plants with large thick fleshy leaves. So, these results indicate the importance of size of the plant and size of the leaves as the main morphological characters suitable for selection of plant types for good yield.

Phytochemical variability vs Morphotypes

The variation in the quantity of various biochemical constituents has also been studied among the various morphological variants, *in vitro* variants and genetic variants. Among the two types of morphotypes the accessions with large sized leaves (LAV) possess higher quantities of various phytochemical constituents than those of (SAV).

Analysis of variance

Analysis of variance (ANOVA) was carried out in different accessions of *Aloe vera* covering various parameters. The ANOVA revealed highly significant differences among various accessions for various characters (Table 5 (a, b). The results indicated adequate genetic variability in the germplasm.

4. Antidiabetic Activity:

Diabetes mellitus belongs to the group of metabolic diseases characterized by hyperglycemia and defective protein metabolism that results from defects in both insulin secretion and insulin action.The literature reports reveal that flavonoids and tannins present in some plant extracts are responsible for antidiabetic activity. The fact that some herbal preparations enhance the beta cell regeneration and peripheral glucose utilization in Alloxan and Streptozotocin induced diabetic rats supports the above assumption. STZ was found to generate reactive oxygen species, which also contribute to DNA fragmentation and evoke other deleterious changes in the cells (Takasu et al., 1991). It is well known that oxygen free radicals are involved in diabetogenic action of alloxan and plants containing flavonoids, isoflavanoids, triterpenoids have been shown to be effective in diabetes due their antioxidants property (Jafri et al, 2000). This suggests that the antihyperglycemic activity of *Aloe CIM-Sheetal* may be due to free radical scavenging activity which enhances the beta cell regeneration against streptozotocin induced free radicals.

One of the major complications of type I diabetes is weight loss. It arises due to the impairment in insulin action in conversion of glucose to glycogen and catabolism of fats, inhibition of lipolysis due to its unavailability because of destruction in beta cells (Gillespie, 2006). Due to this there will be a decrease in the body weight of the animals and death. Treatment with methanolic extract has substantially prevented the body weight loss.

4. A. Mechanism of action of Streptozotocin (STZ) (Junod et al., 1969; Herr et al., 1967):

STZ is widely used to induce experimental diabetes in animals. The mechanism of their action in β cells of the pancreas has been intensively investigated. The cytotoxic action of this diabetogenic agent is mediated by reactive oxygen species however; the source of its generation is different in the case of other diabetogenic agents. STZ enters the β cell via a glucose transporter (GLUT2) and causes alkylation of Deoxyribonucleic acid (DNA). DNA damage induces activation of poly ADP-ribosylation, a process that is vital for the diabetogenicity of STZ than DNA injury itself. Poly ADP-ribosylation ends up in depletion of cellular NAD^+ and ATP. Increased ATP dephosphorylation after STZ treatment provides a substrate for xanthine oxidase resulting in the formation of superoxide radicals. Consequently, peroxide and chemical group radicals are also generated.

Furthermore, STZ liberates toxic amounts of nitric oxide that inhibits aconitase activity and participates in DNA damage. As a result of the STZ action, β cells endure the destruction by necrosis. STZ is approved by the U.S. Food and Drug Administration (FDA) for treating metastatic cancer of the duct gland islet cells. Since it carries a considerable risk of toxicity and rarely cures cancer, its use is generally limited to patients whose cancer cannot be removed by surgery. In these patients, STZ can reduce the tumor size and reduce symptoms (especially hypoglycemia due to excessive insulin secretion by insulinomas (Brentjens and Saltz, 2001). Lifestyle interventions are however more effective than metformin in preventing diabetes regardless of weight loss. Diabetes mellitus is a chronic disease which is difficult to cure.

However, in the present work, we have chosen a miracle plant *Aloe CIM-Sheetal*, an important medicinal plant for studying its antidiabetic activity and histopathology of organs in streptozotocin evoked as diabetic rats. The objective of the present study was to know the anti diabetic affect of leaf extract of *Aloe CIM- Sheetal* and to evaluate its protective role in pancreas; liver and kidney in STZ induced diabetes in SD rats as animal models. World Health Organization (WHO) has recommended that the evaluation of medicinal plants treatment for diabetes were more effective, non- toxic with less or no side effects considered for oral therapy **(**Takamoto and Kadowaki, 2011).

The present study has demonstrated for the first time the anti diabetic property of *Aloe CIM-Sheetal* which caused significant decrease in the blood glucose levels in diabetic rats treated with *Aloe CIM–Sheetal* leaf extract. It may be by stimulation of the residual mechanism of pancreas that could contribute to an increase in due to peripheral utilization of glucose (Erah et al., 1996). STZ was found to generate reactive oxygen species, which also contributes to DNA fragmentation and evokes other deleterious changes in the cells (Takasu et al., 1991). It is well known that oxygen free radicals are involved in diabetogenic action of alloxan and plants containing flavonoids, triterpenoids and isoflavanoids have been shown to be effective in diabetes due to their antioxidants property (Jafri et al., 2000). This study suggests that the antidiabetic activity of *Aloe CIM-Sheetal* may be due to free radical scavenging activity which enhances the β cell regeneration against STZ induced free radicals. The literature reports reveal that flavonoids and tannins present in some plant extracts are responsible for antidiabetic activity. In the present investigation, we have observed that the antidiabetic potential of Aloe leaf extract (ALE) may be due to presence of similar phytoconstitutes which was

evident by preliminary phytochemical screening studies (Table. 9).The different extracts of *Aloe CIM- Sheetal* revealed that the methanol extract was rich in saponins, chloroform extracts was rich in saponins, steroids and tannins. Alkaloids, carbohydrates and glycosides were found to be the present in acetone, methanol and aqueous extracts. Methanol extracts were also found to possess tannins and phenolic compounds.

The results of blood glucose levels and body weights indicate that ALE can be useful in reducing the effects of STZ induced diabetes. One of the major complications of type I diabetes is weight loss. It arises because of impairment in insulin action in conversion of glucose to glycogen and catabolism of fats, inhibition of lipolysis because of its unavailability due to destruction in β cells (Gillespie, 2006). Due to this there will be a decrease in the body weight of the animals and thereby death. Treatment with methanol extract has substantially prevented the body weight loss. Daily treatment of animals with extract for three weeks led to a dose dependent fall in blood glucose levels. Maximum effect was seen in animals which are treated with 300 mg/kg of ALE.

The present study aimed to evaluate the antidiabetic potency of *Aloe CIM- Sheetal* leaf extract in Streptozotocin (STZ) induced diabetic rats and its effect was compared with glibenclamide. 42 adult male SD (Sprague Dawley) rats were used in the experiment for studying the antidiabetic activity and histopathology evaluation was also done. In this study, we address the beneficial effects of *Aloe CIM-Sheetal* (CAL-14), which is a variety of *Aloe barbadensis*, to emphasize the role of active compounds using streptozotocin induced diabetic rats to prove its antidiabetic activity. We have used methanolic extract to study the pharmacological activity. *Aloe CIM- Sheetal* methanolic

extract in different concentrations (100, 200, 300 and 500 mg/kg b.w.) was given to rats which were divided into seven groups, by oral administration for 21 days. It was observed that the fasting plasma glucose levels were reduced in experimental animals when compared to normals / controls and the body weights also increased.

4. B. Histopathology

At the end of 21st day, all the rats were euthanized by pentobarbitone sodium (60 mg/kg) and sacrificed by cervical dislocation. The pancreas, liver and kidney were dissected out, washed immediately in ice-cold saline. In the pancreatic sections of diabetic rats fed with *Aloe CIM-Sheetal*, the islets were comparable to the normal rats. In diabetic rats fed with *Aloe CIM-Sheetal* there were no changes in liver. The changes in liver are shown in Fig.41.The Mesangial matrix is mildly decreased in extra cellular PAS (Periodic Acid Schiff stain) in diabetic rats that were not fed with *Aloe CIM-Sheetal* and also diabetic rats fed with *Aloe CIM-Sheetal.*The results were positive in kidneys as shown in Fig.42 and number of islets was increased in pancreas materiala shown in Fig. 43.

Histology of the pancreatic sections of diabetic rats fed with *Aloe CIM-Sheetal* showed a moderate increase in number of islets with decrease in vaculation and absence of any changes in the liver. Moreover, in diabeties induced rats fed with *Aloe CIM-Sheetal* the kidney sections showed mild decrease in mesangial matrix along with a mild decrease in extracellular PAS positive material which was comparable to diabetic + standard drug treated with Glibenclamide 0.60 mg/kg.

From our studies, we conclude that the methanolic leaf extract of *Ale CIM-Sheetal* has significant antidiabetic activity and histopathological changes are shown in pancreas,

kidney and liver. The glucose lowering activity were observed in the diabetic animals because of stimulation of the β cells of the pancreatic islets and body weights of diabetic animals treated with *Aloe CIM-Sheetal* group were significantly recovered when compared to the diabetic control and diabetic animals treated with glibenclamide groups. Subsequently, the blood glucose levels were decreased at the same time because of the stimulation of β cells of the pancreatic islets naturally in diabetic group followed by increased oxidative levels and no tissue damage were observed by the activity of *Aloe CIM- Sheetal* in STZ treated animals.

It has been observed that only 300 mg/ kg body weight of *Aloe CIM-Sheetal* leaf extract has a protective effect compared to STZ treated and glibenclamide treated groups. It has been found that *Aloe CIM-Sheetal* is a potential antidiabetic candidate in streptozotocin-induced diabetic model by reducing oxidative damage and modulating antioxidant enzymes. Acute and sub-acute toxicity of *Aloe CIM-Sheetal* in SD rats indicated that the methanol extract at the dose of 1, 5, 10, 15, 20 g/kg body weight does not produce significant dose related biochemical and histopathology effects in internal organs. It may be hence concluded that the methanolic extract of *Aloe CIM-Sheetal* does not produce significant toxic impact, in rats during acute and sub-acute treatment in rats. Hence the extract can be used for pharmaceutical industry.

Further, pharmacological and biochemical investigations will clearly elucidate the mechanism of action and will be helpful in protecting this plant as a therapeutic target in cancer research. It can be stated that the crude extract of the leaves of *Aloe CIM-Sheetal* has a potential antidiabetic activity as well as wound healing capacity in herbal treatment. There was no case showing acute hypoglycemic conditions during the administration of

Aloe CIM-Sheetal leave extract or its antidiabetic compounds, and no adverse effect symptoms were observed from the view points of pathological findings. Thus, *Aloe CIM-Sheetal* leaf extract could be useful to prevent Type II diabetes mellitus. This extract may be similar to compounds like acemannan for the treatment of anticancer and further, isolation and establishment of exact mechanism of action of specific compound from *Aloe CIM-Sheetal* has to be carried out in future.

5. Molecular study

Methods of assessment of diversity at gene level have an important role within conservation programmes for genetic sources of crop plants (Newbury and Ford Lloyd, 1993). Most of the ITS (Internal transcribed spacer) based molecular methods rely on the determination of the DNA sequences of the amplified region and then sequence comparison with those of available from Gen Banks databases. Presently, the accessions of *Aloe vera* from different locations have been analyzed using molecular method based on size of the amplicon instead of sequences. Attempt has been carried out to use the fast and precise molecular techniques of RAPD and ISSR.

Methods based on molecular markers are used to identify the putative duplicate accessions and to determine the distribution of genetic variation between individuals, accessions, races etc (Westman and Kresovich, 1997). A number of studies have aimed at assessing the genetic diversity in germplasm collections of crops using various molecular markers such as restriction fragment length polymorphism (RFLP-Dubreuil and Charcosset, 1998), random amplified polymorphic DNA (RAPD-Fofana et al., 1997), amplified fragment length polymorphism (AFLP-Zhu et al., 1998), microsatellite or simple sequence repeat (SSR: Powell et al., 1996). Different authors have demonstrated

the usefulness of the Internal Transcribed Spacers (ITS) of 18S - 5.8S - 25S rDNA gene to assess phylogenetic relationship between cultivated species and their wild relatives (Baldwin, 1992; Adams et al., 2000) investigated the distribution of 5S and 18S - 5.8S - 26S rDNA sequences in as many as 13 *Aloe species* selected from across the geographic range (Arabian peninsula, East and South Africa and Madagascar). They also constructed a phylogenetic tree of twenty eight species including 13 species used for FISH to determine whether patterns of rDNA were reflected in relationships evaluated with nuclear ribosomal DNA (rDNA) internal transcribed.

Yagi et al., 2006 compared the sequence in the ITS region between clonally regenerated *Aloe vera* and the same species in Japan, USA and Egypt. The study revealed the presence of two types of nucleotide sequences, 254bp and 252bp. Orndorff and Steve (1996) developed PCR technique for the detection and identification of Aloe DNA. Considerable attempts have been made to evaluate genetic diversity among *Aloe species* using RAPD and AFLP based marker (Darokar et al., 2003).Such methods have been used successfully for quick identification of strains of pathogens (Casimiro et al., 2004). The method has drawbacks as it lacks the resolution of ITS sequence analysis. Although it might be possible that similar bands of amplified DNA with same molecular weight are of different sequences, therefore, the ALP method can be more improved in its precision through application of the technique double gradient gel electrophoresis (DGGE) that make it possible to discriminate bands of similar weight but different sequences. Many workers have used single stranded confirmation polymorphism (SSCP) technique to identify the sequence variations (Gillman et al., 2001; Kumar and Shukla, 2005).

An understanding of the extent of genetic diversity is important for both plant breeding and germplasm collection. In *Aloe vera* traditional methods like horticultural traits are relatively less reliable and inefficient for precise discrimination of closely related genotypes (Nejatzade et al., 2012). Hence, selection based on genetic information using neutral molecular markers is essential as it is more reliable and consistent. Among different marker systems available, RAPD and ISSR markers became popular in diversity studies because of simplicity, rapid, inexpensive and applicable to any genome without any prior information regarding the genome of the plant.

In the present study, a set of 12 elite *Aloe vera* accessions (Table 4) were analyzed using 64 RAPD and 25 ISSR markers to describe the genetic structure among the accessions. The RAPD primers revealed 71.8% polymorphism with 4.34 polymorphic bands/primer, while ISSR primers revealed 80.9% polymorphism with 4.47 polymorphic bands/primer indicating wide genetic variation among the accessions. ISSR primers detect more polymorphism than RAPD primers because of variability in microsatellite loci due to DNA slippage (Williams et al., 1990). The RAPD markers cover the entire genome in coding and non coding regions including repeated or single-copy sequences, while ISSR markers disclose polymorphism from sequences between two microsatellite primer sites (Williams et al., 1990; Zietkiewicz et al., 1994). The ISSR method has been reported to be more reproducible (Goulao and Oliveira, 2001) and produces more complex marker patterns than the RAPD approach (Chowdhury et al., 2002; Parsons et al., 1997) reported that is an advantageous when differentiating closely related cultivars. Both the marker techniques provides a useful approach for evaluating genetic differentiation, significantly in those species that are poorly known genetically and are

propagated vegetatively like monocot genus in Musa (Bhat Jarret, 1995) and Lilium (Haruki et al., 1998).

The PCO analysis and dendrogram constructed based on RAPD + ISSR polymorphism showed similar clustering pattern and disclosed predominantly two major clusters (I and II). The similarity values ranging from 14% to 85% indicating that there is a remarkable genetic variation among *Aloe vera* accessions used in the present study. The highest similarity (85%) was recorded between IC111280 and IC111279 accessions followed by 82% similarity between wild *Aloe vera* and *Aloe CIM-Sheetal* accessions, which were collected from CIMAP Research Center, Hyderabad, India. Recently, AFLP based characterization of *Aloe vera* accessions from different location of Madhya Pradesh, India has reported modest level of genetic variability (Tripathi et al., 2011). This low level of variability could be because of small sample size collected from the limited geographical regions. Similarly, (Darokar et al., 2003) have also reported the morphological similarity 78.8% to 99% in *Aloe vera* accessions revealed by RAPD and AFLP analysis. While, Nayanakantha et al., (2010) have reported a good amount of genetic variability among *Aloe vera* accessions based on RAPD analysis.

Apparently, the present work constitutes the presence of wide genetic variability among the *Aloe vera* accessions obtained from NBPGR, New Delhi, India. This variability can be used for genetic improvement through breeding programmes and the accession specific bands were identified in this study will provide tags for future genetic improvement and authentication of genotypes. Both the markers techniques (RAPD and ISSR) have been shown to be useful in detecting small genetic variations within and among *Aloe vera* populations.

S UMMA RY

6. SUMMARY

- Aloe accessions obtained from different locations i.e. NBPGR-New Delhi, CIMAP-Hyderabad, India and SANBI, South Africa showed morphological variations on the basis of size of plant, height, gel content, aloin conc. and biomass of leaves. Since all the accessions collected from different places were cytologically diploid with chromosome number 2n=14, they were grown and maintained in the same environmental conditions at Indian Immunologicals Ltd, Hyderabad the observed variability can be related to genetic makeup/genetic diversity.

- Based on morphology, the present studies have revealed that the accessions of *Aloe vera* germplasm can be divided into two morphotypes viz. Small *Aloe vera* (SAV): plant size up to 0- 40 cm, and Large *Aloe vera* (LAV): plants with leaves more than 40 cm in size. The LAV type of accessions exhibited highest incidence.

- Among the two morphotypes, the accessions with LAV possess higher quantities of various phytochemical constituents than those of SAV. Similarly, the characters like gel, biomass content and aloin concentration were associated with higher mean values of various constituents.

- Morphological analysis indicated that all the studied characters have a significant difference at $P<0.01$. Accession IC 111272(4), IC 471883 (8), IC 471882 (7), IC 111267(1) and *Aloe CIM-Sheetal* (11) accessions were found to be tallest (61 - 67 cm range) as they possess a distinct stem (calescent) with long internodes.

- Minimum leaf thickness (18.1 mm) and minimum wideness (3.9 cm) were recorded for accession IC 111271(3) and accession for local *Aloe vera* (12) respectively. Leaf dry weight of local *Aloe vera* (12) was very low (12.20 g) and highest (23.7 g) was in IC 111269 (2).

- Gel fresh weight was recorded 58.52 g in IC 111267 (1) as highest and lowest was 4.10 g in local *Aloe vera* (12). The dry gel weight was recorded 12.80 g in IC 111271 (3) compared to the other accessions.

- Lowest Aloine concentration of 0.86% was recorded from IC 111267 (1) where as highest 1.68% was for accession IC 111280 (6).

- In the present study we standardized the surface sterilization procedure, less expensive media composition with high frequency regeneration. This protocol proves conservation of important miracle medicinal plant *Aloe vera* from extinction, facilitate germplasm conservation and suggest that the developed strategy can be adopted for other endangered species also.

- In our method of tissue culture explant preparation, 35 explants were obtained from single mother plant. Each explants gave rise to 8.84 ± 0.03 shoots on MS medium supplemented with BAP (1.5 mg/l), KIN (0.5 mg/l), NAA (0.2 mg/l), IAA (0.1mg/l) and IBA (0.2mg/l) in 7 weeks in comparison to 30 shoots from 18 explants obtained from 18 mother plants in 8 weeks respectively (Balraj Singh and Neelu Sood, 2009). This is in contrast to earlier work by Meyer and Staden, (1991) who reported better proliferation in *Aloe vera* on medium containing kinetin instead of BA. This

difference may be due to difference in the genotype of plant (Abrie and Staden, 2001).

- The shoot tip of explants initially produces two - three shoots within two weeks after inoculation. But in our method 15-35 shoots/culture were produced from single explant by subsequent 2-3 subcultures with same medium which indicate the high efficiency of this protocol (Fig 14). The average length of shoots per culture was 4.89 ± 0.03 cm.

- Formation of the roots was best observed in *Aloe CIM-Sheetal* and IC111271 in the media containing MS basal media with 3 types of hormones NAA 0.2mg/l, IAA 0.1mg/l, and IBA 0.2 mg/l within four weeks after inoculation for rooting. The highest percentage of shoots that induced roots (91.12%) was observed in MS medium supplemented with NAA (0.2 mg/l) + IAA (0.1 mg/l) followed by IBA (0.2 mg/l) (Table 7).

- The phytochemical variation has been detected among various accessions of *Aloe vera* maintained at Indian Immunologicals Ltd, Hyderabad. Since these accessions are growing in the same environmental and edaphic conditions the presently observed variability may be related to their genetic makeup.

- Thus, in this study *in vitro* antidenaturation of Bovine serum albumin and reducing antioxidant activity was evaluated. The results have clearly demonstrated that all the plant species have moderate to significant antioxidant and anti-denaturation activity.

- It was observed that *Aloe ferox* methanol extract (AFME) showed significant inhibition against carrageenan-induced paw edema in the dose dependent manner (Table 15).
- From the *in vitro* findings, it was clear that both the methanolic and ethyl acetate extracts of *A. ferox* leaves showed good antioxidant and anti-denaturation activities. Among them methanolic extract was more potent than the ethyl acetate extract.
- The phytochemical analysis of *Aloe ferox* leaf extract revealed the presence of flavonoids, flavonols, alkanes, alkaloids, aldehydes, ketones, alcohols, total phenols, saponins, tannins, phytosterols, proanthocyanidins, fatty acids, indoles, pyrimidines, organic acids and dicarboxylic acids. These compounds aloin, aloe-emodin and aloesin may be a contributing factor towards its antioxidant activity.
- Extraction of components from plant material depends on the polarity (Wintola and Afolayan, 2011). Absence of flavonols, proanthocyanidins and tannins were observed in the methanol extracts against ethyl acetate. Phytosterols, fatty acids, indoles, alkanes, pyrimidines, organic acids, aldehydes, dicarboxylicacids and alcohols were absent in ethyl acetate where as present in methanol extract.
- For chemical screening, HPLC coupled with NMR provides a great deal of preliminary information about the content and nature of constituents found in the active extracts. With all these results, we can conclude that *Aloe ferox* leaf extract can be used as a source of safe and natural antioxidant as well as anti-inflammation compound.

- This study propose its potential application as a lead compounds for designing potent anti-inflammatory activity and they can be used for treatment of various diseases such as diabetes, antiviral, antifungal, anti-inflammatory and immunomodulator. Further, pharmacological and biochemical investigations will clearly elucidate the mechanism of action of compound and will be helpful in projecting this plant as a therapeutic target in diabetes and inflammation research.

- The present study for the first time has demonstrated the antidiabetic properties of *Aloe CIM-Sheetal* which caused significant decrease in the blood glucose levels in diabetic rats treated with *Aloe CIM–Sheetal* leaf extract. The results of blood glucose levels and body weights indicate that ALE can be useful in reducing the effects of STZ induced diabetes.

- Treatment with methanol extract has substantially prevented the body weight loss. Daily treatment of animals with extract for three weeks led to a dose dependent fall in blood glucose levels. Maximum effect was seen in animals which are treated with 300 mg/kg of ALE. It was observed that the fasting plasma glucose levels were reduced in experimental animals when compared to normals/controls and the body weights were also increased.

- Histology of the pancreatic sections of diabetic rats fed with *Aloe CIM-Sheetal* showed a moderate increase in number of islets with decrease in vaculation and absence of any changes in the liver. In the pancreatic sections of diabetic rats fed with *Aloe CIM-Sheetal*, the islets were comparable to the normal rats.

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- From our studies, we can conclude that the methanolic leaf extract of *Aloe CIM – Sheetal* has significant anti-diabetic activity and histopathological changes are shown in pancreas, kidney and liver.

- Blood glucose levels were decreased at the same time because of the stimulation of β cells of the pancreatic islets naturally in diabetic group followed by increased oxidative levels and no tissue damage were observed by the activity of *Aloe CIM-Sheetal* in STZ treated animals.

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- Thus *Aloe CIM-Sheetal* leaf extract could be useful to prevent Type II diabetes mellitus. This extract may be similar to compounds like acemannan for the treatment of anticancer and further, isolation and establishment of exact mechanism of action of specific compound from *Aloe CIM-Sheetal* has to be carried out in future.

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- The highest similarity (85%) was recorded between IC111280 and IC111279 accessions followed by 82% similarity between wild *Aloe vera* and *Aloe CIM-Sheetal* accessions, which were collected from CIMAP Research Center, Hyderabad, India.

- The present correlation study revealed that both amount of gel and biomass in the leaves is positively and significantly correlated with morphological characters i.e. plant height and leaf size. The results clearly indicate that selection for yield

component of Aloe depend upon big sized plants with large thick fleshy leaves. So, these results indicate the importance of size of the plant and size of the leaves as the main morphological characters suitable for selection of plant types for good yield.

- The variation in the quantity of various biochemical constituents has also been studied among the various morphological variants, *in vitro* variants and genetic variants. Among the two types of morphotypes the accessions with large sized leaves (LAV) possess higher quantities of various phytochemical constituents than those of (SAV).

- Analysis of variance (ANOVA) was carried out in different accessions of *Aloe vera* covering various parameters. The ANOVA revealed highly significant differences among various accessions for various characters (Table 5 (a, b). The results indicated the presence of adequate amount of genetic variability in the germplasm.

CONCLUSIONS

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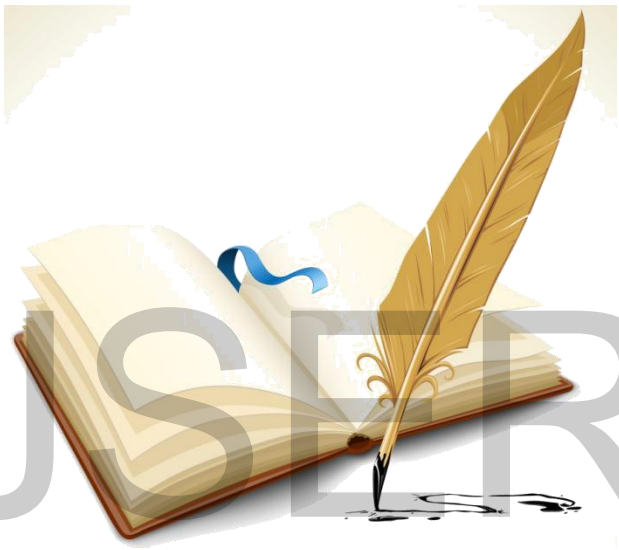

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