FLORAL BIOLOGY, KARYOTYPE, BIOCHEMICAL AND GENOMIC STUDY OF *ETLINGERA* SPECIES – A MEDICINAL AND AROMATIC PLANT

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Dedicated to my beloved parents

Late Hem Chandra Chowdhury

&

Srimati Bharati Bala Chowdhury

ABSTRACT

FLORAL BIOLOGY, KARYOTYPE, BIOCHEMICAL AND GENOMIC STUDY OF *ETLINGERA* SPECIES - A MEDICINAL AND AROMATIC PLANT

The plant is locally known as *Karphul* or *Ka-phul*, a wild perennial aromatic and medicinal herb belonging to the family Zingiberaceae. The plant has been traditionally used as food additive, mouth freshener and also to cure various ailments of mouth, bronchial and stomach. However, of late, due to over exploitation from natural habitats and lack of systematic cultivation as well as propagules, the plant has become a threatened one. Furthermore, no scientific investigation has so far been carried out to validate its worth as well as propagation and improvement.

On the basis of taxonomic description available at the BSI, Shillong, literature survey and field observations the plant has been determined to belong to the genus *Etlingera* having some distinct features as compared to other genera. Such features are: the spike doesn't elongate after flowering, presence of involucral bracts, base of the labellum (lip) adnate to the base of the filament forming a separate and distinct tube. Based on the distinguishing features like tallness (259-300 cm), hairy and serrulate ligule, hairy petiole on inner side, bigger lamina size (50-70 cm x 9-15 cm), shorter peduncle (2.8-4.6 cm), outer bract white with light greenish tinge at tip, 8-14 florets/spike, calyx tube shorter than corolla tube with dentate tip and red in colour, the herb was found to be allied to *E. linguiformis* (Roxb.) Smith; *Alpinia linguiforme* Roxb.; *Amomum linguiforme*

(Roxb.) Benth; *Hornstedtia linguiforme* (Roxb.) Schum; *Achasma linguiforma* (Roxb.) Loesen. Therefore, a probable new name has been proposed to the species.

Karyotype analysis reveal the plant to be a tetraploid with the chromosome number 2n = 48, where, basic chromosome number x = 12. Chromosomes are small in size. The total length of haploid chromosome complement was determined to be 31.2 μm. Variation in chromosome length from 0.5 – 2.4 μm was observed. Chromosomes are either with median or submedian centromere.

Isolation of essential oils from the rhizome of the plant and analysis of its composition through gas chromatography revealed that the oil is a complex mixture of 23 components, of which 4 major components have accounted for 96.2% whereas the minor components 3.8%. Out of the major components, 2 could be identified as anethole accounting for 86.0% and methyl chavicol 5.7% and the rest 2 could not be identified.

Crude dichloromethane-extract from the rhizome of the plant and purification through the TLC yielded four fractions with R_f value 0.35 (F-I), 0.40 (F-II), 0.69 (F-III) and 0.89 (F-IV), respectively. Essential oils and the four fractions of crude dichloromethane-extract exhibited variable levels of antimicrobial activity against all test pathogens *Bacillus subtilis, Staphylococcus aureus, Micrococcus flavus, Escherichia coli, Klebsiella pneumoniae* and *Candida albicans*.

GC-MS analysis of the solvent extract confirmed fragment 1 (F-I) to be a single compound, which matched with the compound 1-methoxy-4-(1-propenyl)-benzene, commonly known as anethole, available in the database of the Saturn 2000 MS Library. Accordingly, the probable structure of the compound was elucidated. Subsequently, the compound along with its structure was reconfirmed by the FTIR and ^{1}H-NMR analysis of

the fraction. The other three fractions could not be analyzed due to their poor composition and yield.

The protocol for the isolation of genomic DNA from the leaf tissue of the plant was optimized, which could yield 28 μg per g of leaf tissue restrictable quality DNA (purity ratio 2). Genome size (DNA in haploid set of chromosome) of the plant was estimated to be 1.55 pg.

In tissue culture of *Karphul,* callus induction was obtained after 25-35 days of culture in MS (Modified) medium supplemented with combinations of 2,4-D (2.0 $mg.l^{-1}$) and BAP (0.5 – 2.0 $mg.l^{-1}$). On the other hand, the medium supplemented with combinations of 2,4-D (1.0 - 2.0 $mg.l^{-1}$), NAA (1.0 – 1.5 $mg.l^{-1}$) and BAP (0.5 – 1.0 $mg.l^{-1}$) produced compact nodular calli after 30 days of culture. Sixty days old compact nodular calli when subcultured in MS (M) medium supplemented with BAP (1.5 $mg.l^{-1}$) and 2,4-D (0.25 $mg.l^{-1}$) produced tiny meristematic protuberances after 7 – 10 days. The calli subcultured in MS (M) medium supplemented with BAP (1.5 $mg.l^{-1}$) and IAA (0.1 $mg.l^{-1}$) produced 10-12 adventitious roots with 3-4 rudimentary shoots. In all cases, shoots and roots did not show any further development. Direct regeneration of 4 – 6 shoots with 3 - 5 roots were observed in MS (M) medium supplemented with BAP (10.0 $mg.l^{-1}$) and 2,4-D (0.25 $mg.l^{-1}$).

Declaration

I here by declare that the thesis entitled "FLORAL BIOLOGY, KARYOTYPE, BIOCHEMICAL AND GENOMIC STUDY OF *ETLINGERA* SPECIES - A MEDICINAL AND AROMATIC PLANT", being submitted to the department of Molecular Biology and Biotechnology, Tezpur University, is a record of original research work carried out by me. Any text, figures, methods or results that are not of own devising are appropriately referenced in order to give credit to the original author(s). All sources of assistance have assigned due acknowledgement. I also declare that neither this work as a whole nor a part of it has been submitted to any other University or Institute for any other degree, diploma or award.

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This is to certify that the thesis entitled "FLORAL BIOLOGY, KARYOTYPE, BIOCHEMICAL AND GENOMIC STUDY OF *ETLINGERA* SPECIES - A MEDICINAL AND AROMATIC PLANT" submitted to the Tezpur University in the Department of Molecular Biology and Biotechnology under the School of Science and Technology in partial fulfillment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology is a record of research work carried out by Mr. Dhiren Chowdhury under my personal supervision and guidance.

All helps received by him from various sources have been duly acknowledged.

No part of this thesis has been reproduced elsewhere for award of any other degree.

Signature of Principal Supervisor
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CERTIFICATE OF THE EXTERNAL EXAMINER

This is to certify that the thesis entitled "FLORAL BIOLOGY, KARYOTYPE, BIOCHEMICAL AND GENOMIC STUDY OF *ETLINGERA* SPECIES - A MEDICINAL AND AROMATIC PLANT" submitted by Mr. Dhiren Chowdhury to the Tezpur University in the Department of Molecular Biology and Biotechnology under the School of Science and Technology in partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology has been examined by us on_____ and found to be satisfactory.

The committee recommends for the award of the degree of Doctor of Philosophy.

Signature of:

Principal Supervisor External examiner

Date:

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LIST OF ABBREVIATION

a.m.	Ante meridiem
A.U.	Absorbance unit
BAP	6-benzylaminopurine
B.C.	Before Christ
BSI	Botanical survey of India
bp	Base pair (s)
C	Dgree cntigrade
cm	Centimeter (s)
CTAB	Cetyltrimethylammonium bromide
cv	Cultivar (s)
dH_20	Distilled water
2,4-D	2,4-dichlorophenoxyacetic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agricultural Organization
Fig.	Figure
g	Gram (s)
h	Hour (s)
HIV	Human immunodeficiency virus
IAA	Indole-3-acetic acid
IUCN	International Union for Conservation of Nature and Natural Resources
Kb	Kilobase (s)
Kin	Kinetin
km	Killometer (s)
l	Litre (s)
M	Molar concentration
mM	Millimolar
µM	Micromolar
MAP	Medicinal and aromatic plants
mg	Milligram (s)
min	Minute (s)
ml	Millilitre (s)
mm	Millimeter (s)
µg	Microgram (s)
µl	Microlitre (s)
µm	Micrometer (s)
MTCC	Microbial Type Culture Collection

N	Normal
NAA	Nephthalene acetic acid
NCIM	National Collection of Industrial Microorganisms
ng	Nanogram (s)
nm	nanometer
No. = no.	Number (s)
O.D.	Optical density
pg	Picogram (s)
pH	Hydrogen ion exponent (a unit symbol for the degree of acidity or alkalinity)
p.m.	Post meridiem
RNA	Ribonucleic acid
RNase	Ribinuclease
rpm	Revolution per minute
sq.	Square
t	Tone (s)
Tris	Trihydroxy-methylaminoethane
USA	United States of America
USD	US Dollar
UV	Ultra violet
W	Watt (s)
WWF	World Wide Fund for Nature
WHO	World Health Organization
V	Volt (s)

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CHAPTER I

INTRODUCTION

Medicinal and aromatic plants (MAPs) play an important role in the healthcare of people throughout the world, especially in developing countries since time immemorial. Until the advent of modern medicine, man depended on plants for treating human and livestock diseases. Human societies throughout the world have accumulated a vast indigenous knowledge over centuries on medicinal uses of plants, and for other related uses including as poison for fishing and hunting, purifying water, and for controlling pests and diseases of crops and livestock. These medicinal plants are well described in the earliest medical writings of 3,000 B.C. and their use might go back well before recorded history. Archaeological evidence indicates that the use of plants for healing dates back into the prehistoric era. Excavation at the Shanidar cave, Northern Iraq revealed a 60,000 year old grave of a Neanderthal medicine man; arrayed around the body were the remains of eight species of flowers. Seven of these flower species even today are used for medicinal purpose by inhabitants of the region. Probably, these medicines were discovered through many years of self-experimentation. It was basically a community-based knowledge that gradually led to the identification of plants yielding drugs used in medicinal practices. About 80% of the population of the developing countries still use traditional medicines derived from plants for treating human diseases (de Silva, 1997). China, India, Brazil, Sri Lanka, Cuba, Thailand and few other countries have endorsed the official use of traditional systems of medicine in their healthcare programmes. For example, the Indian systems of medicine 'Ayurveda', 'Siddha' and 'Unani' entirely, and homeopathy to some extent, depends on plant materials or other derivatives for treating human ailments (Prajapati *et al.*, 2003).

All traditional systems of medicine had their root and origin in folklore medicine and even today large number of rural and tribal populations adopt herbal remedies worldwide for primary healthcare. The ethnotherapeutics refer to the claims, beliefs and medicinal practices of plants and plant products for prevention and treatment of ailments among different ethnic groups. Ethnomedicinal therapeutics have the potential of leading the discovery of new drugs of herbal origin which are easily metabolized in the human body having less or no side effects. The importance of ethnomedicine in search of new potential drugs of herbal origin is gaining momentum throughout the world. The discovery of potential therapeutic compounds like reserpine, quinine, ephedrine, cocain, emetin, khellin, colchicine, dogoxin, taxol, vinblastine, vincristine, artemisinine and gugulipid from medicinal plants with rich ethnobotanical lore gave impetus to ethnomedicinal plant research in the world. The efficacy of a number of phytopharmaceuticals derived from plants, such as atropine (pupil dilator), berberin (for gastrointestinal disorder), caffeine (stimulant), digitoxin (cardiotonic), emetine (antiamoebic), ephedrine (antiasthmatic), morphine (analgesic), papain (protein digestant and anthelmintic), quinine (antimalarial), reserpine (tranquillizer), vinblastine and vincristrine (antileukemic), camptothecine (antitumer drug), forskoline (hypertensive and antispasmodic drug) with rich folklore has been discovered.

At least 130 drugs, all single chemical entities extracted from higher plants, are currently in use, though some of these are now produced synthetically for economic reasons throughout the world. An analysis of the genesis of their induction in modern medicine would show that, invariably, the starting point has been references or clues of these medicinal plants in folklore, or traditional medicine. In fact, plant-derived

compounds are showing promise in the treatment of cancer, malaria, diabetes, human immunodeficiency virus (HIV) etc. As a matter of fact, plants are almost the exclusive source of drugs for a majority of the world population even today. Plant products constitute approximately 25% of all prescribed medicines even in the most advanced countries like U.S.A. (Singh, 2000).

The recent discovery of bioactive compounds viz. artemisinin (antimalarial) from *Artemisia annua*, taxol (anticancer) from *Taxus brevifolia*, hypericin (antiviral) from *Hypericum perforatum*, gossypol (male contraceptive) from *Gossypium* spp. and yuechukene (antifertility agent) from *Murraya paniculata* raised expectations of scientists involved in medicinal plant research all over the world to discover potent herbal medicines.

Worldwide about 12.5% (*ca.* 50,000) of the 4,22,000 plant species documented are reported to have medicinal value. The proportion of medicinal plant to the documented species in different countries varies from 4.4% to 20% (Schippmann *et al.*, 2002). An enumeration of the World Health Organization (WHO) from the late 1970s listed 21,000 plants that have reported medicinal use around the world (Penso, 1980). India has 2.4% of world's area with 8% of global bio-diversity. It is one of the twelve mega-diversity hot spot regions of the world. Almost one fifth (about 20%) of all the plants found in India are used for medicinal purpose. According to an estimate made by the Botanical Survey of India (BSI, 1993), out of *ca.* 17,500 flowering plant species found in India, over 1,600 are used in traditional medicare systems. Forests are estimated to harbour 90% of India's medicinal plants diversity. Only about 10% of the known

medicinal plants of India are restricted to non-forest habitats. But according to Hamilton (2003), 44% of total flora in India is used medicinally.

This rich diversity of medicinal and aromatic plants in the country have resulted from the great range of ecological habitats because of the immense variety of the climatic, edaphic and altitudinal variations. Around 70% of India's medicinal and aromatic plants are found in the tropical forests across the Western and the Eastern Ghats, the Vindhyas, Chotta Nagpur plateau, Aravallis, the Terai region in the foothills of the Himalayas and the North-East region of the country whereas less than 30% of the medicinal and aromatic plants are found in the temperate forests and higher altitudes.

The North Eastern part of the country has been recognized as one of the mega biodiversity centers of the world. The area is lying between 22° - 30° N latitude and 89° - 97° E longitude, and sprawling over 2,62,379 sq km, represents the transition zone between Indian, Indo-Malayan and Indo-Chinese biogeographic regions and a meeting place of the Himalayan Mountains and Peninsular India. It was the part of the northward migrating 'Deccan Peninsula' that first touched Asian landmass after the break up of Gondwanaland in the early Tertiary Period. North East India is thus the geographical 'gateway' for much of India's flora and fauna, and as a consequence, the region is one of the richest in biological values. It is in this lowland-highland transition zone that the highest diversity of biomass or ecological communities is found, and species diversities within these communities are also extremely high. The region possesses more than 2,000 medicinal and aromatic plant species accounting for about 20% of the total plant diversity of the region. According to the recent 'Biodiversity Assessment in the North Bank Landscape' report, the North-East India has the second richest forest reserve in the world

in terms of plant diversity (WWF report, 2005). The area surveyed by the WWF is called the North Bank Landscape, spanning 3,000 sq km of the Himalayan foothills, north of the Brahmaputra river in Assam and parts of Arunachal Pradesh, North Bengal and Bhutan. The report has pointed out that about 107 plant species are grown within an area of 200 sq m, which is second only to the number of species recorded in Sumatra and Indonesia. The richness of forests of the North Bank Landscape is higher than similar lowland forests in other Bio-diversity hotspots like Brazil, Cameroon, New Guinea and Peru.

In India, since the Vedic era *ca.* 5,000 years back, the use of medicinal plants and herbal medicines for alleviating ailments and promotion of healthcare has been reported. However, over the ages, the importance of most of these plants has declined due to the popularity of allopathic medicines developed in the western world. As research activity on the isolation of active principles from important medicinal plants was being carried out, synthetic drugs were made available with the advent of the synthetic Organic Chemistry since 1940s and 50s. These became popular for the treatment of diseases. It was thought, at one point of time that medicinal plants have lost much of their importance. However, this was found to be not true. A survey conducted in the United States of America during 1959 and 1979 revealed that 41% of the total prescriptions contained one or more products of natural origin as therapeutic agents. Out of these prescriptions, 25% were from plants, 13% from microbes and 3% from animal sources. The balance 59% represented synthetic drugs (Cragg *et al.*, 1997 and Dev, 1997). In the recent years, almost all the countries of the world have been giving importance to safer medicines having lesser side affects. Therefore, herbal medicines of plant origin are getting more and more attention.

Demand for medicinal and aromatic plants is increasing in both developing and developed countries due to growing recognition of natural products, being non-narcotic, having no side effects, easily available at affordable prices and sometimes the only source of health care available to the poor. In the developed world, the interest in plant medicines has dramatically surged up to 60% since 1989. As such, medicinal plants play an important role in the modern economy and throughout the world herbal medicines, phytochemicals, nutraceuticals, cosmetics and other herbal products have become a major sector of trade and commerce. It is estimated that the total number of medicinal and aromatic plants in the international trade is around 2,500 species (Schippmann *et al.*, 2002). A recent study indicates that the herbal drug market continues to grow at the rate of 15% annually. As per the estimate of the WHO, the global market of medicinal herbs and herbal products is about USD 62 billion and will hit the market by the year 2050 at the level of USD 5 trillion. The socio-economic development of some countries like Kenya and Brazil is significantly dependent on the trade of medicinal plants. In India, medicinal plant sector has traditionally occupied an important position in the socio-cultural, spiritual and medicinal arena of rural and tribal people. At present, India is exporting herbal materials and medicines to the tune of USD 1.4 billion. According to the data compiled by the International Trade Centre, Geneva; India ranks second amongst the exporting countries, after China. Besides meeting its domestic demand, China earns USD 5 billion per year from herbal trade. It was estimated that India and China could earn USD 14 billion by exporting herbs and herbal products by the year 2005 besides generating employment to 1 crore people (Tiwari, 2000).

The increasing demand and expanding trade on medicinal and aromatic plants worldwide have serious implications on the survival of several plant species due to indiscriminate harvesting of natural flora including those in forests (de Silva, 1997). Consequently, many species become extinct and some are endangered. Forest degradation throughout the tropical world has diminished the availability of widely used medicinal and aromatic plant species. Since the beginning of this century, more than half of the world's tropical forest area has been destroyed. It is estimated that worldwide about 24 ha of the rain forest disappear every minute. During the past 15 years, there has been a substantial loss of habitats, notably tropical forest, which are disappearing at a rate of about 1% per year (FAO, 2003), wetlands and other types of biome as a result of human action. According to an estimate prepared by the Threatened Plants Committee of the "International Union for Conservation of Nature and Natural Resources (IUCN)" about 10% (20,000-30,000) of the wild flowering plants are under threat. Indiscriminate felling of five of the top 12 medicinal trees in the Eastern Amazon region of Brazil for timber purpose has reduced the availability of barks and oils used otherwise for medicinal purposes (Shanley and Luz, 2003). Many other valuable species are also becoming endangered due to over-exploitation. Some examples include '*yohimbe*' (*Pausinystalia johimbe*, bark is used to treat male impotence) in Central Africa (Sunderland *et al.*, 1999), '*goldenseal*' (*Hydrastis canadensis*) collected from hardwood forest in the Eastern part of North America (Hill and Buck, 2000) and '*African Cherry*' (*Prunus africana*, bark used to treat prostatitis) in Cameroon and Madagascar (Cunningham *et al.*, 2002). A survey in the Shinyanga region in the North Western Tanzania indicated that the top 10 priority medicinal species harvested from natural woodlands have become scarce and are near

extinction (Dery *et al.,* 1999). Over-exploitation of these species is leading to unsustainable depletion of natural resources and narrowing of their genetic base.

The situation is even more critical and serious in the Indian context. As the trade of herbs and herbal products has increased by many folds, the Indian herbal industry has touched the annual turnover of more than USD 4000 million. To meet this growing demand, medicinal plants are being harvested every year from some of 1,65,000 ha of forests (Foundation for Revitalization of Local Health Traditions). It has estimated that about 90% collection of medicinal plants is from wild source and since 70% of collections involve destructive harvesting, many plants have become vulnerable or endangered or in the verge of extinction (Anon., 1982). Already 16 medicinal plants including high valued *Atropa acuminata, Dioscorea deltoidea* and *Rauvolfia serpentina* are listed as endangered species in the North Western Himalayas (Gupta, 1986). But, it is only recently that the importance has been focused on collection, conservation, evaluation and characterization of medicinal and aromatic plants along with their valuable constituents. To look into the use and commercial aspects of medicinal plants, the Government of India has formed the Medicinal Plants Board on November 9th, 2000 under the Ministry of Health and Family Welfare. So far, the Board has enlisted 32 medicinal plants for cultivation, characterization, improvement and conservation. These plants are *Amla, Ashok, Ashwagandha, Atees, Bael, Bhumi amlaki, Brahmi, chandan, Chirata, Daruhaldi, Giloe, Gudmar, Guggal, Isapgol, Jatamansi, Kalihari, Kalmegh, Kesar, Kokum, Kuth, Kutki, Makoy, Mukthi, Patterchur, Pippli, Safed musli, Sarpagandha, Seena, Satavari, Tulsi, Vai-vidang* and *Vatsnabh.* As of now, out of 400 industrially useful medicinal species, only 20 are cultivated on commercial basis.

According to a report prepared by R K Hightech Centre (2002), there is an annual business of Rs. 54 crores on 157 wild or cultivated medicinal plants in the state of Assam, the major part of which goes out of the state. It is estimated that 1,836 tones of whole plant, leaves 1,040 t, bark 972 t, fruits 1,728 t, roots 2,700 t, root-like branches 1,080 t, seeds 540 t, thorns 324 t and others 540 t were transported out in the year 2001. As a whole from the entire North Eastern region, annually 35,575 tones of raw medicinal plants and their various parts go to other parts of the country as well as abroad and its value is worth Rs. 165 crores. Because of this exhaustive exploitation, the flora of the North East India in general and medicinal and aromatic plants in particular are facing the threat of existence; many of which are already in the verge of extinction even without having a scientific glance. There are several causes for the dwindling resources of medicinal, aromatic and other economic plants in the region:

1. Shifting cultivation on the vast areas of forestland, which steadily deplete natural resources.
2. Rapid deforestation gives rise to secondary forests, bushy hillocks, grassy or barren hillocks and ultimately leads to soil erosion, landslide and change of climate.
3. Unwise and unsystematic deforestation on a rampant scale for the purpose of timber and firewood by the contractors, forest personnel and local people.
4. Tourists, picnic parties and pilgrims to various wild habitats cause considerable damage to the forest flora of the region.
5. Unsystematic, injudicious and unscrupulous collection of medicinal plants and their parts by the agents of profit oriented business concerns.

6. Lack of systematic cultivation of most of the medicinal and aromatic plants.

In the North Eastern region as a whole and Assam in particular, there is a serious deficiency in conservation, establishment of natural reserves and to locate and protect the plant species of potential medicinal and aromatic use including those unknown and unexploited ones. Medicinal plants of the region so far have not been catalogued. A few books were written on locally available medicinal plants in Assamese language or other local languages, but these books fail to describe phenological and genetic characters, chemical constituents and their pharmacological activities. No systematic effort has so far been made to identify, characterize and conserve the medicinal plant diversity of the region. Confusion has prevailed and has led to the situation where the same plant might be known by different names, or where widely differing species share the same names. This has serious implications. Physicians and manufacturers of Assamese traditional and Ayurvedic medicines have, therefore, had a critical interest in obtaining authentic descriptions of medicinal plants in currently valid taxonomic terms. It is thus necessary to document the local common names, use of specific parts having medicinal value and accurate botanical illustrations. As such a task would be necessary to combine *bej* (local medicine man), systematic botanist, biochemist, biotechnologist, physiologist and experts in modern structural elucidation techniques. However, so far, attempts in this direction are few and incomplete. Thus, there is ample scope to take up a major and systematic research on the medicinal and aromatic plants of the region. So is the case for other economically important plants of the region.

Based on the above facts, one very important but neglected plant species of the region was selected for the present study, which might have tremendous economic

significance in days to come. Medicinal use of this plant has been confirmed through extensive local literature survey and discussion with traditional medicine men of the state. As described earlier, the plant has not been studied systematically at all.

The plant is locally known as *Karphul* or *Ka-phul*, a wild perennial aromatic and medicinal herb under the family Zingiberaceae. It grows wild in fertile sandy loam to clay loam soils of Central and Upper Assam and also in some other parts of the North Eastern region. It possesses long pseudostem and underground creeping rhizomes. Leaves are petiolated, oblong lanceolated, glabrous with prominent mid rib and acuminate apex. The whole plant is aromatic having anise like flavour but the rhizome is more flavoured as compared to other parts of the plant. In Assam, the herb is mostly used for its aromatic and medicinal properties. Local people use its rhizome against various illnesses like stomach upset (ground rhizome paste is mixed in lime water and then consumed); rheumatism (rhizome paste used locally); respiratory complains, bronchial catarrh; cooked with rice to act as a tonic for faster post partum recovery of mothers and also consumed with betel nut and leaf as masticatory for its aromatic and refreshing action. The rhizome is also used as a flavouring agent in many food items.

This plant is endemic to the North Eastern region of the country. Though local people have been using it from time immemorial, the basic scientific information *viz.*, taxonomic characterization *i.e.*, suitable Botanical name; karyotypic information including chromosome number and ploidy level; compounds responsible for flavour and other active principles along with molecular characterization of the plant are lacking. The information regarding habitat, climate, soil type and related parameters required for the growth of the plant are also lacking.

The study of morphological and floral characters would provide valuable information about the plant, its sexual behaviour, which could be of great help in taxonomic characterization and planning breeding programmes for future improvement in desired directions of the plant as well. Likewise, the karyotypic study generates information on ploidy level, chromosome number, centromere position of individual chromosome etc, which would help to understand the evolution pattern, reproduction behaviour and also to identify the species from other related or alien species. As the plant has been in use from time immemorial for medicinal and aromatic purposes, the investigation for active principles might lead to discovery of new medicinal formulations. Isolation and identification of flavoury components from the rhizome would give a new dimension towards the industrial application of the plant in food and medicinal sector as a flavouring agent. Study of the essential oils, which are volatile in nature, could play an important role in aromatherapy against certain aliments. By screening the bioactive potentiality of phytochemicals and essential oils against pathogenic bacteria and fungi would provide vital information in combating the situation arising out of development of resistant bacteria and fungi against common antibiotic and antifungal drugs.

Molecular study including isolation of pure genomic DNA, restriction digestion and genome size determination would provide the basis at molecular level for future improvement in desired direction by utilizing genetic engineering tools.

Moreover, the plant is already in the threatened state, as the natural population of the plant has been decreasing alarmingly due to unsustainable exploitation by man and at certain point of time it would become extinct forever from the region without getting scientific exposure (Gogoi, 2001). This would be an irreversible loss to the natural gene

pool in particular and mankind as a whole. The plant is propagated through rhizome fingers; on the other hand the rhizome is the economic product for medicinal and aromatic uses. The plant does not produce fruits and seeds. Flowers are sterile. Thus, due to large scale harvesting of rhizomes, natural population has dwindled to the present threatened state. Therefore, it is necessary to develop and standardize suitable micropropagation technique to protect and conserve the species from extinction. Further, it is necessary to standardize the package of practices for its cultivation scientifically in a large scale.

Objectives:

On the basis of the facts presented above, the investigation has been taken up with the following objectives:

1. Morphological and floral biology study along with taxonomic characterization of the plant and its tissue culture.
2. Determination of chromosome number, ploidy level and karyotype analysis.
3. Isolation of genomic DNA, purification, restriction digestion and genome size determination.
4. Isolation of essential oil profile and assessment of their antimicrobial activity.
5. Isolation, purification and structure elucidation of aromatic compounds of the plant.

CHAPTER II

REVIEW AND LITERATURE

2.1 General

Zingiberaceae is one of the most widely used plant families for medicinal and aromatic properties. Under the different genera of this family, there are few species, which are yet to get scientific exposure though they have been used for ethnomedicinal purposes for centuries. *Karphul* is one of such threatened plants belonging to the family Zingiberaceae. From the ethnomedicinal point, it is one of the valuable medicinal and aromatic plants of the region, but unfortunately no information regarding the plant's scientific name, morphological and floral characteristics along with karyotype, biochemical and molecular characteristics are available. The present investigation was initiated with a view to explore the plant with respect to its taxonomic, karyotype, biochemical and molecular characterization. Since, there are no reports on the plant species elsewhere, the work done on the related or allied species with respect to taxonomy, karyotype, biochemical, molecular as well as *in vitro* multiplication has been reviewed and reported below.

2.2 Taxonomy

The family Zingiberaceae contains 45 genera and over 750 species that are distributed in the tropical region, chiefly in Indo-Malaysia. In India, this family is represented by about 22 genera and 170 species occurring chiefly in Eastern Himalayas and Western Ghats. The best-known examples of the family are ginger (*Zingiber officinale*), turmeric (*Curcuma longa* L.) and cardamom (*Elettaria cardamomum*). *Etlingera* Giseke is another member of the family, which is known for its ornamental flowers. In India two species have been reported so far that too from the North Eastern region of the country.

Giseke established the genus Etlingera in 1972 with its only species *E. littorale* (Koenig) Giseke. It was wholly based on the description of *Amomum littorale* Koenig

(*Retz. Obs. Bot.* 3:52. 1783) as its type specimens collected from Thailand were not available to Giseke because they were lost at sea. Subsequently, three other genera (allied to *Amomum*) were described, these are: *Geanthus* Reinv. (Syll. Pl. Soc Ratisb. 2:5. 1825), *Achasma* Griff. (Not. Pl. Asiat. 3: 411-426. 1851) and *Nicolaia* Horan. (Prod. Monogr. Scit. 32, 1862). Benthum (1883) and Baker (1890) reduced *Etlingera* and the above three genera as a section of *Amomum.* Valeton (1914) reconstituted the genus *Geanthus* and excluded all the original species of Reinwart with the introduction of new character: the basal part of the labellum and filament are fused to form an internal tube. With the highlighting of the above as a main character, Burtt and Smith (1986) reduced the three genera (viz. *Geanthus, Achasma* and *Nicolaia*) under *Etlingera*, an earliest name for the composite genus. Later, Smith (1986) transferred 57 species to *Etlingera* from *Geanthus* (15 species), *Amomum* (12 spp.), *Achasma* (5 spp.), *Nicolaia* (6 spp.), *Alpinia* (3 spp.) and *Donacodes* (1 sp.). Presently *Etlingera* is known by about 70 species distributing from Himalayas and South West China through Myanmar, Thailand, Malaysia, Indonesia, North Guinea and North Queensland.

In India, the genus *Etlingera* is represented by two species viz., *E. linguiformis* (Roxb.) Smith and *E. loroglossa* (Gagnep.) Smith; occurring both in N.E. India. Prior to *Etlingera,* these species were treated under different genera such as *Amomum* by Baker (1890), *Hornstedtia* by Schumann (1904) and by Rao and Verma (1972 and 1975) and *Achasma* by Larsen (1981). Previous treatment reflected the controversy in generic assignment and also indicated to be inadequate characters for separating them at generic level. Prakash and Tripathi (1998) described the taxonomic delimitation of the genus *Etlingera* (as recircumscribed by Burtt and Smith, 1986) through the study of the above two species based on fresh and pickled collection from Meghalaya and Arunachal Pradesh. They reported that the genus *Etlingera* is closely allied to *Amomum* in having subterranean inflorescence and singly born flowers in the axils of primary bracts with

tubular bracteoles; but differ from it in having a distinct tube formed above the insertion of the petals by the union of the lower parts of the labellum and filament of stamen. In *Amomum*, inflorescence tends to elongate after flowering and lacks an involucral of sterile bracts while in *Etlingera* inflorescence is with sterile involucral bracts and does not elongate after flowering.

However, the circumscription of *Etlingera* as defined by Burtt and Smith (1986) is of a heterogenous nature. The only common constant character for delimiting the complex group is the presence of a tube formed by the union of the basal part of the labellum and filament of stamen, which is visible only after the dissection of the flower. But, there is great diversity in other characters. For instance, the inflorescence is long peduncle (up to 1.0 m tall) and held always well above the ground in several species (not in India), which were treated so far under *Nicolaia* Horan. Similarly, the labellum, in some species (referred so far as *Achasma*) is very long, tri-partite with broad folded base and elongated in central part with entire or bifid apex (as in Indian representatives), while in other species lip is short without distinct lobes and not expanded in central part.

Since, the genus *Etlingera* had not been described till 1998, an artificial key, upto date nomenclatural citations and brief descriptions were provided by before Prakash and Tripathi (1998) distinguish the genus from other allied genera.

2.3 Karyotype study

A karyotype is the complete set of chromosomes of a cell of any living organism. The chromosomes are arranged and displayed (often on a picture or drawing) in a standard format: in pairs, ordered by size. Karyotypes are examined in search for chromosomal aberrations, and may be used to determine other microscopically visible aspects of an individual's genotype. A karyotype is an organized profile of an individual species, in which, chromosomes are arranged and numbered by size from the largest to

the smallest. This arrangement helps scientists to quickly identify chromosomal alterations that may result into a genetic disorder. To make a karyotype, scientists take a picture of chromosomes, cut them out and match them up using size, banding pattern and centromere position as guides.

In general, chromosomal differences reflect in the variation of genetic content of an individual. Based on the karyotype concept most species exhibit a distinct and constant individuality of their somatic chromosomes. It is reported that closely related species have more similar chromosomes than the distinctly related species. According to Sharma (1976) the major variation from the comparison of related species was in absolute chromosome size, staining properties, chromosomal morphology, relative chromosome size and chromosome number including aneuploidy, euploidy and haploidy. She accounted six different criteria to make a comparison of karyotype of different species. There were variations in (i) absolute chromosome size (ii) position of centromere (iii) relative chromosome size (iv) basic number (v) number and position of satellite and (vi) the degree and distribution of heterochromatic region.

Before describing the nomenclature system of chromosomes, Levan *et al.* (1964) suggested the system of calculation of centromeric location. Their system was based on the following:

Difference 'd' = $l - s$,

or, arm ratio (r) = l/s,

or, centromeric index (I) = 100 x s/total chromosome length

where, l = long arm and

s = short arm

They recognized six locations of centromere, such as – median point (M), median region (m), submedian point (SM), subterminal point (St), terminal region (t) and

terminal point (T). Regarding nomenclature, they designated chromosomes as metacentric (M and m), submetacentric (sm), subtelocentric (st), acrocentric (t) and telocentric (T).

Centromeric location provides the basic role for the study of chromosome morphology. Various methods have been used to determine the centromeric locations and nomenclature of chromosomes. There were four primary types of chromosomes based on centromeric location – telocentric, acrocentric, submetacentric and metacentric. According to Stebbins (1971) these four categories were not sharply distinct, but grade imperceptible in to each other.

Using all possible usage of centromeric locations, Adhikary (1974) proposed a system based on arm ratios – R_1 (*s/l*) and R_2 (*l/s*). He recognized four fixed points – median (M), terminal (T), submedian (SM) and subterminal (ST) and four intermediate regions– nearly median (nm), nearly terminal (nt), nearly submedian (nsm) and nearly subterminal (nst). Based on arm ratios and centromeric indices, Abraham and Prasad (1983) proposed a new system regarding centromeric locations. They recognized four fixed points *viz.*, Median (M), sun-median (SM); six intermediate regions *viz.*, nearly median (nm), nearly submedian (nsm^+ and nsm^-), nearly subterminal (snt^+ and snt^-) and nearly terminal.

Verma and Nadkarni (1985) investigated three species of *Glycyrrhiza,* namely, *G. glabra, G. macdonica* and *G. uralensis*, all perennial herbs for chromosome number and karyotypic studies. The basic chromosome number in all the three species reported to have 2n = 16. The total length of haploid chromosome complement was 24.4 μ, 22.6 μ and 20.2 μ in *G. glabra, G. macdonica* and *G. uralensis,* respectively. The chromosome length reported to varies from 4.1 μ to 1.9 μ in *G. macdonica,* 3.7 μ to 1.9 μ in *G. glabra* and 3.5 μ to 1.4 μ in *G. uralensis.* All three species have shown chromosomes either with submedian centromere or with median centromere.

Ruas *et al.* (2001) in Brazil studied karyotypes of fourteen populations including eight species of the genus *Lobelia* using root tip mitotic metaphases. All populations were tetraploid with 2n = 28 chromosomes. The basic chromosome number x = 7 was confirmed for the genus. Karyotype analysis showed that chromosome size varied from 1.05 μm to 2.02 μm with predominance of M and SM chromosome types. The karyotypes were similar among themselves with small intra- and interspecific variations on the size of haploid sets, symmetry indexes and centromere position of some chromosome pairs. These results showed that karyotype of Brazilian lobelias of the subgenus *Tupa* were probably due to polyploidy associated with chromosomal rearrangements probably in small chromatin segments.

Tuna *et al.* (2001) from Turkey reported the development and evaluation a Giemsa C-banding procedure to use in the identification of individual brome grass chromosomes and to develop a kayotype for diploid *Bromus riparius* Rehm. (2n=14; PI 440215). All chromosomes had one or more C-bands, which were located mainly at telomeric regions. A group (I) of four pairs of chromosomes had telomeric bands on only one arm and could be differentiated. In this group, one pair had an interstitial band along with a telomeric band, one pair had a nucleolus organizer region (NOR) at the subtelomeric location on the short arm, and other two pairs could be distinguished by centromere location. The other group (II) of three pairs of chromosomes had telomeric bands on both arms. The unequivocal identification of specific chromosomes of group II was not possible in all cells because of their similarity and differential condensation of chromosomes. Chromosomes of both groups were either metacentric or submetacentric. The total length of individual chromosomes ranged from 5.58 to 6.87 μm and the arm ratios ranged from 1.02 to 1.5 μm. The homologous chromosomes were paired and assigned numbers I to VII in decreasing length. A karyotype was constructed by means of the C-band, mean chromosome length and arm ratio. The C-banding procedure in this

study could be used to develop karyotype for the other species of the genus *Bromus* and these C-banded karyotypes could be used to compare genomes within the genus.

Eksomtramage *et al.* (2002) investigated chromosome number of 22 species belonging to 10 genera of Zingiberaceae family distributed in Thailand and reported that the somatic chromosomes range from 20 to 48 showing diploidy and polyploidy. Ten of these species were firstly reported, i.e. *Alpinia purpurata* (Vielli) K. Schum. (2n = 48), *Boesenbergia* aff. *rotunda* (2n = 20), *Cornukaempferia aurantiflora* J. Mood and K. Larsen (2n = 46), *Curcuma* aff. *oligantha* Trimen (2n = 42), *C. rhabdota* Sirirugsa M.F. Newman (2n = 24), *Etlingera elatior* (Jack.) R.M. Smith (white form) (2n = 48), *E. hemisphaerica* (Bl.) R.M. Smith (2n = 48), *Hedychium gomezianum* Wall. (2n = 34), *H. longicornutum* Bak. (2n = 34) and *Zingiber* aff. *wrayi* (2n = 22).

Fregonezi *et al.* (2004) from Brazil worked with the Asteraceae, one of the largest families of flowering plants, which contains about 1,100 genera and 20,000 species and is well known for its extensive Karyotypic variation. In their study, conventional Feulgen staining, C-CMA_3 banding, and florescence *in situ* hybridization with a 45S rDNA probe were used to determine the chromosome number and the number and physical position of GC-rich heterochromatin and 45S rDNA sites in three Asteraceae weed species (*Crepis japonica, Galinsoga parviflora* and *Chaptalia nutans*). These three species exhibited karyotype differences in chromosome number and shape, as a common feature of Asteraceae. However, the 45S rDNA sites always occurred on the short chromosomal arms, associated with GC-heterochromatin. Even with these differences, it suggests that common features of plant karyotype are maintained.

2.4 Biochemical analysis

Plants were the earliest source of medicines, and until comparatively recent times, they remain mankind's chief method of healing. Even now, in the age of scientific and

technological marvels; drugs and cures, 'botanicals' or their synthetically-derived equivalents account for the majority of prescription and non-prescription medicines. The plant-based drugs are available throughout the world in one or the other form.

Living plant cells are abundantly equipped with the materials for building thousands of compounds having medicinal properties. During the growth of plants, a number of fundamental biological molecules are synthesized including proteins, fats, nucleic acids, carbohydrates (particularly cellulose) and lignins. These materials play vital role in either metabolism of plants (proteins, carbohydrates, fats, nucleic acids) or as structural materials (cellulose, lignin). In addition, many plants synthesize other classes of molecules, called secondary metabolites, including terpenes, acetogenines and alkaloids (basic, nitrogen-containing molecules) whose benefit to the vitality of the plant is less clear. This latter group of materials, whose structures have been intriguing to chemist, have special properties (odour, taste, physiological activity) and have attracted interest for centuries. Thus, certain of these natural products that can be separated from plant materials have served as valuable medicinal agents (e.g., quinine, morphine etc) as flavours or spices (menthol, oil of lemon, oil of clove) or as perfumes (oil of lavender, oil of geranium). Among these natural products, the neutral materials (many of the terpenes and some of the acetogenines) are commonly obtained by steam distillation of appropriate plant materials (e.g., leaves, roots, rhizomes etc.) to separate volatile, water-insoluble oils known as essential oils. These essential oils can also be extracted by maceration, expression, enfleurage and solvent extraction. Many of these essential oils are marketed as flavour or fragrances. Medicinal and aromatic plants possess aromatic compounds, most of which are essential oils, which are volatile in room temperature. These compounds are synthesized and stored in a special structure called gland which is located in different parts of plant such as leaves, flowers, fruits, seeds, barks, stems,

rhizomes and roots. However, from ancient times, these plants have been used as raw materials for cosmetics, pharmaceuticals, botanical pesticides etc.

Tropical and sub-tropical Asia are rich in the number of species of plants due to their varied and suitable ecological conditions. The earliest known records related to aromatic plant were from Asia where the crops are indigenous. Asian people since prehistoric era made use of aromatic plants in various traditional ways. In India, for example, old literatures mention numerous uses of essences obtained from plants in performing religious rites since prehistoric times. Sharma (1996) described India as the traditional home of oriental perfumes. According to Xiao (1996) in China, Emperor Shen Nung, who lived around 2800 BC, held regular spice commodity market and practicing what he preached, consumed large quantities of spices everyday to strengthen his health and prolong his life. Throughout the long history of almost 5,000 years, the Chinese have continued to put faith in spices for medicinal purposes and for preserving and flavouring foods. At present, Chinese people have made use of more than 400 species of aromatic plants, not only for their flavour and fragrance properties, but also as medicines. China now produces more than 120 natural essential oils for domestic consumption as well as for export markets.

Phenolic compounds like phenylpropanoids, which include phenylpropenes, are all aromatic compounds and contribute to the volatile flavours and odours of plants. The phenylpropenes are usually isolated in the essential oil fraction of plant tissues, together with volatile terpenes. They are lipid soluble, as distinct from most other phenolic compounds. Some structures are widespread, such as eugenol, the major principle of oil of clove. Others are restricted to a few families. Anethole occurs in anise and fennel (both Umbelliferae) and myristicin, first described as a principle of nutmeg, *Myristica fragrans*, Myristicaceae, is also found in a number of umbellifers. These compounds are detected, together with essential oils, in either extracts of plant tissues. They are easily separated on

silica gel plates in benzene, mixtures of benzene with chloroform (10%) or light petroleum (20%) or in *n*-hexane – chloroform (3:2).

As no report has been found in the past literature elsewhere on biochemical analysis of the present plant species, the works done on the related and other allied species have been reviewed.

Tiwari *et al.* (1999) while reviewing chemical, biocidal and pharmacological aspects of *Alpinia* species found that the family Zingiberaceae contains 45 genera and over 750 species that are distributed in the tropical region, chiefly Indo-Malaysia. In India, this family is represented by 17 genera and 112 species occurring chiefly in Eastern Himalayas and Western Ghats. The best-known examples of the species are ginger (*Zingiber officinalis),* turmeric (*Curcuma longa* L.) and cardamom (*Elettaria cardamomum*). The plants of the family Zingiberacea are widely used world over as food flavours, speices and for medicinal purposes since the ancient times. *Alpinia* species are used in the oriental part of medicines and food additives, spices and in indigenous system of medicines. In china, *Alpinia galanga, A. globosa, A. officinarum* and in Brazil *A. aromatica* are used as medicine. Altogether 17 species of *Alpinia* are reported in the Wealth of India. Various parts of *Alpinia* species are used in the India system of medicine, Ayurveda and as a foods flavour. It is also used for the treatment of some inflammatory conditions, as an anticancer and anti-calculi agent, as a digestive, spleen and liver tonic, and in dyspepsia, gastrological, seasickness and abdominal colic. Recent studies confirmed that *Alpinia* species possess significant anti-ulcer, anti-calculi and anti-inflammatory activities.

LeFevre (2000) from USA isolated *trans*-anethole from anise seed by stem distillation, purified by flash chromatography and elucidated its structure by one- and two-dimensional (1- and 2-D) NMR spectroscopy.

Tram Ngoc *et al.* (2002) isolated glycosidically bound compounds from the methanol extract of fresh rhizomes of smaller galanga (*Alpinia officinarum* Hance) in Japan. Nine glycosides (1-9) were finally obtained by reversed-phase HPLC and their structures were elucidated by MS and NMR analyses. They were the three known glycosides (1R,.3S, 4S)-trans-3-hydroxy-1,8-cineole β-D-glucopyranoside (1), benzyl β-D-glucopyranoside (3) and 1-O-β-D-glucopyranosyl-4-allylbenzene (chavicol β-D-glucopyranoside, 4); and the six novel glycosides, 3-methyl-but-2-en-1-yl β-D-glucopyranoside (2), 1-hydroxy-2-O-β-D-glucopyranosyl-4-allylbenzene (5), 1-O-β-D-glucopyranosyl-2-hydroxy-4-allyabenzene (demethyleugenol β-D-glucopyranocide, 6), 1-O-(6-O-α-L-rhamnopyranosyl-β-D-glucopyranosyl)-2-hydroxy-4-allyalbenzene (demeth yleugenol β-rutinoside, 7), 1-O-(6-O-α-L-rhamnopyranosyl-β-D-glucopyranosyl)-4-allylbenzene (chavicol β-rutinoside, 8), and 1,2-di-O-β-D-glucopyranosyl-4-allylbenzene (9). Compounds 2-9 were detected for the first time as constituents of galanga rhizomes. Tram Ngoc *et al.* (2003) isolated antioxidative compounds from the methanol extract of fresh rhizome of smaller galanga (*Alpinia officinarum* Hance) in Japan. Seven phenylpropanoids (1-7) were finally obtained by reversed-phase HPLC and their structures were elucidated by MS and NMR analyses. They comprised the two known compounds, (E)-p-coumaryl alcohol γ-O-methyl ether (1) and (E)-p-coumaryl alcohol (6) and the five noval compounds, stereoisomers of (4E)-1,5-bis(4-hydroxyphenyl)-1-methoxy-2-(methoxymethyl)-4-pentene (2a and 2b), stereoisomers of (4E)-1,5-bis(4-hydroxyphenyl)-1-ethoxy-2-(methoxymethyl)-4-pentene (3a and 3b), (4E)-1,5-bis(4-hydroxyphenyl)-1-[(2E)-3-(4-acetoxyphenyl)-2-propenoxy]-2-(methoxy-methyl)-4 pentene (4), (4E)-1,5-bis(4-hydroxyphenyl)-2-(hydroxymethyl)-4-pentene-1-ol (7). Compounds 1-7 were detected for the first time as constituents of galanga rhizomes and exhibited antioxydative activities against the auto-oxidation of methyl linolate in bulk phase.

Chane-Ming *at el.* (2003) isolated essential oils by steam distillation from the rhizomes, leaves and flowers of *Zingiber zerumbet* Smith from Reunion Island and identified sixty-nine constituents. The oils obtained from rhizomes were rich in zerumbone (37%), α-humulene (14.4%) and camphor (13.8%). The oils from leaves and flowers differed appreciably from that of rhizomes by the presence of large amount of (E)-nerolidol (21.4% and 34.9%), β-caryophyllene (6.9% and 10.2%) and linalool (7.7% and 17.1%), respectively. The leaf oils differed from the others by their high level of a- and S-pinenes (10.3% and 31.4%, respectively).

Rout *et al.* (2003) from India obtained essential oils by hydrodistillation from the seeds of green, freshly dried large cardomom (*Amomum subulatum* Roxb.) and were analyzed by GC and GC/MS. A total of 33 components were identified by mass spectra and relative retention indices. The major component of the oil was 1,8-cineole (81.5 – 86%).

Ahmed *et al.* (2004) extracted essential oil from the rhizome of *Hedychium cylindreicum* Ridl. and chemical composition was determined by GC retention indices (on two columns of different polarity) and GC/MS. forty-nine compounds were identified with monoterpenoids accounting for more than 88% of the oil. The major constituents of the oil were terpinen-4-ol (40.5%), sabinene (9.9%), p-cymene (8.5%), limonene (6.0%). [γ]-pinene (5.6%), [α]-terpinene (4.5%) and [α]-terpineol (2.2%).

Behura and Srivastava (2004) isolated essential oils from leaf of *Curcuma caesia* Roxb. and two cultivars of *C. longa* and were investigated by GC. The leaf oils could be characterized by the following major constituents *C. longa* 'Roma': terpinolene (87.8%); *C. longa* 'Kasturi': myrcene (48.8%) and terpinolene (10.1%); *C. caesia*: 1,8-cineole (27%) and camphor (16.8%).

Jantan *et al.* (2004) from Malaysia distilled the galangal oil from the leaves and seeds of *Alpinia galanga* (L.) Willed. And were examined by capillary GC and GC/MS.

the rhizome oil was rich in 1,8-cinole (40.5%). Other compounds that were found in appreciable amounts in the oil were the sesquiterpenoids, [β]-bosabolene (8.4%), (Z,E)-farnseol (3.8%), [β]-caryophyllene (3.0%) and (E)-[β]-farnesene (3.2%). The seed oil wax characterized by its richness in sesquiterpenoids with major ones being [β]-bisabolene (37.6%), (E)-[β]-farnesene (22.7%), (E,E)-fenchyl acetate (7.9%), (Z,E)-farnseol (3.9%) and [β]-caryophyllene (3.0%).

Pino *et al.* (2004) obtained essential oil from the rhizomes of *Zingiber officinale* Roscoe from Cuba and analyzed chemical composition by combined GC and GC/MS. the oil was characterized by the presence of ar-curcumene (22.1%), zingiberene (11.7%), [β]-bisabolene (11.2%) and cadina-1,4-diene (12.5%).

Arambewela *et al.* (2005) from Sri Lanka extracted essential oil from rhizomes, roots and leaves for *Alpinia calcarata* Rose, and analyzed for their chemical composition by capillary GC and GC/MS. They identified around eighteen compounds. The major compound in the rhizome and leaf oil was 1,8-ceneole (33.3% and 24.7%, respectively), whereas in the root oil it was α-fenchyl acetate (39.8%).

Garg *et al.* (2005) from India obtained essential oil by hydrodistillation of *Curcuma zedoaria* (Zingiberaceae) leaves and analyzed by GC and GC/MS. Twenty-three compounds were identified, accounting for 75% of the oil. The oil of *C. zedoaria* was made up mainly of mono- and sesquiterpenoids, monoterpene hydrocarbons (2.3%), oxygenated monoterpenes (8.4%), isoborneol (7%), dehydrocurdione (9%) and selina-4(15),7(11)-den-8-one (9.4%) were the major constitute of the leaf oil.

Kaul *et al.* (2005) isolated essential oils from different parts of *Alpinia calcarata* Rose., (family: Zingiberaceae) in India and were analyzed by capillary GC and GC/MS. the oil yields were: flower 0.06%, leaf sheath 0.03%, stem 0.05% and root 0.18%. Sixty-two compounds accounting for 92.3-98.3% of the oils were identified. The flower oil contained β-pinene (12.5%), 1,8-cinole (18.2%) and (E)-methyl cinnamate (12.3%) as the

major constituents. The important components of the leaf sheath oil were 1,8-cinole (23.2%) and humulene epoxide I (10.6%). The stem oil had β-pinene (11.2%) and 1,8-cinole (33.2%) as the major compounds. On the other hand, the root oil showed camphene (13.6%), 1,8-cinole (15.6%) and α-fenchyl acetate (37.6%) as the main constituents.

Mustafa *et al.* (2005) obtained the steam-distilled oil from the fresh rhizomes of *Curcuma anwda* Roxb. Contained 26 components, of which 24, comprising 96.1% of the oil, were completely identified. The oil contained 10 monoterpenes and 14 sesquiterpenes accounting for 12.2% and 83.9%, respectively. The predominant monoterpene identified was camphor (5.5%). The sesquiterpene fraction consisted mainly of hydrocarbons (*ca.* 83.6%) and the prominent hydrocarbons were (Z)-β-farnesene (21.9%). Guaia-6,9-diene (19.8%), α-guaiene (14.5%). The aromatic constituent contributing to the odor of the oil was thymol (4.9%).

Raina *et al.* (2005) from India isolated essential oils from *Curcuma longa* (Zingiberaceae) rhizome and leaves by hydrodistillation. The oils were analyzed by high resolution GC and GC/MS. Fifty-two constituents were identified from rhizome oil representing 98.6% of the oil. The major constituents of the oil were α-turmerone (44.1%), β-turmerone (18.5%) and ar-turmerone (5.4%). From the leaf oil, 61 compounds were identified constituting 99.8% of the oil and main constitutes were α-phellandrene (53.4%), terpinolene (11.5%) and 1,8-cinole (10.5%).

Zoghbi and Andrade (2005) reported from Amazon that the volatiles of *Etlingera elatior* and *Zingiber spectabilis* (Zingiberaceae), cultivated in the state of Pará were obtained by hydrodistillation and analyzed by GC and GC/MS. The major components identified in the oils of inflorescence and inflorescence axis of *E. elatior* were dodecanol (42.5%, 34.6%), dodecanal (14.5%, 21.5%) and α-pinene (22.2%, 6.3%), respectively.

The inflorescence oil of *Z. spectabilis* was rich in β-phellandrene (45.3%). α-pinene (13.4%) and β-pinene (11.0%).

2.5 Screening of antimicrobial activity

Higher plants have been exploited as a source of biologically active compounds since antiquity. In particular, the ability to inhibit the growth of spoilage and food poisoning bacteria, human, animal and plant pathogens and a number of filamentous fungi has been immense of importance to man over the centuries. Some of these plant antimicrobials also possess antioxidant properties is a welcome bonus in the quest to preserve the food reserves of the world. Natural antimicrobial compounds have been detected in a number of plant genera and have been an inseparable part of human civilization as many food materials like ginger, garlic, etc. are used as medicines. There are large epidemiological and experimental evidences pertaining to worldwide acute microbe-related diseases, which are main causes of infant death, particularly in malnourished communities of poor countries. As no report has been found in the literature regarding the antimicrobial activity of essential oils and other phytochemicals of the present plant species, the works done on the related and other species have been reviewed.

Ahmed *et al.* (1998) reported a total of 82 Indian medicinal plants traditionally used in ethnomedicines were subjected to preliminary antibacterial screening against several pathogenic and opportunistic microorganisms. Aqueous, hexane and alcoholic extracts of each plant were tested for their antibacterial activity using agar well diffusion method at sample concentration of 200 mg/ml. The result indicated that out of 82 plants, 56 exhibited antibacterial activity against one or more test pathogens. Interestingly, extracts of five plants showed strong and broad-spectrum activity as compared to the rest of 51 plant extracts, which demonstrated moderate activity. On the whole, the alcoholic

extracts showed grater activity than their corresponding aqueous and hexane extracts. Among various extracts, only alcoholic extracts of *Emblica officinalis, Terminalia chebula, T. belerica, Plumbago zeylanica* and *Holarrhena antidysentrica* were found to show potentially interesting activity against test bacteria. These active crude alcoholic extracts were also assayed for cellular toxicity to fresh sheep erythrocytes and found to have no cellular toxicity.

Perumal *et al.* (1998) reported a total of 34 plant species belonging to 18 different families, selected on the basis of folklore medicinal reports practiced by the tribal people of Western Ghats, India, were assayed for antibacterial activity against *Escherichia coli, Klebsiella aerogenes, Proteus vulgaris* and *Pseudomonas aerogenes* (gram-negative bacteria) at 1000 – 5000 ppm using disc diffusion method. Of these 16 plants showed activity; among them *Cassia fistula, Terminalia arjuna* and *Vitex negundo* showed significant antibacterial activity against the test bacteria.

Singh *et al.* (2002) evaluated *Curcuma longa* rhizome extracts for antibacterial activity against pathogenic strains for gram-positive (*Staphylococcus aureus, S. epidermidis*) and gram-negative (*Escherchia soli, Pseudomonas aeruginosa, Salmonella typhimurium*) bacteria. Essential oil was found to be the most effective and its activity was compared to the standard antibiotics gentamycin, ampicillin, doxycycline and erythromycin in these strains. Only the clinical isolate of *S. aureus* showed more sensitivity towards the essential oil fraction than the standard strain. The use of essential oil from turmeric as a potential antiseptic in prevention treatment of bacterial infections was suggested.

Somchit *et al.* (2003) tested crude ethanol and water extracts of leaves and bark from *Cassia alata in vitro* against fungi (*Aspergillus fumigeatus* and *Microspoum canis*), yeast (*Candida albicans*) and bacteria (*Staphylococcus aureus* and *Escharichia coli*). *C. albicans* showed concentration-dependent susceptibility towards both ethanol and water

extracts from the bark, but resistant towards the extract of leaves. The degree of susceptibility varied, the water extract from barks showed bigger inhibition zone than the ethanol extracts (12 – 16 mm and 10 – 14 mm diameter, respectively). The growth of *Aspergillus fumigatus* and *Microsporum canis* was not affected by all types of plant extracts. Results were comparable to standard antifungal drug Tioconazole (18 mm diameter) at the equivalent concentration. The antibacterial activity of *C. alata* extracts on *S. aureus* was detected with the leaf extracts only using water and ethanol. The water extracts exhibited higher antibacterial activity than the ethanol extract from leaves (inhibition zones of 11 – 14 mm and 9 – 11 mm diameter, respectively). *E. coli* showed resistance to all types of extracts.

Dusanka *et al.* (2005) worked on the oil of *Calamintha nepeta* (L.) Savi ssp nepata var Suhisodonda (Borb.) Hayek. Hydrodistilled extracts were analyzed by GC and GC/MS. Eleven constituents were identified. The main constituents in the oil were pulengone (75.5%), piperitenoneoxide (6.0%), menthone (5.3%) and menthol (4.3%). The antimicrobial activity of the oil was screened against *Aspergillus niger, Escharichia coli, Staphylococcus aureus, Salmonella enterilidis, Bacillus subtilis* and *Psedomonas aeruginosa.* All microorganisms were sensitive to the oil.

Soumaya *et al.* (2005) analyzed the essential oil of *Cyperus rotundus* tubers obtained through steam distillation by GC and GC/MS. A total of 33 compounds were identified. The oil was characterized by its high content of sesquiterpenes with cyperene (30.9%), being the major. The antibacterial activity of the extract prepared from the tuber of *C. rotendus* showed more important activity against gram positive bacteria specially *Staphylococcus aureus* than gram negative bacteria. The antimutagenic activity was tested by the 'SOS Chromotest' and the 'Ames' test. *C. rotendus* oil acted as an antimutagen against Aflatoxin B1 in both *Salmonella* strains (TA100 and TA 98) and *Escharichia coli* strain (PQ37) and against nifuroxazide in *E. coli strain* (PQ37), where

its mutageneicty was not expressed. The highest rates of AFB1 mutagenesis inhibition tested by Ames assay, ranged from about 82.56% for TA100 strain to 85.47% for TA98 strain at the same dose of 50 μg AFB1 per plate. Whereas, the mutagenic effect of nifuroxazide and AFB1 (50 μg/assay) were reduced by approximately 58.19% and 81.67%, respectively, when tested by the SOS Chromotest assay.

Bouzouita *et al.* (2005) analyzed the oil obtained from dried leaves of *Lavandula stoechas* L. by capillary GC and GC/MS. Fenechone (68.2%) and camphor (11.2%) were the main components of 28 identified molecules. This oil was tested for antimicrobial activity against six bacteria and two fungi. The results showed that this oil was active against all of the tested strains; *Staphylococcus aureus* was the more sensitive strain.

2.6 Molecular characterization

Molecular biology offers promising tools for the creation of novel crop varieties with improved nutritional value, resistance to herbicides, pests and diseases, pollutants and adverse climatic conditions. Using recombinant DNA technology, it is now possible to manipulate the level and composition of active pharmaceuticals and essential oils in medicinal and aromatic plants. However, in order to accomplish this task, it is first crucial to understand the biochemical pathways and the pattern of expression of genes responsible for the synthesis of specific natural products. Novel pathways can be introduced into target plants to confer them with novel traits, and in parallel, endogenous genes can be 'turned off' using proper DNA constructs. The way transgenic plants are obtained, and how the implementation of molecular biology methodologies has improved crops and plant products have been shown practically in different plant species. The emerging opportunities for the incorporation of genetic engineering into existing breeding

programmes for the full exploitation of the biosynthetic potential of aromatic and medicinal plants would be the new trend of research for the days to come.

2.6.1 Isolation of genomic DNA

DNA isolation from the plant tissues is the first and foremost step in molecular biology. Over the last decade, there has been an increased requirement for the isolation of pure genomic DNA that performs well in any downstream application. Such downstream applications include amplification of genes using polymerase chain reaction (PCR), genotyping of single nucleotide polymorphisms (SNPs), Southern hybridization, AFLP, RFLP, microsatellite analysis and Masscode™ technologies. With the expansion of genome analysis research, sample sources are becoming increasingly diverse. Each has its own difficulties associated with the isolation of pure genomic DNA. DNA quality is the single most important factor. Poor quality DNA can lead to sub-optimal results, and DNA that is impure or contaminated will not perform well in downstream applications. The choice of sample preparation method directly affects the results of the downstream applications.

As the medicinal and aromatic plants contain exceptionally high amounts of different polysaccharides, polyphenols and other secondary metabolites having medicinal properties as well as they bind firmly to nucleic acids during DNA isolation and interfere in subsequent reactions. According to Loomis (1974) due to the presence of various complex substances, it is unlikely that just one nucleic acid isolation method suitable for all plants could ever exist.

Lim *et al.* (1997) from Singapore developed a simple and reliable method for the extraction of DNA from orchid species and hybrids. The high quality of DNA obtained was suitable for the amplification via polymerase chain reaction (PCR) for producing random amplified polymorphic DNA (RAPD) markers. Sangwan *et al.* (1998) described

a protocol to obtain high molecular weight, restrictable and amplifiable genomic DNA from the antimalarial plant *Artemisia annua.* The method is a CTAB (cetyl-trimethylammonium bromide) procedure that includes a rapid micro-column chromatography through DE-52 ion-exchange resin. The template should be pure enough for PCR and RFLP-based applications. Restriction of the purified DNA with *Dra* I and *Hind* III resulted in discrete bands that may have arisen from repeat sequences equally spaced between the restriction site(s). This approach might be useful for direct identification of repeat sequences as putative species-specific probes. Khanuja *et al.* (1999) from India reported that the presence of certain metabolites have been observed to interfere with DNA isolation procedures and downstream reactions such as DNA restriction, amplification and cloning. The chemotypic heterogeneity among species may permit optimal DNA yields with a single protocol, and thus, even closely related species may require different isolation protocols. They described the essential steps of a rapid DNA isolation protocol that can be used for diverse medicinal and aromatic plants, which produce essential oils and secondary metabolites such as alkaloids, flavonoids, phenols, gummy polysaccharides, terpenes and quinines. The procedure is applicable to dry as well as fresh plant tissues. This protocol permitted isolation of DNA from tissue of diverse plant species and produced fairly good yields. The isolated DNA proved amenable to PCR amplification and restriction digestion. Pirtilä *et al.* (2001) from Finland described that several protocols already developed for plant DNA isolation fail to produce good quality DNA from medicinal herbs and aromatic plants. These plants contain exceptionally high amounts of secondary metabolites that interfere with the DNA isolation. To address this problem, they developed two DNA isolation methods for Sundew and Tarragon that produce DNA suitable for molecular biological applications. One of the methods also is applicable for Milfoil and Siberian ginseng. Saritnum and Sruamsiri (2003) from Thailand worked on thirty-seven Galanga (*Alpinia* spp.)

accessions, 31 cultivated and 6 wild landraces from different areas in Thailand and evaluated for genetic diversity, using random amplified polymorphic DNA (RAPD) primers. Out of 22 random primers used in the study, eight primers (OPA20, OPB18, OPC09, OPD02, OPD11, OPG13, OPK12 and OPAX17) produced a total of 73 polymophic bands. The UPGMA cluster analysis of genetic similarity estimates (Jaccard's coefficient) separated the accessions in to 5 major clusters. The dendogram showed no relation with their morphological characters such as type, colour of rhizome and collection sites, which were indicated by the regions of Thailand. However, this study illustrated that RAPD analysis could be useful to evaluate genetic diversity in Galanga accessions. The highly informative primers identified in this study could be available for further genetic analysis of Galanga for plant selection and improvement. Sharma *et al.* (2003) isolated DNA from leaves of ten plant species (*Cuminum cyminum, Vigna aconitifolia, Pennisetum typhoides, Tecoma stans, Lycium barbarum, Anogeissus acuminata, Tecomella undulata, Zizyphus mauritiana, Phoenix dactylifera* and *Eruca sativa*) and a fungus (*Fusarium oxyspoyum*) using the CTAB method. Three fixing solutions (alcohol, alcohol and chloroform, alcohol and EDTA) were used to produce high molecular weight DNA (>40 kb). DNA quality and quantity was comparable for 3 fixing solutions and liquid nitrogen grinding. Addition of chloroform or EDTA to fixing solutions offered no advantage over absolute alcohol. Isolated DNA was suitable for RAPD analysis, restriction digestion and cloning. The method does not require liquid nitrogen for fixation, grinding or storage at -80 C, making it advantageous over other common protocols.

2.6.2 Genome size determination

Hinegardner (1976) described genome size as an important biodiversity character with fundamental significance and wide range of modern biological uses. Cavalier-Smith

(1985) described DNA C-value in place of genome size. According to Singh (2003) genome size and DNA C-value are synonymous and the genome size of an organism is the amount of nuclear DNA [measured by weight or number of base pairs; where, 1 picogram (pg) =978 megabases (Mb)] in its unreplicated gametic nucleus, irrespective of the ploidy level of the taxon.

According to Bennett *et al.* (1982), Bennett and Smith (1991), Bennett and Leitch (1995, 2005), Bennet *et al.* (2000) and Zonneveld *et al.* (2005) the genome size of only about 1.7% of the global angiosperm flora has been investigated. Bennett and Smith (1976) and Bennett and Leitch (1997) reported that the genome size varies among the species at least up to about 800-fold (*Fritillaria assyriaca*, 1C = 127.4 pg *vs. Arabidopsis thaliana* 1C = 0.165 pg). The genome size varies greatly between species. But the existence and extent of intraspecific variation in genome size is a particularly difficult and controversial field and is currently receiving much attention. Nevertheless, remarkable intraspecific variations have been reported many times from the late sixtities to the present day by Miksche (1968), Laurie and Bennett (1985), Rayburn *et al.* (1985, 1997) and Graham *et al.* (1994). In contrast to interspecific comparisons when genome size differences are not unexpected, technical variation becomes a much more obvious problem when analyzing intraspecific variation. Clearly, rapid but at the same time reliable methods for genome size estimation are needed.

It has been possible to estimate the amount of DNA in plant nucleus for over 50 years, and since the key role of DNA in biology was discovered in 1953. Several methods have been used to measure the plant genome size or DNA C-value. At present, two most widely used methods are Feulgen densitometry (Fe) and Flow cytometry (FC). But the above-mentioned techniques need sophisticated instruments, which is not possible always in all situations. Moreover, FC easily yields poor data in unskilled hands and by itself does not provide the cytological view of test material(s) that is essential to count

chromosome number(s). More recently, computer-based image analysis (CIA) system, which can estimate DNA amounts using Feulgen-stained cytological preparations in place of a microdensitometer. Although proprietary hard-wired CIA systems have been available since the 1970s (e.g. Zeiss Qusntimet system), they cost much more than microdensitometers, and analysis of the literature shows they have not been used to estimate plant C-DNA values. Therefore, we tried to develop an easy, less expensive, accurate and rapid method for determining the genome size by measuring the DNA content of a single set of chromosomes (gametic chromosomes) in a somatic cell nucleus. The technique of estimating nuclear DNA content of a nucleus using this method is expected to be innovative, unique and first of its kind.

2.7 Tissue culture

Tissue culture is useful for clonal propagation of medicinal and aromatic plants. According to Akerele (1991) and Flores and Medina-Bolivar (1995) propagation and conservation of medicinal and aromatic plants through *in vitro* techniques has been widely accepted and become commercially viable. Plant tissue culture offers some unique advantages over traditional seed propagation by allowing direct multiplication of special phenotypic characters and thereby shortens the time for the introduction of new plants.

The plant *Karphul* doesn't produce seeds. It is generally multiplied through rhizome planting. But mature rhizome being the economic product, its multiplication is a serious problem. As such the plant is becoming highly threatened. An effective tissue culture system could be a viable solution for establishing this plant in the region.

Borthakur *et al.* (1999) worked with emerging buds of rhizome of *Alpinia galanga* Willd, which produced shoots and roots simultaneously when cultured in MS (Murashige and Skoog, 1962) medium supplemented with kinetin 3.0 mg l^{-1}. Each explant-shoot bud produced 8 shoots on average and roots simultaneously within 8

weeks. Shoot proliferation could be continued even after a year by transferring each divided shoot explant to the same medium. Regenerated plantlets could be successfully transferred to soil where they grew well within 10-12 weeks with 80% survivality. Martin *et al.* (2002) reported callus, organogenesis and plant regeneration of *Alpinia calcarata* from rhizome explants in MS medium supplemented with various levels of different auxins and cytokinins either alone or in combination. Transfer of callus to MS medium, having 1.5 mg l^{-1} 6-benzylaminopurine (BAP) and 0.5 mg l^{-1} indol-3-acetic acid (IAA) produced 15-20 shoots per culture along with more than 20 meristematic protuberances within 40 days. Transfer of shoot primordia developed on MS + BAP (1.5 mg l^{-1}) and 2,4-dichlorophenoxy acetic acid (2,4-D) (0.25 mg l^{-1}) to a higher concentration of BAP (10.0 mg l^{-1}) and 2,4-D (0.1 mg l^{-1}) produced 18-25 shoots and 3-5 roots per culture within 40 days. The callus showed potentiality of regeneration along with callus proliferation and formation of meristematic protuberance even after 18 months. Shoots transferred to MS medium with 1.0 mg l^{-1} indole-3-buteric acid (IBA) developed 6-8 roots per shoot. However, shoots in MS full strength hormone free medium produced 3-5 shoots with 3-6 roots within 40 days; 80% of the regenerated plants survived in the field conditions.

Nadgaunda (1991) regenerated about 20,000 plantlets of turmeric (*Curcuma* spp.) within one year from single sprouting bud. Farira and Illg (1995) from Brazil reported bud multiplication of turmeric from rhizome axillary buds in MS medium by combining 10.0 μ M 6-benzyladenine (BA) with 5.0 μ M IAA. Shoot formation occurred when buds were transferred to MS/2 containing 10.0 μ M BA, on a rate of 15 to 20 new shoots every 30 days. Rooting was obtained when the shoots were placed in water or MS/2 with 5.0 μ M naphthalene acetic acid (NAA) or IAA. Shirgurkar *et al.* (2001) reported *in vitro* microrhizome production from turmeric (*Curcuma longa* Linn.). Freshly sprouted buds

with small rhizome portions excised from stored matured rhizomes were cultured on semi-solid culture medium – MS basal + 0.88 μ M BAP + 0.92 μ M kinetin + 5% coconut water + 2% sucrose + 0.5% agar – resulting in bud elongation. Multiple shoots were produced from these elongated buds by culturing in liquid shoot multiplication medium – MS basal medium + 2.2 μ M BAP + 0.92 μ M kinetin + 5% coconut water + 2% sucrose – at 25 ± 1°C and 16 h light (at 11.7 μ mol $m^{-2} s^{-1}$)/8-h dark cycles. Clumps of four to five multiple shoots/single shoot were used in various experiments. Cultures were incubated in the dark at 25 ± 1°C, half strength MS basal medium supplemented with 80 g l^{-1} sucrose was found to be optimal for microrhizome production. Cytokinin BAP had an inhibitory effect on microrhizome production. At the highest concentration of BAP (35.2 μ M), microrhizome production was totally inhibited. Microrhizome production depends on the size of the multiple shoot used. Microrhizomes produced were of a wide range in sizes (0.1-2.0 g) and, readily regenerated when isolated and cultured *in vitro* on culture initiation medium or shoot multiplication medium. Under *in vivo* conditions, small (0.1-0.4 g), medium (0.41-0.8 g) and big (>0.81 g) microrhizomes were regenerated. Plantlets developed from big microrhizomes grew faster.

CHAPTER III

MATERIALS AND METHODS

From time immemorial, *Karphul* has been used by the local people of the North Eastern region for its medicinal and aromatic properties. But up until now, the plant has remained to be more or less unknown to the scientific community. The present investigation is probably the first one to collect systematic information on this unique plant. Accordingly, a holistic approach has been planned to generate information on the plant for taking up future improvement and conservation works in the desired directions.

For the purpose, studies on morphological and floral biology for taxonomic characterization along with karyotype, biochemical and molecular assessment of the plant has been undertaken as per the materials and methods described below:

3.1 Morphology and Floral biology study for taxonomic characterization of *Karphul*

One population of the plant was located at the village Bhalukjharani, Tezpur, District Sonitpur and selected for the present investigation. The population of the plant covers an area of about 35 sq. m with plant density 10 - 12 plants/sq. m. Soil and environmental parameters of the areas are presented below:

Soil	: Fertile, sandy loam to clay loam
Organic carbon	: 0.8 – 0.9%
P_2O_5 (phosphorous)	: 15.0 – 18.0 kg/ha
K_2O (available potash)	: 370.0 – 376.0 kg/ha
pH	: 6.0
Temperature	: 25 – 34°C (summer), 10 -20°C (winter)
Annual rainfall	: 2,500 – 3,000 mm
Relative humidity	: 65 – 95%

From the population, five fully-grown (matured) plants were selected randomly for the investigation. The plants were under constant observation for 24 (twenty-four) months from October 2002 to September 2005 for studying their morphological characters and floral biology. For floral biology study, five spikes were marked randomly from the selected plants and were monitored regularly for measuring and recording the characters.

The *Karphul* plant was grown and maintained since October, 2002 in the Medicinal Plant Park of the Department Molecular Biology and Biotechnology, Tezpur University, Napam, Tezpur – 784 028, Assam, India.

Morphological characters of the selected plants and spikes were measured and recorded as per the procedure described bellow:

Morphological characters of the selected plants

Plant height – Height of the plant was measured in centimeter (cm) from the ground level to the base of the petiole of the top most leaf.

Stem circumference at the base – Circumference was measured in cm at the base of the stem near the ground level.

Stem circumference at 1m height – It was measured in cm at 1m height of the stem from the ground level.

Number of leaves per stem – The total number of green leaves present in a randomly selected stem.

Leaf length– It was the length of leaf blade in cm from the base to the tip of the leaf.

Leaf breadth– It was the width of a leaf at the middle position of the leaf blade in cm.

Ligule length – The length was measured in cm at the middle of the ligule.

Rhizome circumference – It was measured at the internode region of the rhizome in cm.

Characters of selected spikes

Spike length – It was measured in cm from the base of the peduncle to the tip of the labellum.

Number of florets per spike – The total number of flowers present in a single inflorescence or spike was counted.

Peduncle length – The length was measured in cm from the attachment point with rhizome to the top of the peduncle on which the florets are born.

Bract length – The length was measured in cm from the point of attachment with the peduncle to the tip of the bract.

Bract breadth – The width in the middle position of the bract was measured in cm.

Floret length – The length was measured in cm from the point of attachment with the peduncle to the tip of the floret.

Floral bract length – The length was measured in cm form the point of attachment with the ovary to the tip of the bract.

Floral bract breadth – The width in the middle position of the bract was measured in cm.

Bracteole tube length – The length of the tube was measured in cm from the point of attachment with the ovary to the portion of the floret it covers.

Calyx tube length – The length was measured in cm from the point of attachment with the ovary to the portion of the corolla it covers.

Corolla tube length - The length of the tube from the point of attachment with the ovary to the base of the labellum was measured in cm.

Petal length – The length was measured in cm from the point of attachment with the corolla tube to the tip of the petal.

Petal breadth – The width of the petal at the middle position was measured in cm.

Filament length – The length from the point of attachment with the inner wall of the corolla tube to the point of attachment with the anther was measured in cm.

Anther length – Anther length was measured in cm from the lower end to the top end of the anther.

Anther breadth – The width of the anther was measured in cm at the middle position.

Pollen grain diameter – The diameter was measured in μm under the compound microscope at 100x magnification.

Style length – The measurement between the ovary and the stigma was measured in cm.

Ovary length – The measurement was taken in cm between the point of attachment of the perianth and the style.

Ovary diameter – The diameter was measured in cm making a cross-section of the ovary.

In addition to the above-mentioned characters, the following qualitative characters like colour of the spike, colour of the bract and colour of the floret were recorded. The time of anthesis, peak anthesis hour, duration and time of the highest anthesis were recorded after examining 50 florets.

From the recorded data for each character mean, range of variation and standard error were calculated.

An extensive literature survey was carried out for taxonomic characterization of this plant. For distinguishing the genus from other allied genera, the key to *Etlingera* and allied genera (Tribe Alpineae) suggested as well as up-to-date nomenclatural citations provided by Prakash and Tripathi (1998) were followed. Comparison of taxonomic characters of the plant with the herbarium specimens of similar plants under the same genus reported from Assam and adjoining states and maintained in the BSI, Shillong and Kolkata as well as those in Royal Botanic Garden, Kew was made to ascertain identity of the plant. The field observations were also used for the speciation of the plant.

3.2 Karyotype study

Karyotype analysis is the principal means of detecting anomalies in living organisms caused by chromosomal irregularity due to missing/altered segments. Size of chromosomes measuring their length, type of chromosomes on the basis of centromeric position and their number represent karyotype of a plant. The illustrative presentation of chromosomes incorporating these information depicts karyotype of the plant. Karyotype study of the plant was carried out using the following steps and procedures:

3.2.1 Solutions used

I. Preparation of Aceto-Carmine stain (1 and 2 per cent)

One of the most widely used dyes for chromosome staining is Aceto-Carmine. It is prepared by mixing the carmine powder in acetic acid. Carmine powder is the powdered form of the dead and dried bodies of the female *Cochineal* insect (*Coccus*

cacti), a tropical American homoptera living on the plant *Opuntia coccinellifera.* The dye is crimson-coloured.

II. Materials required

Components	For 1% solution	For 2% solution
Carmine powder	1 g	2 g
Glacial acetic acid	45 ml	45 ml
Distilled water	55 ml	55 ml

III. Preparation procedure

1. In a 250 ml conical flask, a volume of 45 ml glacial acetic acid was added to 55 ml distilled water to prepare 100 ml of 45% acetic acid solution.
2. The mouth of the flask was loosely closed with cotton plug and heated the solution to boiling. Carmine powder (2 g for 2% and 1 g for 1% solution) was weighed and added to the boiling acetic acid solution slowly and stirred gently with a glass rod.
3. Gentle boiling was continued till the dye dissolved properly in the solution.
4. The solution was allowed to cool down to room temperature and then filtered using two layers of the Whatman (size 42) filter paper fitted in a glass funnel.
5. The filtrate was transferred in to dark bottles and stored at 4°C for subsequent uses.

IV. Pre-treatment of the tissue

For chromosomal study, pre-treatment of the plant tissue is necessary for several reasons, such as:

(a) Clearing the cytoplasm

(b) Separation of the middle lamella causing softening of the tissue

(c) Brining about scattering of chromosomes with clarification of constriction regions.

(d) Pre-treatment may also be needed to achieve rapid penetration of the fixative by removing undesirable deposits on the tissue as well as for the study of the spiral structure of chromosomes.

V. Pre-treatment procedure

1. An aqueous saturated solution of *p*-dichlorobenzene (*p*-DB) was used.
2. The *p*-DB solution was prepared by adding slowly *p*-DB crystals in to 100 ml distilled water taken in a 200 ml conical flask till the solution became saturated.

VI. Preparation of the fixative (Carnoy's fluid)

The purpose of fixation is to keep the cells in the stationary phase, so that they cannot progress further. Hence, the tissue is killed by using a suitable chemical, which does not allow further distortion of the cellular components to be studied, as far as practicable. The fixative solution also plays a preservative role of cellular compounds. For fixing the material, Carnoy's fluid was used. It was prepared by mixing one part of glacial acetic acid with three parts of absolute ethanol (Choudhury and Choudhury, 1989).

Glacial acetic acid	1 part
Absolute ethanol	3 parts

3.2.2 Materials for cytological study

Root tips of the plant were used to undertake the cytological investigation.

3.2.3 Collection of root tips, pre-treatment and fixation

1. Rhizomes of the plant with intact roots were dug out from soil using a hoe and cleaned very carefully giving attention not to damage the roots.
2. The rhizomes were washed under tap water.gently to remove the dirts.
3. Intact roots were excised out from the rhizome and washed thoroughly using distilled water in a beaker.
4. Actively growing healthy apical tips of the roots measuring about 1 to 2 cm in length were excised out in water using a sharp scalpel for chromosome study.
5. Root tips were collected in the morning over a period of 4 h from 4.15 am to 8.15 am at half-hourly interval. Timely collection of root tips was done to find out the proper time of cell division of the plant species.
6. The excess water adhering to the root tips was wiped out using a piece of blotting paper and then transferred into a conical flask containing the aqueous saturated pretreatment solution of *p*-DB and kept for 4 h at 12°C.
7. After pretreatment for 4 h, root tips were washed thoroughly with distilled water to remove the chemical.
8. The root tips were then transferred to the conical flask containing the Carnoy's fluid (fixative) and then kept at 12°C for 24 h.
9. After fixing for 24 h, root tips were taken out of the Carnoy's fluid and washed with distilled water.

10. The material was then stored in 70% ethyl alcohol at 12°C for subsequent studies.

3.2.4 Tissue hydrolysis and staining of chromosome

1. After the fixation, root tips were taken out from storage solution of 70% ethyl alcohol and washed thoroughly with distilled water.
2. Hydrolysis of root tips and simultaneous staining of chromosomes were done by transferring the root tips in to 5 - 8 ml of 2% aceto-carmine stain and then kept at room temperature for 4 h, followed by heating in a heating mantel at 60°C for 10 min.

3.2.5 Squashing and slide preparation

1. The root cap of the hydrolyzed-cum-stained tips was removed using a surgical blade and forceps.
2. The meristematic part of the root tip was excised and transferred in to a small drop of 1% aceto-carmine stain on a clean slide.
3. The meristematic tissue was then pressed by using needle and scalpel to convert into a thin layer of cell mass.
4. The cell mass was covered with a clean cover-slip and squashed carefully by applying uniform pressure on top of the cover-slip with the thumb by putting on top a piece of blotting paper.
5. Enough care was taken while putting the cover slip on the cell mass to get rid of the air bubble below the cover slip.
6. Finally all the sides of the cover slip were sealed with euperol to make it airtight.

Slide preparation was carried out following the 'Squash method' described by Sharma and Sharma (1980).

3.2.6 Viewing of slides

1. The semipermanent slides thus prepared were observed under a compound microscope (Leica ATC 2000 Model) using the low magnifiction of 10x X 10x.
2. Slides revealing distinct cells were viewed under the high power of 10x X 40x for tracking the nucleus and chromosomes.
3. Finally cells were viewed under the oil emersion with 10x X 100x for individual chromosome studies.
4. A total of 20 well-scattered and perfectly stained cells having the chromosomes in the metaphase plate were marked for karyomorphological analysis of the chromosomes following the standard method described by Levan *et al* (1964).
5. Microphotographs of the well-illustrated cells were taken by using a C-5060 Wide Zoom Digital Camera attached on the Olympus – Bx 41 Microscope at 1000X magnification.
6. Measurement of chromosomes was taken using an ocular micrometer.

Parameters recorded to describe karyomorphological traits are presented below:

Total number of chromosomes - Number of chromosomes was counted in twenty distinctly revealing random cells and the average was recorded.

Chromosome pairs - With the total count of chromosomes, the number of pairs (2n) was determined.

Length of long arm - It was measured as the length between the rear end of the long arm and the primary constriction of an individual chromosome and measured in μm.

Length of short arm - It was the length between the rear end of the short arm and the primary constriction of an individual chromosome and measured in μm.

The long and the short arms of an individual chromosome depend on the position of the primary constriction or centromere.

Total length of the individual chromosome - Individual chromosome length was measured in μm by adding the length of the long arm and the short arm.

Relative length of chromosome (percentage length of individual chromosome) - It was the ratio between the length of the individual chromosome and the total chromosome length of the haploid set expressed in percentage.

Total length of haploid chromosome complement - It was calculated by adding the length of all chromosomes in the haploid complement.

$$\text{Relative chromosome length} = \frac{\text{Length of the individual chromosome}}{\text{Total chromosome length of the haploid set}} \times 100$$

Chromosome arm ratio: The arm ratio 'R' of each chromosome was calculated as -

$$R = \frac{L}{S}$$

Where, L is the length of the long arm and S is the length of the short arm

Centromeric index (I) - The location of the centromere on the chromosome was expressed as the percentage of ratio between the short arm to the total

length of the chromosome and was calculated as the centromeric index or I (Levan, *et al.*, 1964).

$$\text{Thus, Centromeric index or I} = \frac{\text{Length of short arm}}{\text{Total length of the chromosome}} \times 100$$

Centromeric position - Based on the centromeric index, centromeric position of an individual chromosome was determined and the chromosomes were classified into two groups - metacentric (M) and submetacentric (SM) following the nomenclature system of Levan *et al.* (1964).

3.3 Biochemical analysis of *Karphul* rhizome

3.3.1. Essential oils

Aromatic plants are those possessing flavoury compounds, which are mostly essential oils being volatile in nature at the room temperature. These compounds are synthesized and stored in a special structure called as gland which is located in different parts of a plant such as leaves, flowers, fruits, seeds, barks, stems, rhizomes and roots. However, from the ancient times, these plants have been used as raw material for cosmetics, pharmaceuticals, botanical pesticides etc. Among the natural products, the neutral materials (many of the terpenes and some of the acetogenines) are commonly obtained by steam distillation of appropriate plant material, such as leaves, roots, rhizome etc to separate volatile and water-insoluble oils known as the essential oils. The essential oils can also be extracted by maceration, expression, enfleurage and solvent extraction. Many of these essential oils are marketed as flavours or fragrances.

I. Extraction of essential oils

(a) Plant material used

Rhizomes were collected from Bhalukjharani village, Tezpur, Assam during Nov-Dec, 2003 for extracting the essential oils. Voucher specimens were authenticated and then deposited in the Herbarium of the Department of Molecular Biology and Biotechnology. Herbarium samples were also deposited in the Botanical Survey of India, Shillong.

(b) Apparatus used

Clevenger apparatus was used for extracting the essential oils from the rhizomes.

(c) Hydro-distillation of rhizomes

Fresh rhizome of *Karphul* was washed thoroughly to remove the dirt and ground into paste in a mixer grinder. The essential oils were extracted by hydro-distillation (200 g fresh rhizome paste) for 4 h using a Clevenger apparatus, the supernatant was separated by decantation, dried over anhydrous $Na_2 SO_4$ overnight and kept in sterile glass bottle at 4°C for subsequent analysis.

(d) Gas Chromatography

The essential oils obtained by hydrodistillation were analyzed by Gas Chromatography. Constituents present in the oil were separated in 15% SE 52 packed column (2 mm id x 2 M glass) using multi ramp temperature programming mode from 800 – 2250. The detector (FID) signal output was integrated by the help of a Hewlett

Packard HP 3395 date integrator. The constituent components were identified with comparing with their respective authentic standard reference compound under identical conditions.

3.3.2. Aromatic compound

Phenolic compounds like phenylpropanoids, which include phenylpropenes are aromatic compounds and contribute to the volatile flavours and odours of plants. The phenylpropenes usually come out along with the isolated in the essential oil fraction of plant tissues, together with volatile terpenes. They are lipid soluble, and distinct from most of the other phenolic compounds. Some structures are widespread, such as eugenol, the major principle of oil of cloves. Others are restricted to a few families. Anethole is present in anise and fennel seeds (both Umbelliferae) and myristicin in nutmeg, *Myristica fragrans* (Myristicaceae), also found in a number of umbellifers.

I. Plant material used

Rhizomes of the plant were collected from the same site for the preparation of the crude extract for subsequent isolation of aromatic compounds.

II. Apparatus used for the extraction

Soxhlet apparatus was used for the extraction.

III. Solvent used

Dichloromethane (CH_2Cl_2) (Merck, Mumbai) was used as the solvent for the preparation of the crude extract.

IV. Extraction procedure

1. Rhizomes were washed thoroughly under the tap water to remove the dirt and drained off the water. The adhering moisture was allowed to dry off keeping the rhizomes in shade for 1 – 3 h.
2. The shade-dried rhizomes were ground in to a fine mixture using a mixer grinder (REMI, Auto-Mix-Blender).
3. The ground rhizome (80 g) was subjected to solvent extraction by treating with 250 ml of dichloromethane (CH_2Cl_2) taken in a soxhlet apparatus for 8 h.
4. Following 8 h extraction, the solvent was evaporated to dryness in a rotary evaporator.

V. Purification

a) Thin Layer Chromatography

1. The crude extract obtained after solvent evaporation was resuspended in dichloromethane in a clean vile and mixed properly to dissolve.
2. Samples in low concentrations were subjected to the thin layer chromatography (TLC) for the fractionation of the constituent compounds.
3. The TLC plates were prepared with silica (Silica gel G, Merck, Mumbai) in small plates (size: 5 X 20 cm). The solvent system used for the purpose was comprised of

n-hexane : dichloromethane (v/v) at the proportionate combinations of 1:5, 1:2, 1:1, 2:1, 5:1 and 6:0.

4. After the TLC, all plates were allowed to dry for 3-4 min (for evaporating the solvent) and placed in to a chamber full of iodine vapour to detect the spots.
5. The R_f (Retention Factor) value of the spots under standard conditions was determined.

b) Preparative TLC

1. Based on the TLC experiment, the preparative TLC was performed with the solvent system comprised of *n*-hexane : dichloromethane 5:1 (v/v) following the procedure described by LeFevre (2000) in big TLC plates (size: 20 X 20 cm).
2. Before loading, the sample was prepared by dissolving the crude at the rate of 300 mg in 5 ml of dichloromethane.
3. After the preparative TLC and subsequent detection of spots under the iodine vapour, four fractions were collected from the TLC plate by stripping out spots carefully.
4. Each fraction was eluted with dichloromethane separately using silica (60-120 mesh size; Sisco Research Laboratory Pvt. Ltd., Mumbai)-packed columns to elute the compound from the silica.
5. The solvent was evaporated to dryness on a rotary evaporator from each of four fractions separated. Out of these four factions, only one fraction was kept for subsequent analysis and the rest three were discarded due to poor yield.

VI. Identification of TLC purified fractions

The crude dichloromethane extract of *Karphul* rhizome fractionated through the TLC in to four fractions were collected separately. Out of these four fractions, only the major fraction (Fraction I) was subjected to further analysis (GC-MS, FTIR and ^{1}H-NMR) for the identification of the compound present in it, rest could not be analyzed due to their poor yield.

a) Gas Chromatography and Mass Spectroscopy (GC-MS)

1. The sample was analyzed on a Varian GC-MS 3800, Saturn 2000 system (USA).
2. An aliquot of 20 µl sample with the concentration of 1mg.ml^{-1} was injected in the split-less mode in to a Chrompack Capillary GC column CP-Sil 8 low bleed (30 m x 0.25 mm x 0.25 µm) coupled with a CP-Sil 5 CB low bleed/MS (30 m x 0.25 mm x 0.25 µm) column using helium as the carrier gas. The column temperature was 80 to 240°C and maintained for 30 min. The injector temperature was 240°C and the transfer line temperature was 300°C.
3. The mass spectrometric data were acquired in the FID mode.
4. The mass spectra of the unknown compound obtained were compared on the basis of the retention time of the authenticated compounds, using Saturn 2000 MS library where 99% matching was observed (Rana and Blazquez, 2003).

b) IR Spectroscopy

1. The IR spectrum of the isolated fraction was recorded using KBr pellets in a Nicolet Model Impact 410 FTIR spectrometer.

2. Samples were prepared by dispersing the solid uniformly in a matrix of dry (KBr) mull and compressed to form an almost transparent disc.
3. The spectra showing the functional groups were used to study the composition of the compound.
4. Absorption spectra were plotted using a built-in plotter. The IR spectra were collected using 500-4000 wave number (cm^{-1}).

c) ^{1}H-NMR

1. The isolated fraction was subjected to the ^{1}H-NMR spectroscopy.
2. The isolated fractions were recorded on a Varian 400 MHz NMR spectrometer (Model: Mercury Plus 400 NMR, Varian, USA) using tetramethylsilane (TMS) as the internal standard and $CDCl_3$ as the solvent.

3.4 Antimicrobial (antibiotic) bioassay

Higher plants have been exploited as a source of biologically active compounds since antiquity. In particular, the ability to inhibit the growth of spoilage and food poisoning bacteria, human and animal pathogens and a number of filamentous fungi has been of immense importance to man over the centuries (Zaika 1989, Deans and Svoboda, 1990a and Deans *et al.*, 1990). Some of these plant antimicrobials also possess antioxidant properties is a welcome bonus in the quest to preserve the food reserves of the world. Natural antimicrobial compounds have been detected in a number of plant genera.

3.4.1 Test pathogens

The essential oils were tested for their activity against six pathogens. Out of these six pathogens used, three were gram-positive bacteria – *Bacillus subtilis* (NCIM 2063), *Staphylococcus aureus* (MTCC 737) and *Micrococcus flavus* (collection from Downtown Hospital, Guwahati); two gram-negative bacteria – *Escherichia coli* (MTCC 739) and *Klebsiella* sp. (MTCC 109) and one was fungal pathogen *Candida albicans* (MTCC 3017). Likewise, four fractions of aromatic crude obtained after purification by the TLC were also tested for their activity against the same pathogens except *M. flavus*. All pathogens except *M. flavus* were obtained through the courtesy of the MTCC and the Gene Bank, IMTECH, Chandigarh, India. *M. flavus* was collected from Downtown Hospital, Guwahati, Assam, India.

3.4.2 Medium

The bacterial test pathogens were maintained on nutrient Agar (NA) medium. The composition of the medium is given below:

Composition of the Nutrient Agar Medium

Component	g/l
Peptone	10.0
Beef extract	10.0
Sodium chloride	5.0
Agar	12.0

pH = 7.3 ± 0.1

The fungal pathogen *C. albicans* was grown on M2 Agar Base medium. The composition of the M2 Agar Base Medium is given below:

Composition of the M2 Agar Base Medium

Component	g/l
Sodium chloride	10.0
Yeast extract	5.0
Monopotassium phosphate	0.1
Magnesium sulphate	0.05
Dextrose	10.0
Agar	15.0

pH = 7.1 ± 0.2

For antimicrobial susceptibility test, Mullar Hinton Agar (MHA) Medium was used. The composition of Mullar Hinton Agar Medium is given below:

Composition of the Mullar Hinton Agar Medium

Component	g/l
Beef infusion	300.0
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17.0

pH = 7.3 ± 0.1

The compositions of all the media used were procured from Hi-Media Private Limited, Mumbai, India.

3.4.3 Evaluation of antimicrobial activity

There are various tests for the evaluation of the potency of essential oils and other phytochemicals in inhibiting the growth of bacteria and fungi. For screening the efficacy of essential oils and other phytochemicals, 'agar well diffusion' method was used. The steps followed in agar well diffusion method are described below:

1. Spore suspensions required were prepared in Ringer's solution (sterile saline water) and adjusted to a final inoculum size of 3×10^5 cells/ml. One ml of the inoculum was added to 20 ml media at 45°C for bacterial and fungal strain. The suspension was prepared thoroughly and poured in to the sterile petriplates.
2. After solidification of media, wells of 6 mm in diameter were cut in the agar plates.
3. For screening essential oils, 250 µl of the oil was dissolved in 1 ml dimethyl sulfoxide (DMSO) and then 200 µl of it was loaded into each well on the respective petriplates having bacterial and fungal cultures. DMSO (200 µl) alone was loaded as the control into a different well of each petriplate to observe whether there was any activity of this chemical against the tested microorganism.
4. The fractions (4) fractionated by the TLC were also dissolved in DMSO at 5 mg/ml concentration and then loaded 200 µl of it in each wells along side the control as in the case of essential oil screening against the tested bacterial and fungal culture petriplates.

5. After loading the samples into respective wells, the petriplates were kept 60 min under the laminar hood to allow the test solution to diffuse in to the agar prior to their incubation.
6. The plates were incubated at 37°C in the dark for 24 h for bacterial and 48 h for fungal pathogens.
7. The inhibition zones following required incubation around the wells and their diameter were measured and recorded. The same experiments were repeated for three times for confirmation.
8. To test the significance of activity between the fractions with respect to their zone of inhibition against the individual pathogen, paired 't' test was calculated.

3.5 Molecular characterization

3.5.1 Genomic DNA isolation

Traditionally, members of the Zingiberaceae family have been used for their medicinal and aromatic properties. Also they have been used as spices and condiments; flavouring agent in food, confectionery and perfume industries. Hence, medicinal and aromatic plants are the subject of extensive genetic study worldwide. Isolation of clean, restrictable and amplifiable genomic DNA is a crucial step prior to the application of molecular marker technologies to these plants for achieving target-oriented goals. Currently, there are several methods of DNA isolation, available as variant of the principal protocols (Saghai-Maroof *et al.*, 1984; Dellaporta *et al.*, 1985; Doyle and Doyle, 1987; Webb and Knapp, 1990). Experience has shown that the plant protocols need to be tailored to each plant species due to the presence of secondary metabolites,

which vary in nature and/or composition. These phytochemicals might not only hinder the *su moto* application of the other methods, but also interfere with the subsequent amplification and/or restriction-digestion of the isolated DNA. Therefore, it is necessary to standardize the DNA isolation protocol for each plant species.

3.5.2 The plant material

For the isolation of genomic DNA of *Karphul* plant, tender leaves were collected from the plant in the morning, washed thoroughly with the distilled water. The excess water adhering to the leaves was soaked off using tissue paper. The leaves were then packed in polythene bags and stored in darkness at room temperature for the subsequent DNA extraction work.

3.5.3 Equipment and implements

Autoclave	Sorval
Mortar and pestel	Tabletop centrifuge
Mettler Electronic balance	Magnetic stirrer
Microscope	Ultra violet (UV) light box
Micropipettes – (2-20 µl,	Waterbath
20-200 µl and 200-1000 µl)	-20°C Deep freezer,
Refrigerator	Freeze-drier
Polypropelene tube	Gel Doc system
Oven	Incubator (37°C)
Vertical Gel Apparatus	UV Spectrophotometer

3.5.4 Reagents, chemicals and solutions

1.0 M Tris-HCl (pH 8.0) – 121.1 g of Tris base was dissolved in 800 ml of dH_2O. The pH was adjusted to 8.0 by adding concentrated HCl. The solution was allowed to cool to room temperature. The volume was adjusted to 1 liter and sterilized by autoclaving. The solution was stored at room temperature.

0.5 M EDTA (pH 8.0) – 186.1 g of $Na_2EDTA.2H_2O$ was dissolved in 700 ml of dH_2O. The pH was adjusted to 8.0 with 10 M NaOH (~ 50 ml). The volume was adjusted to 1 litre and sterilized by autoclaving. The solution was stored at the room temperature.

5.0 M NaCl - 292 g of NaCl was added to 900 ml of dH_2O. the volume was adjusted to 1.0 litre.

CTAB (20%)

Chloroform: Isoamyl alcohol (24:1 v/v) mixture

Polyvinylpyrolidone (PVP)

B-mercaptoethanol

Liquid Nitrogen

Loading dye (Bromophenol blue)

Ethidium bromide

Isopropanol

RNase

Agarose

Hind III digested λ DNA molecular weight marker

3.5.5 CTAB extraction buffer

For 27.0 ml CTAB extraction buffer

100 mM Tris-HCl (pH 8.0)	– 2.7 ml of 1 M Tris-HCl (pH 8.0)
25 mM EDTA	- 1.35 ml of 0.5 M EDTA (pH 8.0)
1.5 M NaCl	- 8.1 ml of 5.0 M NaCl
2.5% CTAB	- 3.375 ml of 20% CTAB
0.2% B-mercaptoethanol (v/v)	- 54 μl added immediately before use
PVP	– 300 mg of PVP (100 mg/g leaf sample) added immediately before use

The volume was adjusted to 27.0 ml by adding sterile dH_2O and stored at the room temperature.

3.5.6 High salt TE buffer

For 100 ml High salt TE buffer

1 M NaCl	- 20.0 ml of 5 M NaCl
10 mM Tris-HCl (pH 8.0)	- 1.0 ml of 1M Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)	- 200 μl of 0.5 M EDTA (pH 8.0)

The volume was adjusted to 100 ml by adding sterile dH_2O and stored at the room temperature.

3.5.7 50 X TAE electrophoresis buffer (Tris Acetate EDTA)

For 100 ml 50 X TAE stock solution

0.5 M EDTA (pH 8.0)	- 3.7 g $Na_2EDTA.2H_2O$

Tris base	- 24.2 g Tris base
Glacial acetic acid	- 5.71 ml of glacial acetic acid

The volume was adjusted to 100 ml by adding sterile dH_2O and stored at the room temperature.

3.5.8 Loading dye (Bromophenol blue)

6 X Loading dye (for 4.0 ml):

Bromophenol blue	- 10 mg of Bromophenol blue
Xylene cyanol	- 10 mg of Xylene cyanol
Glycerol	- 1.2 ml Glycerol (autoclaved)

The volume was adjusted to 4 ml by adding sterile dH_2O and stored at 4^0C.

3.5.9 Ethidium bromide (10 mg/ml)

Ethidium bromide 100 mg was dissolved in 10 ml sterile dH_2O, mixed properly and then stored at 4^0C in darkness.

3.5.10 0.8% agarose gel

For 400 ml stock solution of 1 x TAE

1 x TAE	- 8.0 ml of 50 X TAE buffer

The volume was adjusted to 400 ml by adding 392 ml sterile dH_2O.

Agarose 400 mg was dissolved in 50 ml of 1 X TAE and completely melted by boiling for several minutes. To facilitate visualization of DNA fragments during the run, 2 µl ethidium bromide (10 mg/ml) was added in the gel.

3.5.11 DNA extraction protocol

A modified CTAB-PVP DNA isolation protocol was standardized on the basis of the method described by Khanuja *et al.* (1999). On the other hand, Khanuja *et al*'s method was simply the extension of the original protocols described by Dellaporta *et al.* (1983), Doyle and Doyle (1987) and Porebski *et al.* (1997). The new modified protocol is described below:

1. Fresh tender leaves were collected (3 g) in the morning hours from the plant, washed thoroughly with sterile dH_2O, blotted the excess water with tissue paper and then surface sterilized with 70% alcohol.
2. The sterilized leaves were cut in to smaller pieces.
3. The leaf material was ground in to a fine powder treating with liquid nitrogen and using a pre-chilled pestle and mortar.
4. The ground powder was transferred directly in to a 25-ml polypropylene tube and added 9 ml freshly prepared pre-warmed (56°C) extraction buffer and mixed gently by several inversions.
5. The sample was incubated at 60°C in a shaking waterbath (100 rpm) for 2 h with occasional mixing to avoid reaggregation of the homogenate.
6. To the homogenate, 9 ml of chloroform: isoamyl alcohol (24:1) was added and mixed gently by inversion for 10 minutes.
7. The extract was centrifuged at 10,000 rpm for 10 minutes with SS34 rotor in a Sorval RC-5C centrifuge at 25°C. The supernatant was transferred in to a clean 15 ml polypropelene tube and the process (adding of chloroform: isoamyl alcohol, mixing and centrifuge) was repeated twice to clear the aqueous phase.

8. A volume of 4 ml of 5 M NaCl was added to the aqueous phase and was mixed properly without vortexing.
9. Isopropanol was added to the mixture at 0.6 volume and mixed by inversion. The mixture was incubated at room temperature overnight to precipitate the nucleic acid.
10. The sample was centrifuged at 10,000 rpm for 10 minutes at 25°C. The supernatant was poured off and washed the pellet with 80% ethanol and then carefully transferred the pellet in to a clean microfuge tube. The pellet was again washed with 80% ethanol.
11. The pellet was dried in a speed vacuum (Maxi dry plus, Hoefer Pharmacia Biotech Inc., USA) for 15 minutes and dissolved in 0.5 ml of high salt TE buffer. RNase 5 µl was added to the solution and then incubated at 37°C for 2 h.
12. After incubation, the sample was extracted with equal volume of chloroform: isoamyl alcohol (24:1). The aqueous layer was transferred to a fresh 1.5 ml microfuge tube and was added 2 volumes of cold ethanol.
13. The sample was then centrifuged at 10,000 rpm for 10 minutes at 25°C to precipitate the DNA.
14. The pellet was rinsed with 80% ethanol and then dried in a speed vacuum system. The pellet was dissolved in 200 µl of TE buffer and then stored at -20°C.

3.5.12 DNA quantification and purity test by UV spectroscopy

DNA quantification is an important step. It is necessary to know the amount of DNA extracted while carrying out restriction digestion or performing different downstream applications, such as, PCR and RAPDs. There are several methods for

quantification of DNA and the most widespread being: (i) UV-Spectroscopic determination (ii) the comparison of an aliquot of the extracted sample with standard DNAs of known concentration using gel electrophoresis and (iii) flourimetric determination. In this investigation, the first method was used as described below:

3.5.13 UV-spectroscopic determination yield and purity of DNA

1. From the stored sample, an aliquot of 5 µl of DNA was transferred in to a quartz cuvete and made up the volume to 1 ml with dH_2O.
2. The cuvete was placed in the UV-spectrophotometer (Beckman DU® 530 Life Science UV/Vis Spectrophotometer) and absorption was measured at 260 nm and 280 nm.
3. DNA concentration was calculated by using the relationship of soluble standard DNA being 1 O.D. at 260 nm = 50 µg/ml.
4. The ratio between the absorption data at 260 nm and 280 nm was calculated to check the purity of the isolated DNA. A good DNA preparation exhibits a value in between 1.8 - 2.0.

3.5.14 Agarose Gel Electrophoresis

Procedure

1. Agarose gel 0.8% was prepared in 1XTAE.
2. Ethidium bromide 2.0 µl (10 mg/ml) was added to the agarose gel.

3. DNA sample 10 µl was added with 2 µl of Bromophenol blue dye and mixed. The sample was then loaded into the wells of the agarose gel along side the standard *Hind* III digested λ DNA molecular weight marker.
4. Electrophoresis was carried out at 60 – 70 V for about 90 min.
5. The electrophoresed gel was exposed to UV light using a UV transilluminator for visualization of the DNA. DNA bands seen in the gel were documented by taking photographs with a Gel Doc system (BIO RAD Gel Doc 1000).

3.5.15 Restriction digestion of isolated DNA to check the quality of DNA

The presence of contaminants like polysaccharides, polyphenolic compounds and other sticky and resinous materials in DNA preparations often makes the samples viscous and renders DNA unrestrictable by the restriction endonuclease enzymes. To check the purity, the extracted DNA samples were single digested with *Eco*R I, *Hind* III and double digested with *Eco*R I/*Hind* III restriction endonuclease enzymes. For the purpose, first 1.5 ml Eppendorf tubes were labeled appropriately for the restriction enzymes and the extracted DNA to be digested. The DNA samples, each measuring 15 µl were aliquoted in to the labeled tubes. The final volume of each sample was made to 20 ml and 25 ml for single and double digestions, respectively. The following were the reaction mixtures:

3.5.15.1 Single digestion with *Eco*R I

Restriction mixture (on ice)	Volume per sample
Eco RI buffer (10X)	2.0 µl
Eco RI enzyme (10 units/µl)	1.0 µl

Sterile distilled water	2.0 µl
DNA (2.1 ng/µl)	15.0 µl
Total volume	20.0 µl

3.5.15.2 Single digestion with *Hind* III

Restriction mixture (on ice)	**Volume per sample**
Hind III buffer (10X)	2.0 µl
Hind III enzyme (10 units/µl)	1.0 µl
Sterile distilled water	2.0 µl
DNA (2.1 ng/µl)	15.0 µl
Total volume	20.0 µl

3.5.15.3 Double digestion with *Eco*R I and *Hind* III

Restriction mixture (on ice)	**Volume per sample**
Buffer B (10X)	4.0 µl
Eco RI enzyme (10 units/µl)	1.0 µl
Hind III enzyme (10 units/µl)	1.0 µl
Sterile distilled water	4.0 µl
DNA (2.1 ng/µl)	15.0 µl
Total volume	25.0 µl

The reaction mixtures were incubated overnight at 37°C. After the incubation, 2 µl of the loading dye was added and reincubated for another 15 min. After the digestion,

the reaction mixtures were electrophoresed through ethidium bromide stained 0.8% agarose gel along with standard *Hind* III digested λ DNA molecular weight marker. The digested DNA was visualized under a Gel Doc system (UV-transilluminator) and photographed.

3.5.16 Genome size determination (Estimation of DNA C-values)

Genome size (DNA C-value is often used synonymously, Singh, 2003) is an important biodiversity character with fundamental significance and for many other uses. The genome size or DNA C-value of an organism is the amount of nuclear DNA in its unreplicated gametic nucleus (Swift, 1950), irrespective of the ploidy level of the taxon. The genome size of a plant or animal can be determined by measuring the DNA content of the nucleus.

We have tried to develop an easy, accurate and rapid method for the determination of genome size of *Karphul* plant by measuring the DNA content of a single somatic cell nucleus. In this method, the average volume of a cell from the leaf tissue was calculated. The number of cells present in a particular leaf tissue with known volume and weight (less 7 – 70% cells as intercellular space depending upon the species and the habitat of the particular plant, Turrell, 1934) was calculated. If, the amount of DNA per g fresh tissue, number of cells per g tissue and ploidy level of the plant (by Karyotype study) are known, the amount of DNA in a single cell can be calculated easily by dividing the amount of DNA present in that tissue by the total number of cells in the same tissue. Thus, the genome size of a particular plant can be worked out as the amount of DNA present in the haploid set of its chromosomes.

3.5.16.1 Cell volume determination in leaf tissue

For measuring the average volume of a single cell of *Karphul* leaf tissue, fine transverse and longitudinal sections were made with the help of a sharp razor blade and mounted on a slide for observations under a compound microscope (Leica ATC 2000 Model). By studying the *Karphul* leaf anatomy, it was found that there is a single layer of rectangular shaped epidermis cell on both surfaces of the leaf and in between, cylindrical and spherical shaped mesophyll cells (Fig. 3.1 a). The cell parameters: length, breadth and thickness (for rectangular cell); length and radius (for cylindrical cell) and radius (for spherical cells) (Fig. 3.1 b-c) means were measured from five randomly selected cells of five different sections and volumes were calculated for each case with mounted micro scale having 400x magnification. The average volume of 15 cells was calculated. Photographs of the leaf section (at 10 X 40x magnification) were also taken with a camera attached to the microscope.

Within the tissue, there are intercellular spaces. The ratio of intercellular space to total cell volume is species-specific and habitat-specific. The intercellular space in leaf tissues of plants ranges from 7-70% of the total volume. The volume of intercellular space in the leaf tissues of plants grown under the shady condition is higher than those grown under sunny condition (Turrell, 1934). Since, this perennial herb *Karphul* is mostly grown under semi shady condition, the intercellular space in its leaf tissue was considered to be medium. The intercellular space of the leaf tissue of the plant was measured from photographs (10) of different leaf sections taken with the help of graph paper and then expressed in percentage.

Mathematical deduction of cell number in the leaf tissue of *Karphul*:

Average volume of a single cell (l x b x t as in Fig. 3.1 b-c) = $x\ \mu^3$

Volume of the tissue (L x B x T as in Fig 3.1d) = $t\ \mu^3$

Volume of the intercellular space (32% of total vol.) = $s\ \mu^3$

Actual cell mass = $(t\text{-}s) = v\ \mu^3$

Total number of cells in the cell mass = $v\ \mu^3 / x\ \mu^3 = y$

Now, weight of the tissue section = w g

Or, w g tissue contains = y cells

So, 1 g tissue will contain cells = (y/w)

DNA yield per g of leaf tissue = d µg DNA

= d x 10^6 pg DNA

So, one cell contains d x 10^6 /(y/w) pg DNA

Since, the species is tetraploid (from karyotypic study), so, the genome size or DNA content of the haploid set of chromosome will be $½\{d \times 10^6 / (y/w)\}$ pg DNA

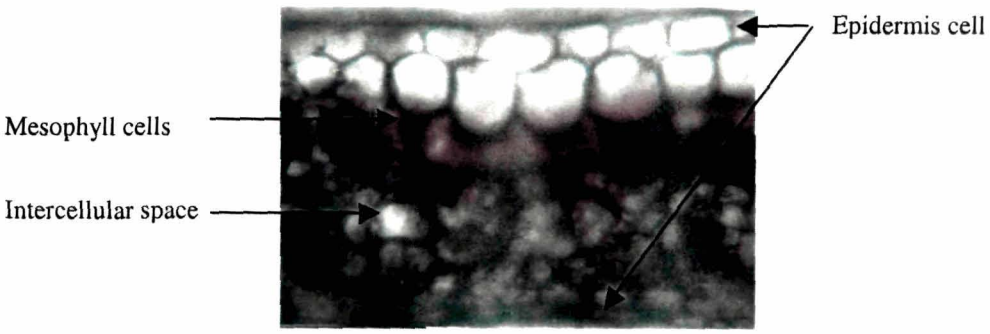

Fig. 3.1 a Cell types in a typical leaf section of *Karphul*

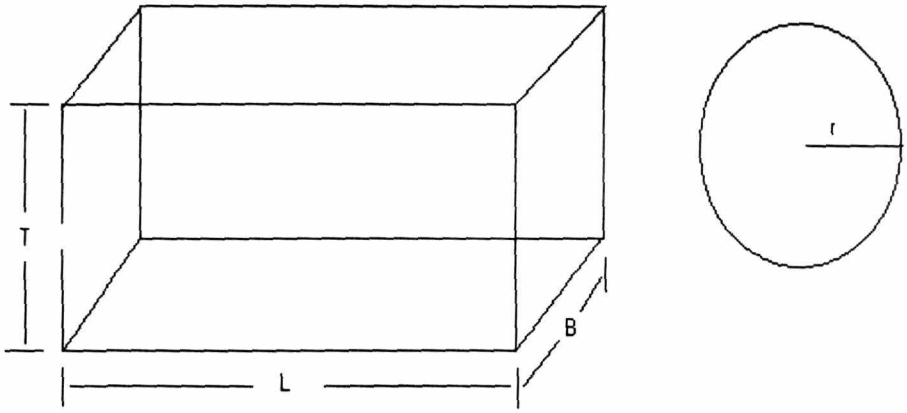

Fig. 3.1 b Length (L), breadth (B) and thickness (T) of rectangular cell
Fig. 3.1 c Radius (r) of cylindrical and spherical cell

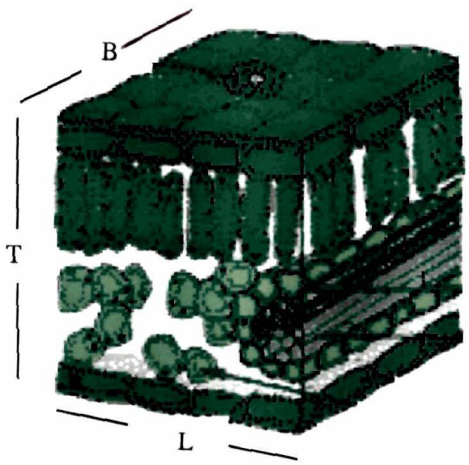

Fig. 3.1 d Leaf cross section

3.6 Tissue culture technique

Due to large-scale deforestation, rapid industrialization and indiscriminate exploitation, the population and diversity of medicinal and aromatic plants have been decreasing day by day. *In vitro* techniques could effectively be used for the multiplication, maintenance and conservation of these plant species as well as production of a large number of phytochemicals.

Though *in vitro* propagation has been reported in different plants belonging to the family Zingiberaceae by various workers (Nirmal Babu *et al.*, 1992; Chang and Criley, 1993 and Agretious *et al.*, 1996), it is necessary to develop a standard protocol for propagating this endangered plant. Tissue culture of *Karphul* plant is of more significance, as the plant does not produce seed and its propagule, the rhizome is used as the economic product.

3.6.1 Equipment and materials required

Mettler top loading balance	Mettler electronic balance
Digital pH meter	Autoclave
Laminar flow cabinet	Magnetic stirrer
Micropipettes (2, 20, 200, 1000 µl)	Micropipette tips (Sterile)
Eppendorf tube (sterile)	Plastic tray
Ovens (37°, 60°, 80°, 100°C)	Sharp sterile blade
Glass beaker (250, 500, 1000 2000 ml)	Erlenmeyer flask (100, 250, 500, 1000 ml)
Glass petri dishes (medium size)	

3.6.2 Solutions used

3.6.2.1 Preparation of media and solutions

Almost all the necessary solutions were prepared with distilled water. However, some growth regulators were first dissolved in ethanol or sodium hydroxide and made up the required volume with distilled water. *In vitro* culture media were prepared with Millipore (Mili Q) water. Various stock solutions were used for media preparations. Usually, the required quantities of chemicals were weighed on a Mettler top Loading balance (Sartorius GM 3101, Sartorius Ag Gottingen, Gemany). However, for weighing micro quantities (less than 10 mg) the Mettler electronic balance (Sartorius BP 121 S, Sartorius Ag Gottingen, Gemany) was used.

3.6.2.2 pH adjustment

The pH of media was tested prior to autoclaving or filter sterilization with a Cyberscan 510 digital pH Meter and adjusted according to the requirement with NaOH and HCl.

3.6.2.3 Stock solutions

Stock solutions of various chemicals were prepared for the following reasons:

1. When a chemical was common to a number of media and solutions, a stock of it was prepared to save time and labour.
2. To reduce the volume of solutions requiring filter sterilization, e.g., antibiotics, some of the growth regulators etc.
3. To reduce the loss during repeated weighing and dissolving.

4. To use micro quantities of chemicals, e.g., growth regulators, vitamins etc.

Stock solutions were prepared for micro-inorganic salts (10 ml each) with different concentrations (1 mg/ml, 10 mg/ml and 100 mg/ml) depending upon the amount to be added to the final medium. For the antibiotics and different growth regulators, stock solutions of 10 ml each were prepared with a concentration 10 mg/ml. The inorganic macro-salts and vitamins were added fresh during media preparation.

3.6.3 Aseptic procedures

In vitro plant tissue culture media are equally suitable for the growth and development of microorganisms, such as, bacteria, fungi, yeast etc. Thus, all solutions, glasswares, equipment and plant materials were sterilized by different means and all manipulations and culture works were carried out in an aseptic environment.

3.6.3.1 Autoclaving

Solutions without thermolabile components, all polypropylene and polycarbonate (eppendorf tube, micropipette tips, centrifuge buckets and tubes) equipment, *in vitro* culture vessels (conical flask, tubes, wide mouthed glass containers) were sterilized by autoclaving at pressure of 1.5 kg/cm^2 for 20-35 min. No contamination was observed in autoclaved media and solutions.

3.6.3.2 Dry sterilization

All glassware (conical flasks, glass pipettes, petri dishes) metallic equipment and tools (forceps, steel needles, scissors, blades, steel knifes, scalpels), which can tolerate

high temperatures were sterilized by heating at 100°C over 6 h in an oven. Usually the equipment and glassware were wrapped with several layers of aluminum foil before putting in the oven.

3.6.4 Storage of solutions and media after autoclaving

Solutions were autoclaved in borosilicate glass bottles, allowed to cool in a laminar flow cabinet and stored at room temperature or in a refrigerator. Nutrient agar media were autoclaved in Erlenmeyer flask, sealed with non-absorbent cotton plug and 2 – 3 layers of aluminum foil. The media were poured into culture tubes and vessels after allowing them to cool down to *ca.* 40°C in the laminar flow cabinet. Growth regulators, antibiotics (all filter-sterilized) from stock solutions were added to the autoclaved media on cooling to about 40 ° C. Media containing antibiotics were not kept longer than two weeks because of their gradual inactivation (Mossel, 1971).

3.6.5 Filter sterilization

Solutions, such as, growth regulators, antibiotics etc were sterilized using sterile syringe and autoclaved-sterilization units containing Whatman cellulose – nitrate membrane filter with 0.2 μm pore size. Filter sterilized solutions were stored in sterile Terson (15 ml) tubes at 4 ° C.

3.6.6 Aseptic manipulation

Aseptic handling of plant materials (explants) and sterile media and solutions was done inside a laminar flow cabinet. The laminar flow cabinet was switched on at least 30

min prior to use. The bench was swabbed with rectified spirit. Forceps, scalpel and other tools were kept in a jar containing rectified spirit and were flamed and cooled before use. Sound laminar flow bench practice included working in an unobstructed laminar air flow, keeping bottles, dishes, jars closed as much as possible, flaming their openings before and after use, and reflaming tools at regular intervals.

3.6.7 Culture media and growth regulators

MS (Murashige and Skoog, 1962) modified medium was used for *in vitro* culture of *Karphul*. The composition of the medium is presented in Table 3.1.

The growth regulators and their concentration regimes used for *in vitro* culture of *Karphul* are presented in Table 3.2.

Table 3.1. Composition of modified MS [MS (M)] medium used for *in vitro* cultures

Components	MS (mg/l)
(A) INORGANICS	
Macro salts	
NH_4NO_3	1650.0
KNO_3	1900.0
$MgSO_4.7H_2O$	370.0
KH_2PO_4	170.0
$CaCl_2.2H_2O$	440.0
Minor salts	
H_3BO_3	6.0
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$FeSO_4.7H_2O$	27.8
Na_2EDTA	37.3
KI	0.83

(B) ORGANICS	
Vitamin	
Pyridoxin – HCl	0.5
Nicotinic acid	0.5
Thiamine – HCl	0.1
Glycine	2.0
Myo – inositol	100.0
Sucrose	30,000.0
Agar	7,500.0
pH	5.8
Growth regulators	As required

Table 3.2 Growth regulators and their concentrations (mg/l) used in *in vitro* cultures of *Karphul*

Hormones / Conc.	BAP	2, 4 – D	NAA	Kin	IAA
	0.5	0.1	0.1	0.5	0.1
	1.0	0.25	0.25	1.0	0.25
	1.5	1.0	0.5	2.0	0.5
	2.0	1.5	1.0	5.0	
	5.0	2.0	1.5	7.0	
	7.0	2.5	2.0	10.0	
	10.0	3.0			

To prepare 1.0 litre medium, the required quantities of inorganic macro salts (w/v), vitamins (w/v), stock solutions of inorganic minor salts (v/v) and sucrose (w/v) were added and the volume was made up by adding the Mili Q water to 900 ml. After mixing the solution thoroughly, the pH was adjusted to 5.7 using 1N sodium hydroxide (NaOH) and 1N hydrochloric acid (HCl); made up the volume to 1.0 litre. Agar 7,500.0 mg (0.75% w/v) was added to solidify the medium and then autoclaved at a pressure of

1.5 kg/cm^2 for 30 min. After autoclaving, the medium was allowed to cool to *ca.* 40°C and added the growth regulators (v/v) to the required concentrations from their filter-sterilized stocks.

3.7 Plant material

3.7.1 Source plant materials

Young rhizome explants consisting of vegetative buds were collected from plants grown in the Medicinal Park of the Department of Molecular Biology and Biotechnology, Tezpur University, Napam.

3.7.2 Sterilization and preparation of explants

The explants were washed thoroughly in running water for 1 h to remove the dirt materials and microbes followed by detergent Extran for 20 min. The explants were then washed briefly with sterile distilled water. Later on the explants were treated with 70% ethyl alcohol for 30 - 40 sec and surface sterilized with 0.1% $HgCl_2$ (5 - 7 min). The explants were rinsed thrice in sterile distilled water to remove the excess $HgCl_2$. Each explant was then cut in to small pieces (0.5 –1.0 cm) with sterile dissecting blade in a Petri dish under the laminar flow cabinet and kept ready for inoculation.

3.7.3 Inoculation of explants

The aseptically prepared explants were inoculated quickly by opening the cotton plug of the flask in front of a flame under the operational laminar flow cabinet. After the inoculation, the culture flasks were maintained at 25 ± 2°C under 16/8 h photoperiod.

3.7.4 *In vitro* callus, shoot and root development

For *in vitro* tissue culture of *Karphul,* the young rhizomes were cultured in MS (M) medium supplemented with the above-mentioned growth regulators (Table 3.2) for induction of callus, plantlets and roots. Browning of the medium due to phenolic compounds was observed. The problem was controlled successfully in media by adding ascorbic acid 10 mg/l.

After the identification of growth regulators and their specific concentrations suitable for the production of callus, plantlet and root from axenic plant parts, explants, calli and plantlets were cultured separately on shooting and rooting media.

CHAPTER IV

RESULTS

The investigation was carried out with a view to explore the plant *Karphul* scientifically with respect to its taxonomy, karyotype, biochemical characterization, antimicrobial activity of the active compounds isolated, molecular characterization and tissue culture. The investigation generated interesting results with respect to above-mentioned aspects, which are presented below.

4.1 Taxonomy

Taxonomic study of a plant is important for its identification and proper description. Description on morphophenological characters is an integral part of the conventional taxonomic study and these two aspects were studied systematically on the plant *Karphul.*

4.1.1. Morphological study

Morphological characters of the selected plants were recorded during October 2002 to September 2005 and mean data are presented along with standard error (SE) in Table 4.1. The photographic plates of the plant, its leaves and rhizomes are presented in Fig. 4.1. The characters studied and recorded were plant height (cm), circumference of the stem at the base (cm), circumference of the stem at 1.0 m height (cm) from the base, number of leaves, length of leaf (cm), breadth of leaf (cm), length of ligule (cm), length of petiole (cm) and circumference of rhizome (cm). For each character the standard error was calculated.

Table 4.1 Morphological characteristics of *Karphul*

Morphological characters	Mean plant characteristic ± S.E.	Range
Plant height (cm)	274.5 ± 7.98	250-300
Circumference of the stem at the base (cm)	12.5 ± 0.82	10.0-15.0
Circumference of the stem at 1 m ht (cm)	10.0 ± 0.63	8.0-12
Number of leaves	10.4 ± 1.56	6.0-15.0
Length of leaf (cm)	60.0 ± 3.16	50.0-70.0
Breadth of leaf (cm)	11.9 ± 1.34	9.0-17.0
Length of ligule (cm)	1.06 ± 0.05	0.9-1.2
Length of petiole (cm)	0.44 ± 0.02	0.4-0.5
Circumference of rhizome (cm)	7.0 ± 0.81	5.0-9.5

In addition to the above-mentioned characters other qualitative characters were also recorded and are presented in Table 4.2.

Table 4.2 Qualitative morphological characters

Morphological characters	Qualitative features
Pseudostem	Hard and green in colour.
Base of the pseudostem	Pubescent and swollen.
Leaf blade	Oblong-lanceolated, glabrous, green in colour.
Rhizome	Strong, pubescent, fibrous, aromatic, brownish with the skin and white inside.
Root	Stout, hard and strong, penetrating up to 30-35 cm deep into the soil.

4.1.2. Floral biology study

Another important criterion of taxonomy is the description of floral or reproductive biology of the plant. During the period of floral biology study, October 2002 to September 2004, various features of the selected spikes like length of spike (cm), number of florets per spike, length of peduncle (cm), length of bract (cm), breadth of

bract (cm), length of floret (cm), length of floral bract (cm), breadth of floral bract (cm), length of bracteole tube (cm), length of calyx tube (cm), length of corolla tube (cm), length of petal (cm), breadth of petal (cm), length of filament (cm), length of anther (cm), pollen grain diameter (μ m), length of style (cm), length of ovary (cm) and diameter of ovary (cm) were recorded and are presented in Table 4.3. Photographs of spike, a floret and its various parts are presented in Fig. 4.1.

Table 4.3 Characteristics of *Karphul* spike and its different parts

Floral characteristics	Mean ± S.E.	Range
Length of spike (cm)	13.50 ± 0.82	11.0-16.0
No. of florets/spike	10.00 ± 0.98	8.0-14.0
Length of peduncle (cm)	3.58 ± 0.29	2.8-4.6
Length of bract (cm)	3.80 ± 0.67	2.0-6.0
Breadth of bract (cm)	1.94 ± 0.29	1.2-3.0
Length of floret (cm)	9.46 ± 1.02	6.6-12.8
Length of floral bract (cm)	4.00 ± 0.63	2.0-6.0
Breadth of floral bract (cm)	1.52 ± 0.15	1.2-2.0
Bracteole tube (cm)	3.34 ± 0.13	3.0-3.8
Length of calyx tube (cm)	4.80 ± 0.08	4.5-5.0
Length of corolla tube (cm)	6.34 ± 0.51	4.8-8.0
Diameter of corolla tube at base (cm)	0.2 ± 0.01	0.15-0.25
Diameter of corolla tube at top (cm)	0.4 ± 0.01	0.40-0.45
Length of petal (cm)	2.10 ± 0.26	1.5 – 3.0
Breadth of petal (cm)	0.94 ± 0.02	0.9 – 1.0
Length of labellum with tail (cm)	5.16 ± 0.24	4.5 – 6.0
Length of filament (cm)	0.36 ± 0.04	0.3-0.5
Length of anther (cm)	0.68 ± 0.02	0.6-0.7
Breadth of anther (cm)	0.18 ± 0.002	0.15 – 0.2
Pollen grain size (μm)	48.6 ± 0.36	48.0-50.0
Length of style (cm)	5.48 ± 0.45	4.3-7.0
Ovary length (cm)	0.56 ± 0.04	0.5-0.7
Ovary diameter (cm)	0.1 ± 0.002	0.09-0.1

Fig 1: A: Habitat, B: Rhizome with inflorescence, C: Inflorescence, D: Outer bract, E: Inner bract, F: Bracteole, G: Calyx, H: Peduncle, I: Corolla tube, labellum, petals, J: Anther, K: Gynoecium, L: Leaf, M: Ligule, N: Ovary in TS

Apart from the quantitative characters, qualitative characters of a spike were also recorded and the same are presented in Table 4.4.

Table 4.4 Qualitative characters of a spike of *Karphul*

Spike characters	Qualitative features
Spike	Club-shaped, solitary. Creamy white with light greenish tinge at base, top portion red with yellow margin.
Involucral bract	Sessile, imbricately appressed, oblong, hard, acute and white in colour with light greenish tinge at tip.
Floret	Sessile, complete, bisexual, zygomorphyic and epigynous. Creamy white at base and red towards top with yellow lip.
Floral (inner) bract	Lanceolate, membranous, striate; white in colour with light greenish tinge towards the tip.
Bracteole	Tubular, membranous, tube ends up into two unequal parts with pointed tips. Bottom portion white in colour and top portion light reddish.
Calyx	Tubular, membranous, tube opens up with a dentate tip. Bottom portion light reddish in colour and towards the tip the colour intensity increases to deep red.
Corolla	Gamopetalous, tubular and apically tridentate with three membranous petals. Base of the tube white in colour and light reddish at the top.
Petal	Ovate and deep red colour.
Labellum	Tongue-shaped, fleshy, long, lanceolated and emarginated tip; deep red blotches in middle with incurved frilled yellow margin.
Filament	Short, flat, fleshy, stiff and white in colour.
Anther	Bi-lobed, divided by deep longitudinal connecting groove, light reddish-white at the base as well as inner side and deep red at the top as well as outer side.
Style	Single, slender and white in colour.
Stigma	Triangular with three lobes, deep red in colour.
Ovary	Villous, inferior, trilocular, axile placentation with many ovules.

The percent anthesis of *Karphul* florets numbering 50 over the hours of the day was studied and data are graphically presented in Fig. 4.3. Anthesis behaviour of the plant as well as the duration of anthesis was also studied in those 50 marked florets and data obtained are presented in Table 4.5.

Table 4.5 Anthesis behaviour of *Karphul* florets

Nature of anthesis	Time and duration
Initiation of anthesis	5.30 am
Early anthesis	5.30 – 6.30 am
Peak anthesis	6.30 – 7.30 am
Duration of anthesis	5.30 am –1.30 pm

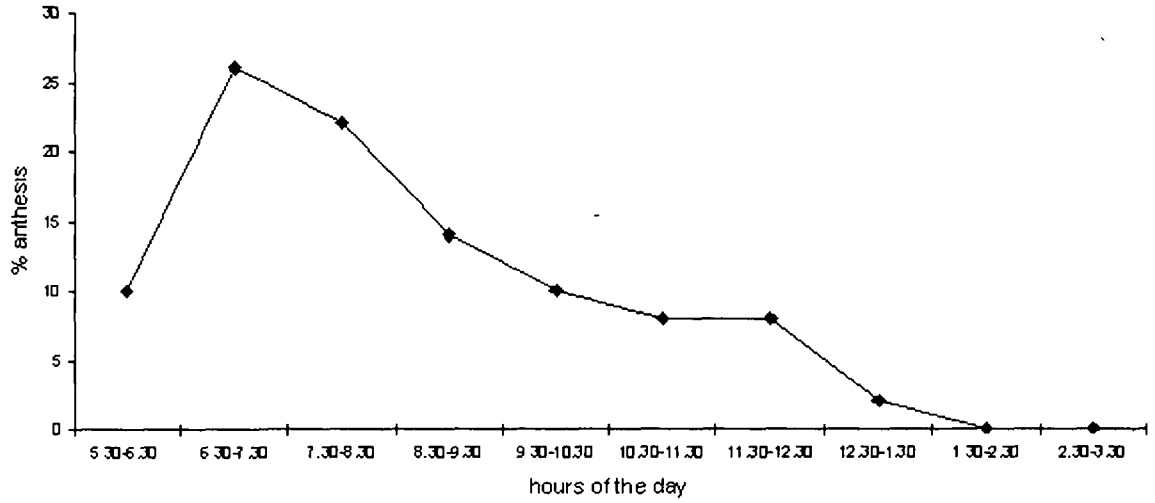

Fig. 4.2 Percent anthesis over the hours of the day of *Karphul* floret

4.2 Karyotype study

An experiment was designed to find out the actual time of cell division having the chromosomal movement of *Karphul* somatic cells through fixation of root tips from early morning 4.15 h to 8.15 h with 30 min interval. Time of collection, fixation of root tips and active cell division observed are presented in Table 4.6.

Table 4.6 Time of collection/fixation of root tips and active cell division

Time of collection/fixation	Active cell division
4.15 am	None
4.45 am	None
5.15 am	Few
5.45 am	Some
6.15 am	Maximum
6.45 am	Maximum
7.15 am	Some
7.45 am	Few
8.15 am	None

Actively dividing cells and the stages of cell division are presented in Fig. 4.3. Camera Lucida drawing and ideogram representing chromosomes and their features are presented in Fig. 4.4 and Fig. 4.5, respectively.

The detailed karyotype including the measurement and other features of somatic chromosomes of *Karphul* are presented in Table 4.7.

4.3. Biochemical analysis of *Karphul* rhizome

4.3.1. Isolation and composition analysis of essential oils from *Karphul* rhizome

The essential oils extracted from the fresh rhizome paste of *Karphul* by hydrodistillation (200 g fresh rhizome paste) for 4 h using a Clevenger-type apparatus were dried over anhydrous sodium sulfate (Na_2SO_4) overnight and kept in sterile glass bottle at 4°C in a refrigerator for subsequent analysis. Hydrodistillation yielded 0.14% essential oil (w/w).

Table 4.7 Chromosome features of *Karphul*

Chromo-some	Long arm (µm)	Short arm (µm)	Total length (µm)	Arm ratio r=l/s	Relative length*	Index i = 100XS/C	Centro-meric position	Group
1	1.2 ± 0.05	1.2 ± 0.06	2.4 ± 0.07	1.0 ± 0.08	8.0	50.0	M	I
2	1.2 ± 0.09	1.2 ± 0.09	2.4 ± 0.18	1.0 ± 0.20	8.0	50.0	M	I
3	1.4 ± 0.14	0.6 ± 0.06	2.0 ± 0.17	2.3 ± 0.35	6.0	30.0	SM	II
4	1.4 ± 0.06	0.6 ± 0.09	2.0 ± 0.13	2.3 ± 0.36	6.0	30.0	SM	II
5	0.8 ± 0.06	0.8 ± 0.11	1.6 ± 0.12	1.0 ± 0.20	5.0	50.0	M	I
6	0.8 ± 0.06	0.8 ± 0.15	1.6 ± 0.11	1.0 ± 0.32	5.0	50.0	M	I
7	0.8 ± 0.11	0.5 ± 0.00	1.3 ± 0.11	1.6 ± 0.22	4.0	38.0	SM	II
8	0.8 ± 0.13	0.5 ± 0.06	1.3 ± 0.19	1.6 ± 0.05	4.0	38.0	SM	II
9	0.9 ± 0.13	0.4 ± 0.14	1.3 ± 0.17	2.3 ± 1.50	4.0	31.0	SM	II
10	0.9 ± 0.13	0.4 ± 0.14	1.3 ± 0.17	2.3 ± 1.50	4.0	31.0	SM	II
11	0.6 ± 0.11	0.6 ± 0.06	1.2 ± 0.15	1.0 ± 0.14	4.0	50.0	M	I
12	0.6 ± 0.00	0.6 ± 0.13	1.2 ± 0.13	1.0 ± 0.27	4.0	50.0	M	I
13	0.6 ± 0.00	0.6 ± 0.00	1.2 ± 0.00	1.0 ± 0.00	4.0	50.0	M	I
14	0.6 ± 0.00	0.6 ± 0.00	1.2 ± 0.00	1.0 ± 0.00	4.0	50.0	M	I
15	0.6 ± 0.07	0.6 ± 0.08	1.2 ± 0.06	1.0 ± 0.24	4.0	50.0	M	I
16	0.6 ± 0.14	0.6 ± 0.08	1.2 ± 0.08	1.0 ± 0.08	4.0	50.0	M	I
17	1.0 ± 0.13	0.2 ± 0.00	1.2 ± 0.13	5.0 ± 0.63	4.0	17.0	SM	II
18	1.0 ± 0.00	0.2 ± 0.06	1.2 ± 0.06	5.0 ± 0.92	4.0	17.0	SM	II
19	0.5 ± 0.00	0.5 ± 0.00	1.0 ± 0.00	1.0 ± 0.00	3.0	50.0	M	I
20	0.5 ± 0.00	0.5 ± 0.00	1.0 ± 0.00	1.0 ± 0.00	3.0	50.0	M	I
21	0.5 ± 0.00	0.2 ± 0.00	0.7 ± 0.00	2.5 ± 0.00	2.0	29.0	SM	II
22	0.5 ± 0.00	0.2 ± 0.00	0.7 ± 0.00	2.5 ± 0.00	2.0	29.0	SM	II
23	0.4 ± 0.00	0.1 ± 0.00	0.5 ± 0.00	4.0 ± 0.00	2.0	20.0	SM	II
24	0.4 ± 0.00	0.1 ± 0.00	0.5 ± 0.00	4.0 ± 0.00	2.0	20.0	SM	II

*Relative length = Percentage length of individual chromosomes, M - Median, SM –Submedian

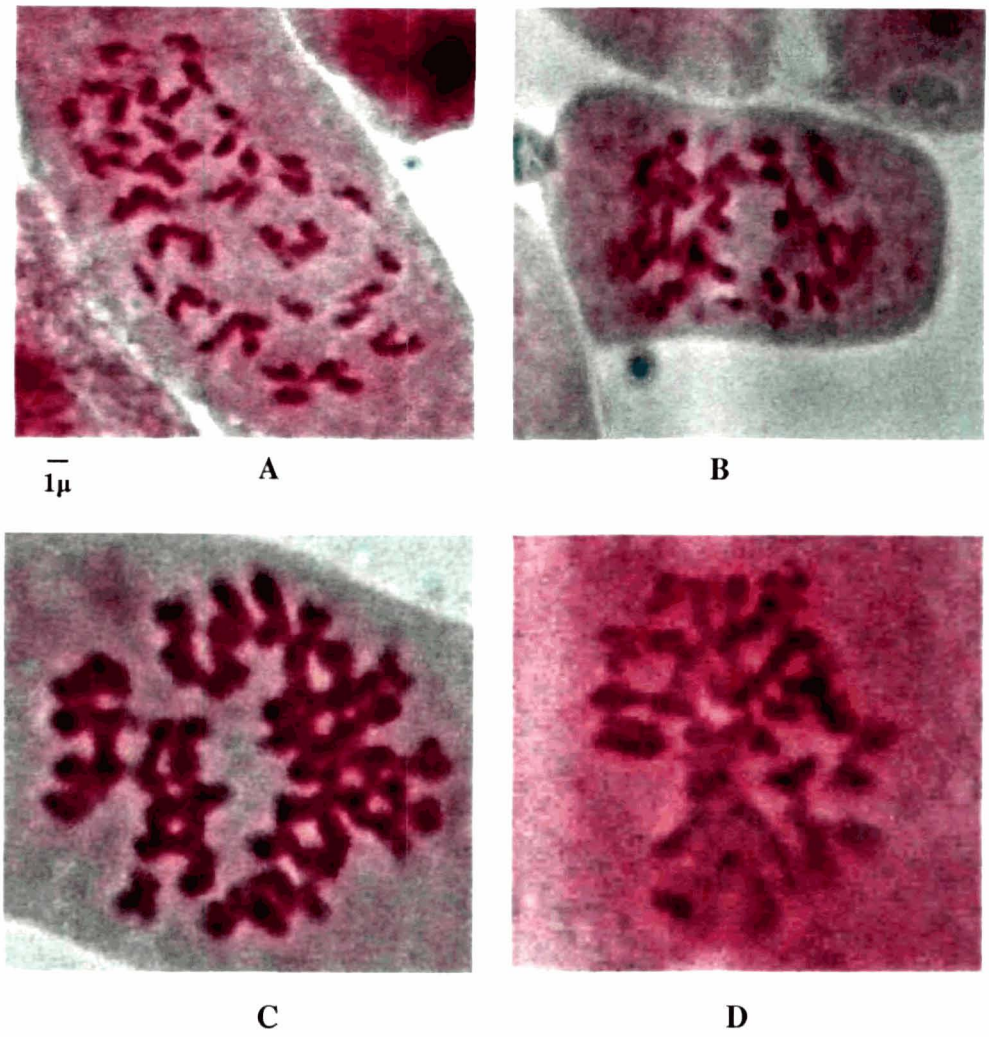

Fig. 4.3 Somatic chromosomes *Karphul* plant A, B, C – Prophase; D – Early Metaphase (1000x magnification)

Fig 4 4 Camera Lucida drawing of somatic chromosomes of *Karphul* Plant

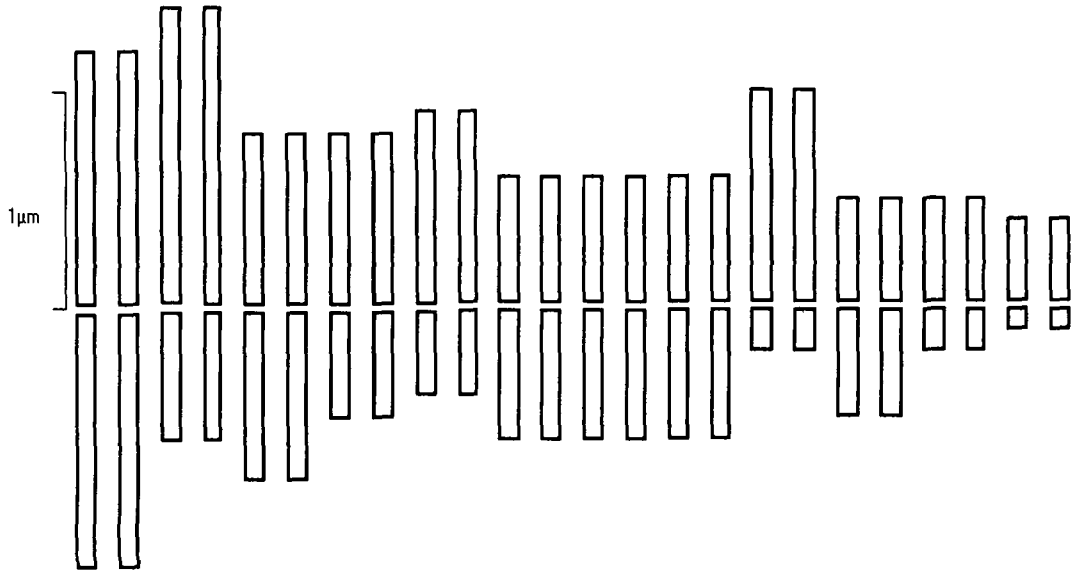

Fig 4 5 Idiogram of *Karphul* chromosomes

The chemical constituents of the extracted oils were identified by comparing with their GC spectra, retention time and retention area with respective authentic standard reference compounds under identical conditions. The results obtained from the qualitative and quantitative analysis of the oils are presented in Table 4.8. The chromatogram is presented in Fig. 4.6.

Table 4.8 Percent composition of components of essential oils extracted from *Karphul* rhizome

Sl. No.	Retention Time (min)	Area	Width	Area %	Compound
1.	0.645	125112	0.079	0.18396	-
2.	1.036	18117	0.049	0.02664	-
3.	11.367	39991	0.363	0.05880	-
4.	13.782	84996	0.439	0.12498	-
5.	17.047	220432	0.422	0.32412	-
6.	19.972	10636	0.376	0.01564	-
7.	21.550	12626	0.356	0.01857	-
8.	22.487	1112871	0.442	1.63637	(I)
9.	25.715	3865614	0.435	5.68400	Methyl chavicol (II)
10.	27.565	58423936	0.488	85.90662	Anethole (III)
11.	31.340	599797	0.401	0.88194	-
12.	33.005	52960	0.397	0.07787	-
13.	35.444	554936	0.527	0.81598	-
14.	36.675	219495	0.498	0.32275	-
15.	37.806	103931	0.337	0.15282	-
16.	38.524	2007671	0.607	2.95208	(IV)
17.	40.393	52544	0.298	0.07726	-
18.	40.810	183182	0.398	0.26935	-
19.	41.260	171262	0.376	0.25235	-
20.	41.806	68660	0.457	0.10096	-
21.	43.023	33891	0.393	0.04983	-
22.	47.732	24305	0.607	0.03574	-
23.	50.395	21394	0.445	0.03146	-

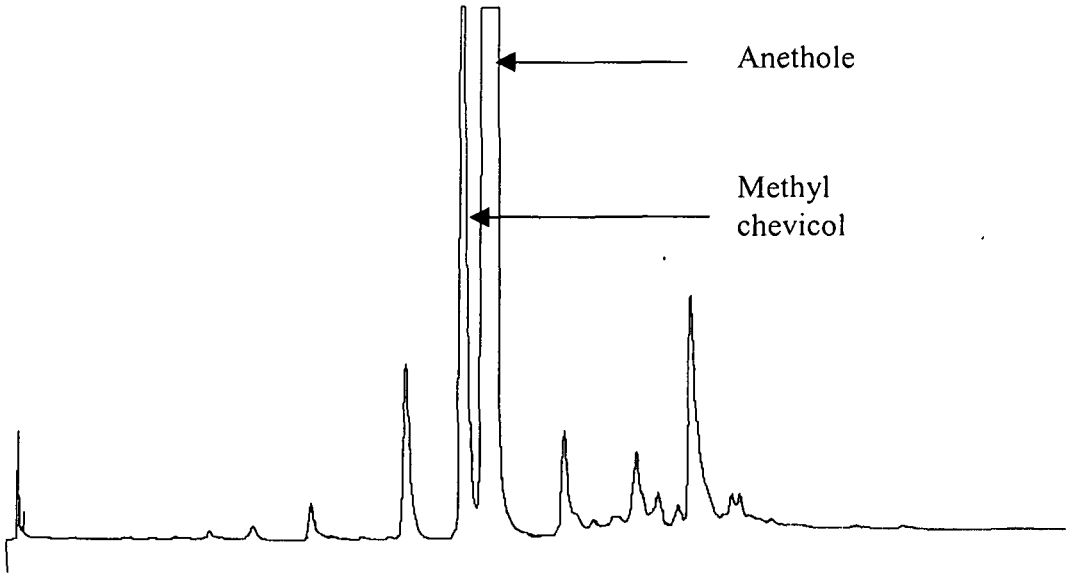

Fig. 4.6 GC chromatogram of essential oil from *Karphul* rhizome.

4.3.2 Antimicrobial bioassay of essential oils

Despite differences in the methods of assessment, it is clear that many higher plants contain compounds of interest and value to mankind. Secondary metabolites of the plants have always been shown to be active against food poisoning bacteria such as *Staphylococcus aureus, Salmonella enteritidis* and *Clostridium botulinum,* in addition to food spoilage organisms like lactic acid bacteria, *Bacillus cereus, Pseudomonas fluorescens* etc. Culinary compounds such as garlic, vanillin and eugenol from clove have been demonstrated to inhibit the growth of pathogenic and dimorphic yeast *Candida albicans, Cryptocovvus neoformans,* etc.

A study was undertaken to test the activity of essential oils (isolated through hydrodistillation) of *Karphul* rhizome against five pathogenic bacteria *viz.*, *Bacillus subtilis, Staphylococcus aureus, Micrococcus flavus*, *Escherichia coli* and *Klebsiella pneumoniae* and one fungal pathogen *viz.*, *Candida albicans* by following the methods described in Chapter III (Materials and Methods). The antimicrobial spectra showing zone of inhibition in millimeters (mm) against each microorganism was recorded and the results are presented in Table 4.9 and Fig 4.7.

Table 4.9 Zone of inhibition (mm) in culture plates of pathogenic microorganisms by essential oils isolates from *Karphul* rhizome

Microorganisms	Zone of inhibition (mm) ± S.E.	
	Essential oils	Control (DMSO)
Bacillus subtilis (gram +ve)	18.0 ± 0.24	0.0 ± 0.0
Staphylococcus aureus (gram +ve)	36.5 ± 0.24	0.0 ± 0.0
Micrococcus flavus (gram +ve)	14.5 ± 0.24	0.0 ± 0.0
Escherichia coli (gram -ve)	21.2 ± 0.28	0.0 ± 0.0
Klebsiella pneumoniae (gram -ve)	17.4 ± 0.15	0.0 ± 0.0
Candida albicans (fungus)	40.2 ± 0.14	0.0 ± 0.0

(Data are average of three repetitions)

4.3.3 Aromatic compounds

a) Extraction of crude from *Karphul* rhizome

The ground rhizome paste of *Karphul* (80 g) extracted with 250 ml of dichloromethane for 8 h in a Soxhlet apparatus and then evaporated the solvent to dryness in a rotary evaporator. The yield of crude flavoury compound was determined on the dry weight basis.

b) Thin Layer Chromatography (TLC) for fractionation

The TLC was performed to determine the suitable solvent systems and their effective concentrations for separating the specific compounds from the prepared crude extract. The solvent system used was *n*-hexane : dichloromethane with the proportionate combinations of 1:5, 1:2, 1:1, 2:1, 5:1 and 6:0. The retention factor (R_f) values of these four spots were determined and the values obtained are presented in Table 4.10.

Table 4.10 Fractions separated by TLC from the crude extract of *Karphul* rhizome and their R_f values

Fractions	R_f values
Fraction – I	0.35
Fraction – II	0.40
Fraction – III	0.69
Fraction – IV	0.89

c) Preparative TLC for collecting the fractions

Based on the above TLC experiment, the preparative TLC was performed to collect the separated fractions. Four fractions obtained were eluted separately; solvents were evaporated to dryness and the compounds weighed. The isolated fractions with their amount are shown in Table 4.11.

Table 4.11 The isolated fractions with their weight (mg)

Fractions	Weight (mg)
I	103
II	21
III	22
IV	21

4.3.4 Antimicrobial bioassay of the TLC purified fractions

An experiment was designed to test the activity of four TLC purified fractions from dichloromethane extract of rhizome of the plant against four pathogenic bacteria *viz., Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumoniae* and one fungal pathogen *viz., Candida albicans* as described in Chapter III. The results showing zone of inhibition in millimeters against each microorganism were recorded and the same are presented in Table 4.12 and Fig. 4.8.

Table 4.12 Zone of inhibition in culture plates of different pathogenic microorganisms by different TLC purified fractions from crude extract of *Karphul* rhizome

Fractions	Zone of inhibition (mm) ± SE				
	B. subtilis	*S. aureus*	*E. coli*	*K. pneumoniae*	*C. albicans*
F-I	0.0 ± 0.0	17.0 ± 0.47	18.3 ± 0.72	20.7 ± 0.27	15.5 ± 0.24
F-II	0.0 ± 0.0	21.3 ± 0.14	15.2 ± 0.49	31.5 ± 0.41	20.3 ± 0.27
F-III	0.0 ± 0.0	12.5 ± 0.24	16.8 ± 0.14	29.8 ± 0.49	0.0 ± 0.0
F-IV	13.5 ± 0.62	21.3 ± 0.72	13.2 ± 0.59	24.5 ± 0.41	22.1 ± 0.05
Control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

(Data are average of three repetitions)

4.3.5 Identification of TLC purified fraction

The crude dichloromethane extract of *Karphul* rhizome fractionated through TLC in to four fractions were collected separately. Out of these four fractions, only Fraction I was subjected to further analysis (GC-MS, FTIR and ^{1}H-NMR) for the identification of compounds present in it, rest could not be analyzed because of their poor yield.

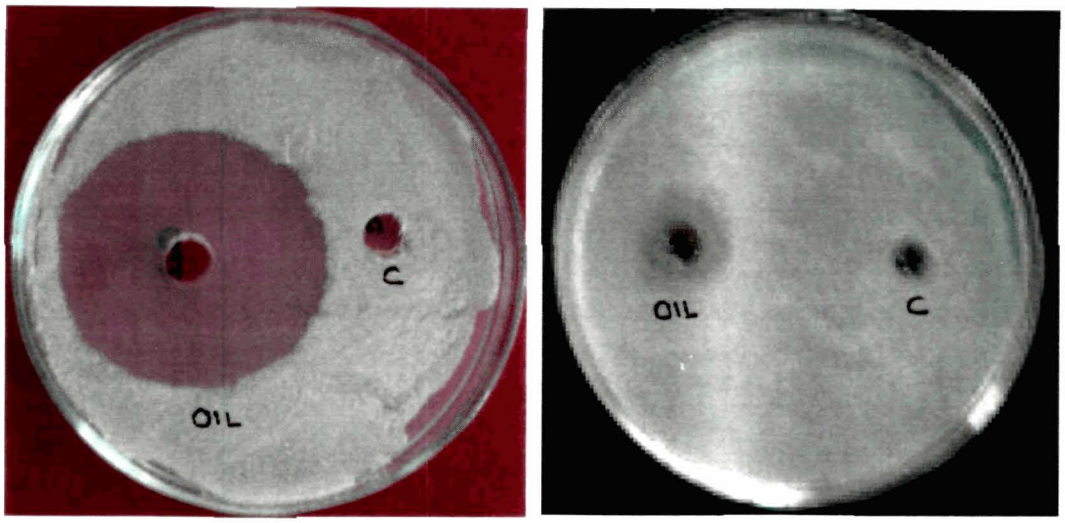

Fig. 4.7 Activity of essential oils against *C. albicans* and *M. flavus*

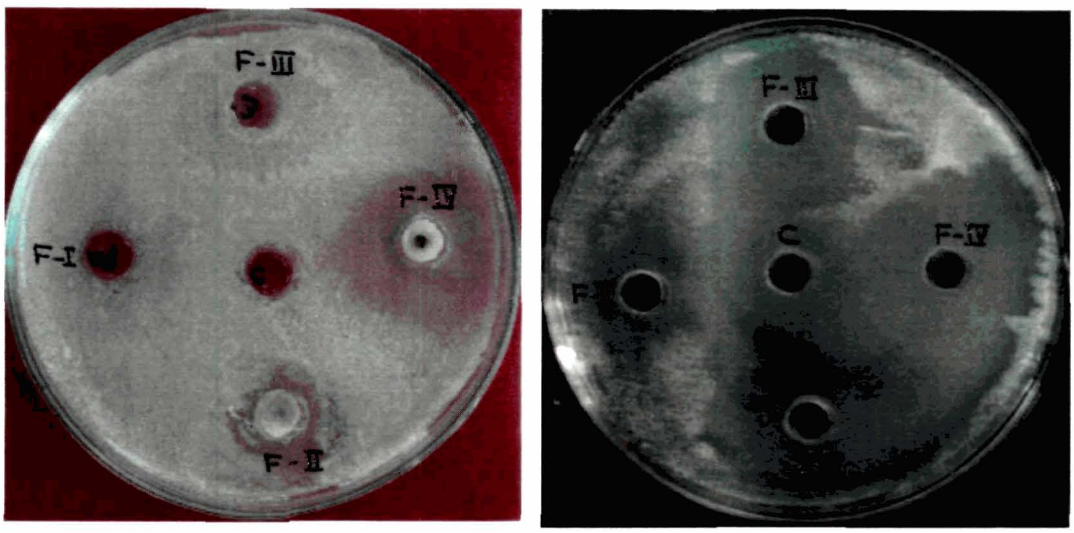

Fig. 4.8 Activity of different fractions against *S. aureus* and *K. pneumoniae*

a) Gas Chromatography and Mass Spectroscopy (GC-MS)

The results of the Gas Chromatography-Mass Spectroscopy analysis of the fraction for the identification of the compound are presented in Fig. 4.9 and Fig. 4.10.

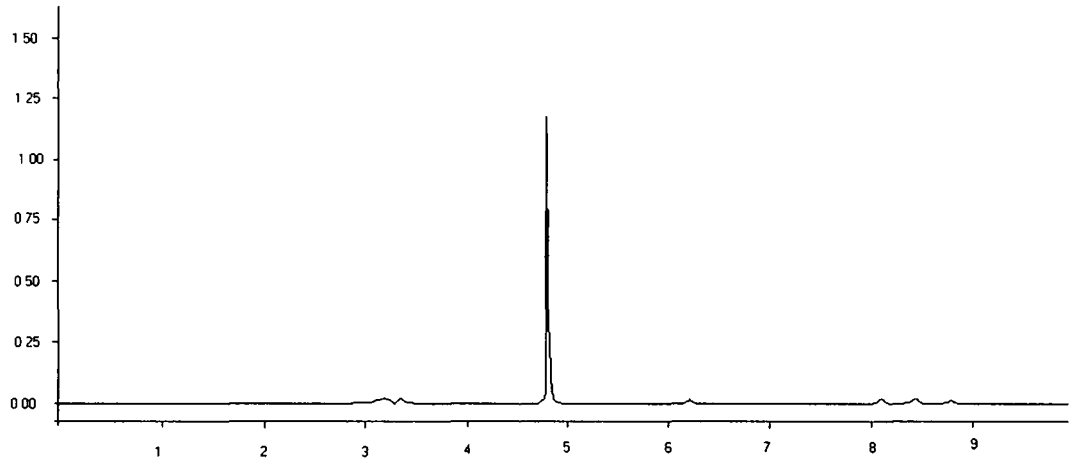

Fig. 4.9 GC chromatogram of the Fraction I purified through TLC

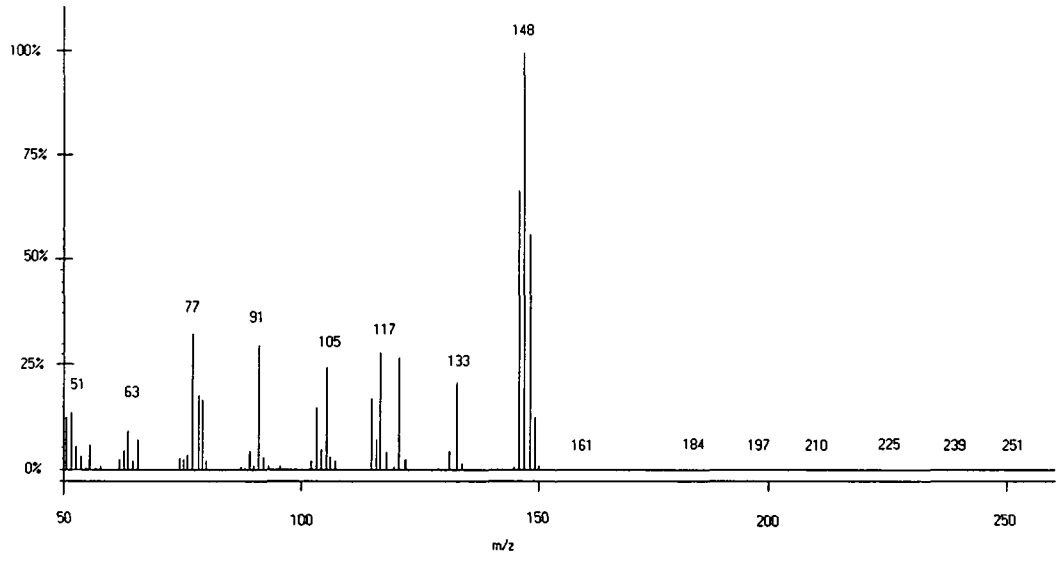

Fig. 4.10 Mass Spectra of the Fraction I

Comparing the mass spectra results with that of the Saturn 2000 MS Library data and reference data from the NIST, USA, a probable chemical structure was elucidated and the same is presented in Fig. 4.11.

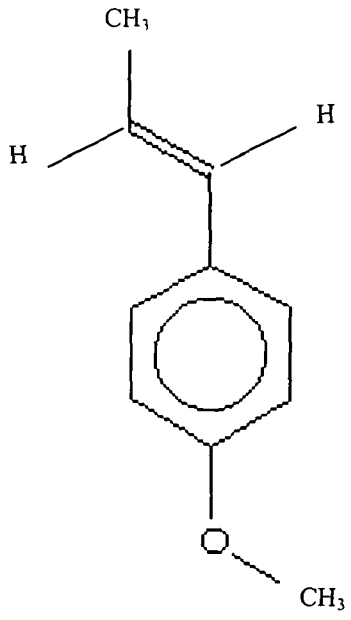

Fig. 4.11 Probable chemical structure of 1-methoxy-4-(1-propenyl)-benzene (from the Saturn 2000 MS Library)

b) FTIR spectroscopy

To support the above chemical structure obtained from the MS spectra, the compound was subjected to FTIR spectroscopy. The FTIR spectrum of the compound is presented Fig. 4.12.

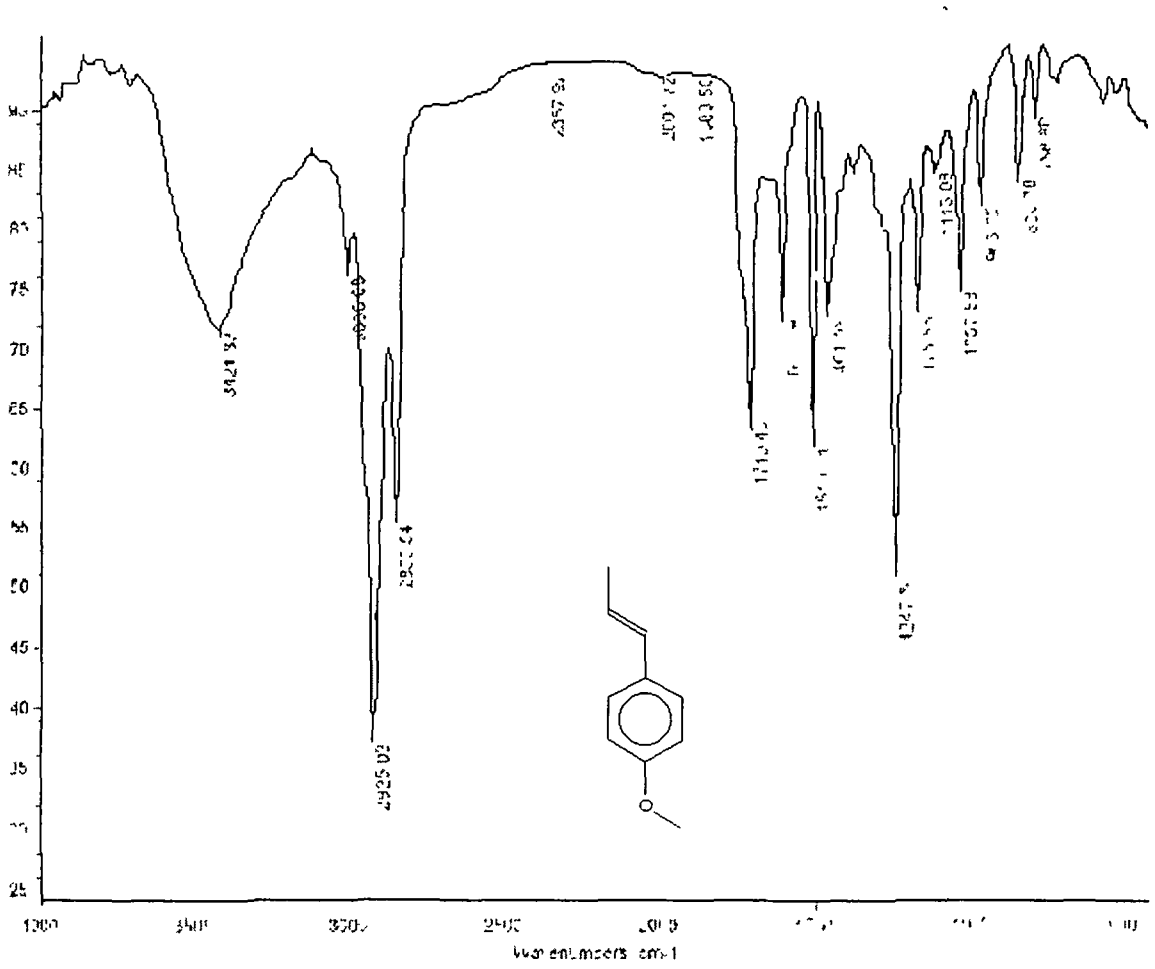

Fig. 12 FTIR spectra of 1-methoxy-4-(1-propenyl)-benzene

c) ^{1}H-NMR spectroscopy

For further confirmation of the structure, the compound was subjected to the ^{1}H-NMR analysis The ^{1}H-NMR spectrum generated is presented in Fig. 4 13.

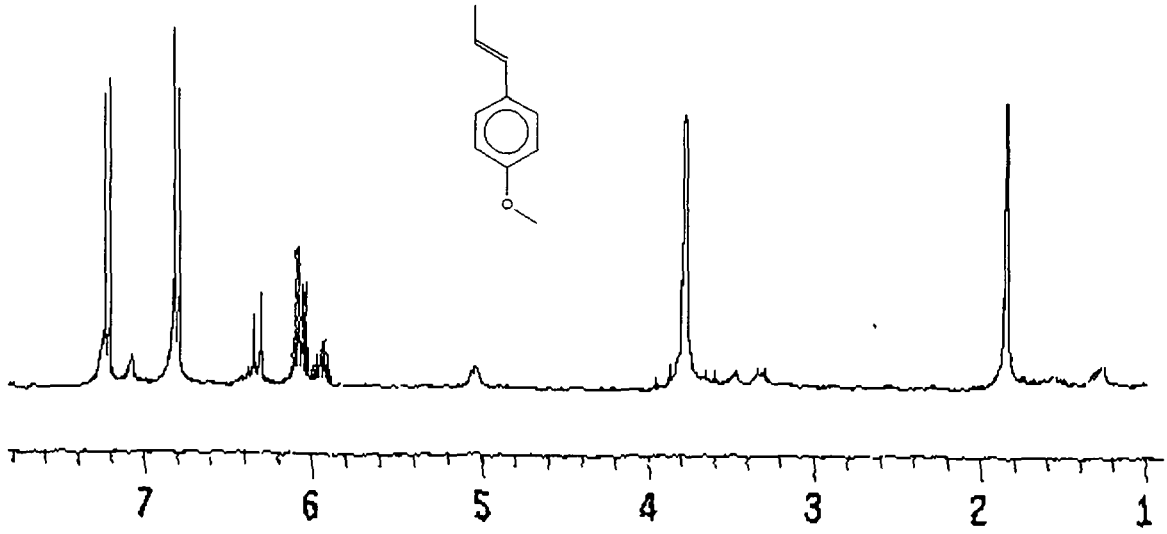

Fig. 4.13 ^{1}H-NMR spectra of 1-methoxy-4-(1-propenyl)-benzene

4.4 Molecular characterization

4.4.1 Genomic DNA isolation

A modified CTAB-PVP isolation protocol was standardized. From 3 g fresh tender leaf sample of *Karphul* the genomic DNA was isolated.

4.4.2 DNA quantification and purity test by UV spectroscopy

For quantification, O.D. (optical density) at 260 nm of isolated genomic DNA, was recorded and the concentration of isolated DNA was calculated by the formula

1 O.D. (at 260 nm) = 50 μg/ml

The purity of the isolated DNA was checked by the ratio $\lambda_{260}/\lambda_{280}$. The value of the ratio should be between 1.8 to 2.0.

4.4.3 Agarose Gel Electrophoresis

The isolated and purified genomic DNA of *Karphul* plant was electrophoresed through ethidium bromide-stained 0.8% agarose gel running along side the standard molecular weight marker λ DNA digested with *Hind* III. Photograph of the gel was taken with the help of Gel Doc System and the same is presented in Fig. 4.14.

4.4.4 Restriction digestion

The genomic DNA of *Karphul* was digested separately by restriction endonuclease enzymes *Eco*R I and *Hind* III and also by combination of both for double digestion to confirm the purity of the isolated DNA. After the digestion, all these samples were electrophoresed. The photograph of the gel was taken and the same is presented in Fig. 4.15.

Fig. 4.14 Isolated genomic DNA from *Karphul* plant resolved in 0.8% agarose gel. Lane 1: *Hind* III digested λ DNA, Lane 2: Genomic DNA

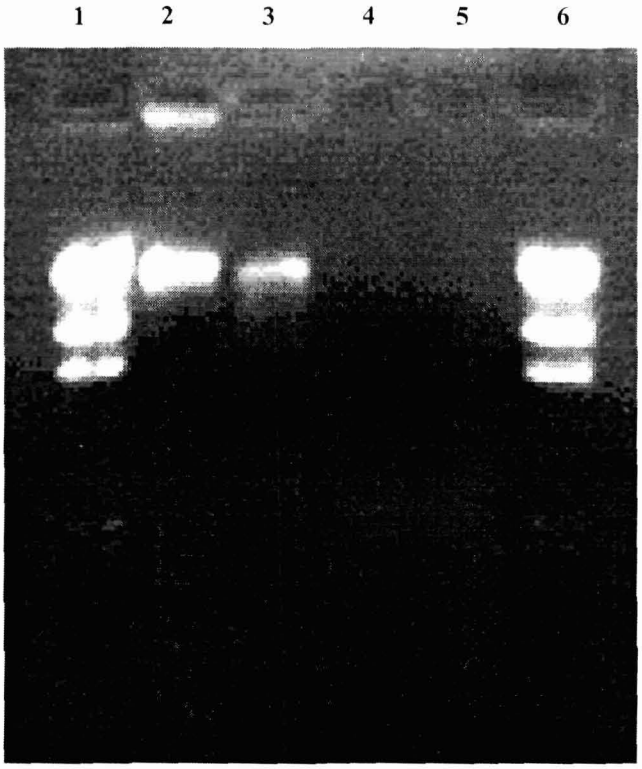

Fig. 4.15 *Karphul* genomic DNA restriction pattern using the restriction endonuclease enzyme *Eco*R I and *Hind* III. Lane 1 & 6: *Hind* III digested λ DNA, Lane 2: Undigested genomic DNA, Lane 3: *Eco*R I digested λ DNA; Lane 4: *Hind* III digested DNA and Lane 5: *Eco*R I-*Hind* III digested DNA.

4.4.5 Genome size (DNA C-value) determination

The genome size (DNA C-value) of an organism is the amount of nuclear DNA in the haploid or single set of chromosome present in the nucleus. For determining the amount of DNA in the nucleus of a cell of *Karphul,* a novel method was employed, where the average volume of a cell of leaf tissue (Table 4.13) and other related parameters were calculated by the method as described in Materials and Methods.

Table 4.13 Mean length (L), breadth (B), thickness (T), radius (r) and volume of the randomly selected cells of leaf tissues of *Karphul*

Cell No.	L (μ) Mean	B (μ) Mean	T (μ) Mean	r (μ) Mean	Volume (μ^3)
Epidermal cells*					
1.	58.85 ± 3.48	33.75 ± 2.80	60.25 ± 2.74	-	1,19,668
2.	70.00 ± 6.78	45.00 ± 4.95	62.50 ± 2.99	-	1,96,875
3.	47.50 ± 4.14	32.50 ± 3.64	52.00 ± 4.49	-	80,275
4.	37.50 ± 3.40	30.00 ± 4.11	50.00 ± 5.62	-	56,250
5.	65.50 ± 5.78	45.00 ± 4.21	59.50 ± 4.82	-	1,75,406
Spherical mesophyll cells**					
6.	-	-	-	20.0 ± 2.20	33,510
7.	-	-	-	12.5 ± 2.36	8,181
8.	-	-	-	30.0 ± 3.41	1,13,098
9.	-	-	-	24.9 ± 3.34	64,668
10.	-	-	-	28.0 ± 2.51	91,953
Cylindrical palisade parenchyma cell***					
11.	40.0 ± 6.09	-	-	10.0 ± 1.51	12,566
12.	65.5 ± 4.19	-	-	20.0 ± 2.87	82,310
13.	70.0 ± 5.37	-	-	24.8 ± 3.60	1,35,586
14.	52.5 ± 3.58	-	-	13.5 ± 2.02	30,059
15.	69.5 ± 6.53	-	-	26.5 ± 5.04	1,53,330
				Average volume	90,249

(*Volume of rectangular cell = L x B x T, **Volume of a spherical cell = 4/3 x л x r^3 and *** Volume of cylindrical cell = л x r^2 x L, where л = 3.1416.)

The average volume of a cell in the leaf tissue (Av. vol. of 15 randomly selected cells presented in Table 4.13) $= 90{,}249\ \mu^3$

Vol. of the leaf tissue (1.0 cm x 1.0 cm x 300 μ) $= 3.0 \times 10^{10}\ \mu^3$

Vol. of intercellular space (32% of total vol.) $= (3.0 \times 10^{10} \times 0.32)\ \mu^3$

$= 9.6 \times 10^{9}\ \mu^3$

Actual cell mass $= (3.0 \times 10^{10} - 9.6 \times 10^{9})\ \mu^3$

$= 2.04 \times 10^{10}\ \mu^3$

Total number of cells in the cell mass $= \dfrac{\text{Actual cell mass}}{\text{Average volume of single cell}}$

$= \dfrac{2.04 \times 10^{10}}{90{,}249}$

$= 2{,}26{,}041$

Now, wt. of the tissue section $= 0.025$ g

Or, 0.025 g tissue contains cells $= 2{,}26{,}041$

So, in 1.0 *g* tissue, number of cells $= (2{,}26{,}041 \times \dfrac{1}{0.025})$

$= 90{,}41{,}640$

Genomic DNA isolated from 1.0 *g* leaf tissue $= 28.0\ \mu g$

$= 28.0 \times 10^{6}$ pg

i.e., 90,41,640 cells contain DNA $= 28.0 \times 10^{6}$ pg

Therefore, one cell contains DNA $= \dfrac{28.0 \times 10^{6}}{90{,}41{,}640}$ pg

$= 3.096$ pg

$\cong 3.1$ pg

Since, the species is tetraploid (2n = 48) (from karyotypic study)

So, the genome size or DNA content of the haploid set of chromosome (n) will be

$$= \frac{3.10}{2} \text{ pg DNA} = 1.55 \text{ pg DNA} = 1.52 \times 10^{9} \text{ base pair}$$

$$(1.0\, pg \text{ DNA} = 9.78 \times 10^{8} \text{ bp})$$

4.5. Tissue culture

4.5.1 Induction of callus from young rhizome

Young rhizome explants of *Karphul* consisting of vegetative buds were cultured for the induction of callus on MS (M) medium supplemented with 2,4-D, NAA, BAP and Kin either singly or in combinations. The responses of different hormones are presented in Table 4.14 and Fig. 4.16 A.

Swelling of cultured tissue explants was observed subsequent to culturing for 10 - 15 days in the medium supplemented with 2,4-D (1.0 – 2.0 mg/l) and Kin (0.5 – 1.0 mg/l) alone and combinations of 2,4-D (1.0 and 2.5 mg/l), NAA (1.0 and 2.0 mg/l) and BAP (0.5 and 1.5 mg/l) but failed to show callus formation. But these tissue explants subsequent exhibited die back after 50 days of culture. Combinations of 2,4-D (2.0 mg/l) and BAP (0.5 – 2.0 mg/l) showed swelling of tissue explants cultured for 10 – 15 days and subsequently induced callus after 30 days. The calli thus produced were green, friable and loosely arranged. The MS medium supplemented with combinations of 2,4-D (1.0 - 2.0 mg/l), NAA (1.0 – 1.5 mg/l) and BAP (0.5 – 1.0 mg/l) produced compact nodular calli after 30 days culture. But medium supplemented with 2,4-D (3.0 mg/l) and NAA (0.5 – 2.0 mg/l) failed to show response.

Table 4.14 Effect of different growth regulators on callus induction from rhizome explants of Karphul on MS (M) medium

Growth regulators (mg/l)				Response
2,4-D	NAA	BAP	Kin	
1.0	-	-	-	Swelling, no response afterward
2.0	-	-	-	Swelling, no response afterward
3.0	-	-	-	No response
-	0.5	-	-	No response
-	1.0	-	-	No response
-	2.0	-	-	No response
2.0	-	1.0	-	Swelling and green loose callus formation after 30-35 days
2.0	-	1.5	-	Swelling and green loose callus formation after 25-30 days
2.0	-	2.0	-	Swelling and small amount of green loose callus formation after 35-40 days
-	-	-	0.5	Swelling, no response afterward
-	-	-	1.0	Swelling, no response afterward
1.0	1.0	0.5	-	Swelling, no response afterward
1.5	1.0	0.5	-	Swelling and formation of compact nodular callus
1.5	1.0	1.0	-	Swelling and formation of compact nodular callus
2.0	1.5	1.0	-	Swelling and formation of compact nodular callus
2.5	2.0	1.5	-	Swelling, no response afterward

(Each figure represents a mean of 10 replicates; growth period 50 days)

4.5.2 Organogenesis

Sixty days old compact nodular calli developed in 2,4-D (1.0 - 2.0 mg/l), NAA (1.0 – 1.5 mg/l) and BAP (0.5 – 1.0 mg/l) – supplemented medium were transferred to MS medium containing different levels of cytokinin and auxin for the differentiation of calli in to shoots and roots. The results are presented in Table 4.15, Table 4.16 and Fig. 4.16 A - D.

4.5.3 Direct multiple shoot regeneration from young rhizome explants

Young rhizome explants of *Karphul* consisting of vegetative buds were also cultured for multiple shooting and rooting in MS (M) medium supplemented with BAP, NAA, IAA and KIN either singly or in combinations. Results obtained in different hormone supplemented media are presented in Table 4.17 and in Fig. 4.16E.

Table 4.15 Effect of different growth regulators on organogenesis from calli of *Karphul*

Growth regulators ($mg.l^{-1}$)					Response
BAP	2,4-D	NAA	KIN	IAA	
1.0	-	-	-	-	No response
1.5	-	-	-	-	3 – 4 nos. of adventitious roots and 2 – 3 nos. of rudimentary shoots
2.0	-	-	-	-	3 – 4 nos. of adventitious roots and 2 – 3 nos. of rudimentary shoots
1.5	0.1	-	-	-	3 – 4 nos. of adventitious roots and 2 – 3 nos. of rudimentary shoots
1.5	0.25	-	-	-	Produced tiny meristematic protuberances
2.0	0.25	-	-	-	No response
5.0	0.25	-	-	-	No response
-	-	-	1.0	0.1	No response
			2.0	0.5	No response
1.0	-	-	-	0.5	No response
1.5	-	-	-	0.1	10 – 12 nos. of adventitious roots and 3 – 4 nos. of rudimentary shoots
1.5	-	-	-	0.5	No response
2.0	-	-	-	0.5	No response
1.0	-	0.5	-	-	No response
1.5	-	0.5	-	-	No response
2.0	-	1.0	-	-	No response

(Each figure represents a mean of 10 replicates; growth period 60 days)

Table 4.16 Subculture of rudimentary shoots on media supplemented with high concentrations of cytokinin and low concentrations of auxin

Growth regulators ($mg.l^{-1}$)					Response
BAP	KIN	2,4-D	IAA	NAA	
10.0	-	-	-	-	No response
10.0	-	0.1	-	-	No response
	10.0	0.1	-	-	No response
10.0	-	-	0.1	-	No response
-	10.0	-	0.1	-	No response
10.0	-	-	-	0.1	No response
-	10.0	-	-	0.1	No response
-	10.0	-	-	-	No response

(Each figure represents a mean of 10 replicates; growth period 60 days)

Table 4.17 Effect of different growth regulators on multiple shoot and root formation from *Karphul* rhizome

Growth regulators ($mg.l^{-1}$)					Response
BAP	KIN	2,4-D	IAA	NAA	
2.0	-	-	-	-	No response
5.0	-	0.25	-	-	1 – 2 shoots with 2 – 3 roots developed
7.0	-	0.25	-	-	2 - 4 shoots with 3 – 4 roots developed
10.0	-	0.25	-	-	4 - 6 shoots with 3 – 5 roots developed
-	2.0	-	-	-	No response
-	5.0	-	0.25	-	1 – 2 shoots with 1 – 3 roots developed
-	7.0	-	0.25	-	1 – 2 shoots with 1 – 3 roots developed
-	10.0	-	0.25	-	1 – 2 shoots with 1 – 3 roots developed
5.0	-	-	-	0.25	1 – 2 shoots with 2 – 3 roots developed
7.0	-	-	-	0.25	1 – 2 shoots with 2 – 3 roots developed
10.0	-	-	-	0.25	1 – 2 shoots with 2 – 3 roots developed

(Each figure represents a mean of 10 replicates; growth period 60 days)

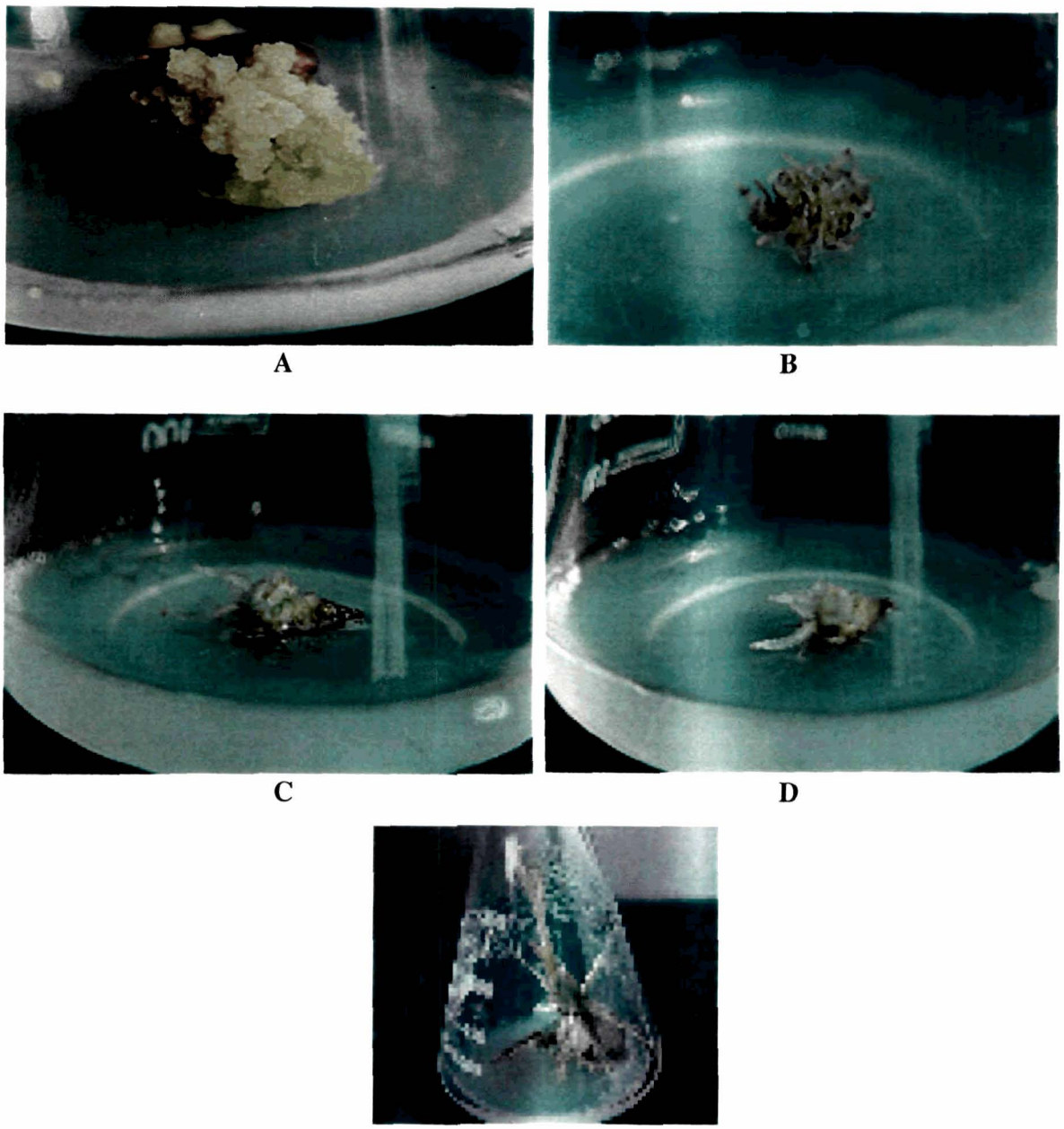

Fig. 4.16 A - Green friable callus, B - 10-12 adventitious roots and 3-4 shoots C and D – Rudimentary shoots and adventitious roots generation and E - Direct shoot and roots regeneration from rhizome

CHAPTER V

DISCUSSION

Motivated by the preliminary observations, based on field study, literature survey and visit to the herbarium maintained by the Botanical Survey of India, Shillong we explored the *Karphul* plant scientifically with respect to its taxonomy, karyotype, biochemical characterization, antimicrobial activity, molecular characterization and tissue culture. Results obtained from the investigation are discussed below.

5.1 Taxonomy

For proper identification, it is necessary to describe a plant taxonomically and compare the same with the herbarium specimen, literature available and field verification. In this context, taxonomic study including morphology and floral biology was carried out. Results, thus obtained were compared with the closest specimen under the same family present at the herbarium of the Botanical Survey of India, Shillong and also verified with the literature available to identify the species as well.

The results of morphological study (Table 4.1, Table 4.2 and Fig. 4.1) revealed that the plant possesses a hard-green pseudostem having 250 - 300 cm in height. The pseudostem is surrounded by several leafy sheaths arising out of the nodal positions. The base of the stem is pubescent and somewhat swollen having circumference 10 - 15 cm which gradually decreases to 12 - 8 cm at a height of about 1.0 m from the base. The plant bears 6 - 15 leaves, each measuring 50 - 70 cm in length and 9 - 15 cm in breadth. Leaves are petiolated, distichous, sheathing and with distinct ligules (0.9 - 1.2 cm). Ligule is hairy, serrulate, green in the young leaves and brown in the older leaves. The mean petiole length is 0.44 cm, hairy in the inner surface. Leafblade is green, glabrous and oblong-lanceolated. Mid-rib is prominent and grooved. Venation is parallel, veins are

distinct. The plant possesses a long modified underground stem called as rhizome covered with numerous scales. The rhizome is strong, thick, pubescent and fibrous having a strong but pleasant aroma. The colour of the rhizome is brownish outside and white inside with circumference 5.0 - 9.5 cm. New shoots appear from the eyes of the rhizome.

The floral biology study of the plant revealed that conspicuously it bears a limited number of spikes (3 - 4 spikes per year) and stops flowering if disturbed frequently due to collection of rhizomes, damage by stray cattle etc. During the investigation, it has been found that the plant flowers year round throwing new spikes from the nodal position of the mature rhizome. The duration from floral bud initiation to full bloom was found to be 69 - 81 days. However, no fruit and seed formation was observed during the study period.

The results obtained from the study of inflorescence (Table 4.3, Table 4.4 and Fig. 4.1) revealed that the inflorescence is a spike, club-shaped, solitary, sub-terranean and 11.0 - 16.0 cm long arising from the rhizome. The spike is creamy white with light greenish tinge in colour at the basal portion while the top portion is red with yellow margin and it is thick like a thumb. Single spike bears 8 - 14 florets arranged in a concentric circle on a slightly curved peduncle. Two to four florets open at a time. Peduncle is short, 2.8 - 4.6 cm long and embedded with the ground, thick like the little finger, covered with numerous light brown-sheathed scales. Sometimes, the spike remains invisible as being covered by soil, stem, weeds etc. The spike doesn't elongate with blooming of flowers. The base is surrounded by 4 - 9 numbers of sterile involucral bracts. Bracts are sessile, imbricately appressed, oblong, hard, acute and 2.0 - 6.0 cm in

length and 1.2 - 3.0 cm in breadth. They are white in colour with light greenish tinge at the tip.

The elongation of spike and the presence of involucral bracts are the two most important distinguishing features to *Etlingera* and allied genera (Tribe Alpineae) of the family Zingiberaceae (Burtt and Smith 1986; Prakash and Tripathi 1998).

Flowers of the plant are sessile, complete, bisexual, zygomorphyic and epigynous with single bract. Flowers are creamy white in colour at the base and the rest part is red with a yellow lip, 6.6 - 12.8 cm in length. The individual floret is enclosed at the base covering half diameter by a lanceolate, membranous, striate inner bract (floral bract) white in colour with light greenish tinge towards the tip. The length of the floral bract is 2.0 - 6.0 cm and width 1.2 - 2.0 cm. The bracteole is a tubular, membranous structure and it surrounds the floret upto a length of 3.0 - 3.8 cm from the base then it opens up into two unequal parts with pointed tips whose length ranges from 1.9 - 2.5 cm. The bottom portion is white in colour while the top portion is light reddish.

Calyx is an elongated tubular and membranous structure, which encloses the corolla tube upto a length of 4.5 - 5.0 cm from the base then it opens up and extends upto 2.5 - 3.0 cm with a dentate tip. The bottom portion of the calyx is light reddish in colour and the intensity increases to deep red towards the tip.

Corolla is gamopetalous and tubular, its length is 4.8 - 8.0 cm. The diameter of the tube increases gradually from its base (0.2 cm) towards the end (0.4 cm) of the tube. The tube is white at the base and it becomes light reddish at the top having three membranous petals at a height of 2.8 - 5.5 cm from the base. Petals are ovate, deep red in colour having the size 1.5 - 3.0 x 0.9 - 1.0 cm with an acute apex. At the top of the corolla

tube, half of it fused with the stamen forming a staminal tube with the labellum, slightly incurved towards the centre of the tube, which bears an elongated anther. The other half of the tube, which is opposite to the stamen, has a modified fleshy tongue-shaped labellum with a long lanceolated tail having the length of 4.5 - 6.0 cm. Deep red blotches in the middle of the labellum having incurved frilled yellow margin and emarginated at the tip. The base of the labellum is fleshy and expanded, forming a collar surrounding the stamen and adnate to the base of the filament forming a separate and distinct tube like structure (staminal tube). This is also an important distinguishing key to *Etlingera* and allied genera (Tribe Alpineae) of the family Zingiberaceae (Burtt and Smith 1986; Prakash and Tripathi 1998).

The lateral staminodes are obsolete. The stamen is opposite to the labellum and the filament is short, flat, fleshy, stiff and white in colour. The size of the filament is 0.3 - 0.5 x 0.5 cm and it bears an anther at the tip. The anther is 2-lobed, divided by a deep longitudinal connecting groove, the top of the lobe is flat but slightly slanting towards the connecting groove. It is longer than the filament having the size of 0.7 x 0.4 cm, keeled on the back, light reddish to white at the base as well as inner side and deep red at the top as well as the outer side.

Style is single and slender, 4.3 - 7.0 cm long, white in colour and it passes through the anther lobe. At the base, the style is enclosed by two appendages *ca.* 0.4 x 0.2 cm in size. The appendages are free from each other and attached with the ovary. The stigma is deep red in colour and triangular with three lobes. Ovary is 0.5 - 0.7 cm in length, 0.1 cm in diameter, villous, inferior and trilocular with many ovules on axile placenta. Pollen grains are spherical, 48 - 50 μm in diameter.

The study on anthesis (Table 4.5) revealed that the florets of the herb start anthesis at around 5.30 in the morning and continues up to 1.30 in the afternoon. The percent anthesis of florets shows a sharp increase which attains peak (26%) in between 6.30 – 7.30 am and gradually starts decreasing and completely ceases at 1.30 pm as shown in Fig. 4.3.

The florets are sterile and the reason for the sterility may be due to the higher position of the stigma over the anther. The lower position of the anther prevents pollen grains to reach the stigma during dehiscence resulting no pollination. Moreover, the florets remain closed due to the formation of the tube like structure of the corolla at the top part by the base of the labellum and the filament. This arrangement of the corolla restricts the movement of natural pollinating agents resulting in lack of cross-pollination too. Probably, due to these reasons, the plant fails to produce seeds; however, this needs further investigation.

The description of the herb presented above with respect to its morphological and floral characteristics and comparison with the characteristics of other species under the genus *Etlingera* available at the BSI Herbarium, Shillong; thorough literature survey (A.S. Rao and D.M. Verma, 1972 and 1975; Prakash and S. Tripathi, 1998) and field observations revealed that the herb is allied to *E. linguiformis* (Roxb.) Smith in general habit, but distinct from the later in having taller plant height (259-300 cm), hairy and serrulate ligule, hairy petiole on the inner side, bigger lamina size (50-70 cm x 9-15 cm), shorter peduncle (2.8-4.6 cm), outer bract being white with light greenish tinge at the tip, 8-14 florets/spike, the calyx tube being shorter than the corolla tube with dentate tip and red in colour. In contrast, *E. linguiformis* is 200 cm tall with ligule being entire without

hair, smaller lamina (48 – 58 x 5 –9 cm), longer peduncle (± 8 cm), outer bract white-pink in colour, less number of florets/spike (7-9), calyx much longer than the corolla tube, apically tridentate, pink in colour (Prakash and Tripathi, 1998). Therefore, the present herb might be a new species under the genus *Etlingera* reported from this part of India apart from *E. linguiformis* (Roxb.) Smith and *E. loroglossa* (Gagnep) Smith.

5.2 Karyotype study

The experiment carried out to determine the actual time of cell division with the starting of chromosomal movement in somatic cells through the collection and fixation of root tips in the early morning from 4.15 h to 8.15 h (Table 4.6) with 30 min interval revealed maximum dividing cells in the root tips collected between 6.15 – 6.45 am. The root tips collected till 4.45 am did not reveal any dividing cell, whereas, those collected at 5.15 am and 5.45 am showed some dividing cells. This indicated that the active cell division in this plant starts at around 5.15 am in the morning. On the other hand, the root tips collected at 7.15 am and 7.45 am showed few dividing cells; those collected at 8.15 am and thereafter (not shown in table) failed to show any dividing cell. This proved the fact that the active cell division in this herb lasts for 150 - 180 min (2.30 - 3 h) between 5.15 to 7.45 am.

Observations of slides prepared following the 'Squashing Method' (Sharma and Sharma, 1980) under the microscope revealed the somatic chromosome number of *Karphul* to be 2n = 48 (Fig. 4.3; Fig. 4.4 and Fig. 4.5). The detailed study of chromosomes revealed tetraploid nature of the herb with the basic chromosome number (x) being 12. Beltran and Kiew (1984) also reported tetraploid *Etlingera* with basic

chromosome number (x) 12. Eksomtramage *et al.* (2002) reported chromosome number of 22 species belonging to 10 genera of Zingiberaceae distributed in Thailand to be in the range of 20 - 48 showing diploidy and polyploidy. The first ten species were *Alpinia purpurata* (Vielli) K. Schum. (2n = 48), *Boesenbergia* aff. *rotunda* (2n = 20), *Cornukaempferia aurantiflora* J. Mood and K. Larsen (2n = 46), *Curcuma* aff. *oligantha* Trimen (2n = 42), *C. rhabdota* Sirirugsa M.F. Newman (2n = 24), *Etlingera elatior* (Jack.) R.M. Smith (white form) (2n = 48), *E. hemisphaerica* (Bl.) R.M. Smith (2n = 48), *Hedychium gomezianum* Wall. (2n = 34), *H. longicornutum* Bak. (2n = 34) and *Zingiber* aff. *wrayi* (2n = 22). Further, they reported the chromosome numbers of 5 species viz., *A. henryi* K. Scum.; *A. purpurata* (Vielli) K. Schum.; *E. Elatior* (white form) (Jack.) R.M. Smith; *E. hemisphaerica* (B1.) R.M. Smith and *Hornstedtia leonurus* (Koenig) Retz in Alpineae (2n = 48) were tetraploid (2n = 4x = 48).

Karyotype data confirmed size (Table 4.7) of the chromosomes of the herb. The total length of haploid chromosome set was 31.2 μm. The length of each chromosome varied from 0.5 to 2.4 μm. The species showed chromosomes either with median or submedian centromere. On the basis of length, arm ratio and centromeric position, chromosomes of the herb are divided in to two groups. Group I consists of twelve (12) chromosome pairs (chromosome pairs 1, 2, 5, 6, 11, 12, 13, 14, 15, 16, 19 and 20) with median centromere and Group II consists of twelve (12) chromosome pairs (chromosome pairs 3, 4, 7, 8, 9, 10, 17, 18, 21, 22, 23 and 24) with submedian centromere. Studying chromosome number and karyotype of *Glycyrrhiza glabra, G. macdonica* and *G. uralensis*, (all perennial herbs) Verma and Nadkarni (1985) reported the basic chromosome number to be 2n = 16 in these three species. They reported the total length of haploid chromosome complement to be 24.4 μ, 22.6 μ and 20.2 μ in *G. glabra, G. macdonica* and *G. uralensis,* respectively. The length of chromosomes varied from 4.1 μ

to 1.9 μ in *G. macdonica*; 3.7 μ to 1.9 μ in *G. glabra* and 3.5 μ to 1.4 μ in *G. uralensis*. Chromosomes were either with submedian or median centromere.

5.3 Biochemical analysis of *Karphul* rhizome

5.3.1 Extraction and composition analysis of essential oils from *Karphul* rhizome

The essential oils extracted from the fresh rhizome paste of *Karphul* by hydrodistillation yielded 0.14% oil (w/w). The gas chromatography results revealed that the oil is a complex mixture of 23 components, of which 4 components accounted for 96.2%, while the minor or trace components constituted the remaining 3.8% (Table 4.8 and Fig. 4.6). Out of these 4 components, only 2 could be identified, one as anethole accounted for 86.0% and the other methyl chavicol accounted for 5.7% while the rest 2 components could not be identified. Ahmed *et al.* (2004) isolated essential oils and analyzed their constituents using GC and GC-MS from the rhizome of *Hedychium cylindreicum* Ridl. Similarly, Behura and Srivastava (2004) isolated essential oils from the leaves of *Curcuma* spp.; Chane-Ming *et al.* (2003) from rhizomes, leaves and flowers of *Zingiber zerumbet* Smith; Jantan *et al.* (2004) from leaves and seeds of *Alpinia galanga* (L.) Willed.; Pino *et al.* (2004) from the rhizome of *Zingiber officinale* Roscoe; Rout *et al.* (2003) from the seeds of green, freshly dried large cardamom (*Amomum subulatum* Roxb.); Arambewela *et al.* (2005) from rhizomes, roots and leaves of *Alpinia calcarata* Rose; Garg *et al.* (2005) from *Curcuma zedoaria* leaves; Kaul *et al.* (2005) from different parts of *Alpinia calcarata* Rose.; Mustafa *et al.* (2005) from the fresh rhizomes of *Curcuma amada* Roxb.; Raina *et al.* (2005) from rhizome and leaves of *Curcuma longa* and Zoghbi and Andrade (2005) from *Etlingera elatior* and *Zingiber spectabilis* isolated essential oil and analyzed using the same procedure.

5.3.2 Antimicrobial bioassay of the isolated essential oils of *Karphul* rhizome

Essential oils of aromatic and medicinal plants have been used in industries for the production of soaps, perfumes and toiletries. Many of them are also used in traditional medicines. The present investigation carried out to evaluate the antimicrobial activity of the essential oils isolated from *Karphul* rhizome (hydrodistillation) revealed that the diameter of the zone of inhibition of the essential oils varied from 40.2 mm 14.5 mm (Table 4.9). The essential oils showed largest zone of inhibition 40.2 mm against *C. albicans* (Fig 4.7) followed by 36.6 mm against *S. aureus*, 21.2 mm against *E. coli*, 18.0 mm against *B. subtilis*, 17.4 mm against *K. pneumoniae* and 14.4 mm against *M. flavus* (Fig 4.7). This clearly indicated the presence of differential levels of antimicrobial activity of *Karphul* essential oils against the tested pathogen as compared to the control (only DMSO) having no response at all. The essential oils of *Karphul* rhizome exhibited the highest antimicrobial activity against the fungus *C. albicans*. Thus, the essential oils of the herb could be a potential antifungal drug. Singh *et al.* (2002) reported antibacterial activity of essential oils of *Curcuma longa* rhizome against *S. aureus, S. epidermidis, E. coli, Pseudomonas aeruginosa, Salmonella typhimurium* and its activity was compared to standard antibiotics like gentamycin, ampicillin, droxycycline and erythromycin. Sartoratto *et al.* (2004) screened essential oils from aerial parts of *Mentha piperita, M. Spicata, Thymus vulgaris, Origanum vulgare, O. applii, Aloysia triphylla, Ocimum gratissimum, O. basilicum* against antibacterial and anti-*C. albicans* activity using bioautographic method. Most of the essential oils were effective against *Enterococcus faecium* and *Salmonella cholerasis. A. triphylla* and *O. basilicum* presented moderate inhibition against *S. aureus* while only *A. triphylla* and *M. piperita* were able to control

the yeast *C. albicans*. Khalighi-Sigaroodi *et al.* (2005) isolated essential oils from the aerial parts of *Ferulago bernardii* and evaluated antimicrobial activity by the broth dilution method in comparison to Gentamycin and Fluconazole as standard showed week activity against *S. aureus, B. subtilis, E. coli, C. albicans* and *Aspergillus niger*. The essential oils did not show any activity against *P. aeruginosa*.

5.3.3 Aromatic compounds

a) Extraction of crude from *Karphul* rhizome

The yield of the crude extract in dichloromethane from the ground rhizome paste of *Karphul* (80 g) was 0.49% following evaporation of the solvent. The solvent free extract was oily and light brownish in colour with strong anise flavour.

b) Thin Layer Chromatography for fractionation

From the preliminary TLC experiment to determine the suitable solvent systems and their effective concentrations for the fractionation of crude extract revealed that the chemicals *n*-hexane : dichloromethane with 5:1 combination was the best. This solvent system yielded four different clear spots. The rest of the combinations like 1:5, 1:2, 1:1, 2:1 and 6:0 failed to provide good separation. The experiment further revealed that the fractions were separated on the TLC plate according to their polarity. In the TLC plate, the fraction nearer to the starting point possessed the highest polarity but with the lowest R_f value as compared to the fractions away from the starting point (Table 4.10). Fraction-I possessed the highest polarity with lowest the R_f value (0.35) as compared to the other three fractions. The gradual increase of R_f values to 0.40, 0.69 and 0.89 in the fractions II,

III and IV, respectively revealed the gradual decrease in their polarity as was evident from their positions on the TLC plate (Table 4.10).

c) Preparative TLC for collecting the fractions

In the case of the preparative TLC experiment, the highest yield of 103 mg was obtained in the case of Fraction-I, whereas the Fraction-II, III and IV yielded 21.0 mg, 22.0 mg and 21.0 mg, respectively (Table 4.11).

5.3.4 Antimicrobial bioassay of the TLC purified fractions

The four TLC purified fractions from dichloromethane extract of *Karphul* rhizome were also evaluated for their antimicrobial activity against five pathogenic microorganisms viz., *B. subtilis, S. aureus*; *E. coli, K. pneumoniae* and *C. albicans* by following the 'Agar Well Diffusion' method. All four fractions showed differential antimicrobial activity against all the microorganisms except Fraction I, II and III against *B. subtilis* and Fraction III against *C. albicans* (Table 4.12 and Fig. 4.8).

Fraction IV showed differential size of zone of inhibition against all the microorganisms tested as compared to the control (DMSO), indicating the presence of antimicrobial activity in the fraction. The biggest zone of inhibition (24.5 mm) was observed against *K. pneumoniae* revealing the presence of the strongest activity against it followed by *C. albicans* (22.1 mm) and *S. aureus* (21.3 mm). Against *B. subtilis* and *E. coli,* it exhibited 13.5 mm and 13.2 mm inhibition zone, respectively, indicating the presence of weak activity against these pathogenic microorganisms as compared to *K. pneumoniae*, *C. albicans* and *S. aureus* (Table 4.12). The Fraction I showed the biggest

zone of inhibition against *K. pneumoniae* (20.7 mm) followed by *E. coli* (18.3 mm), *S. aureus* (17.0 mm) and *C. albicans* (15.5 mm) indicating the presence of antimicrobial activity. Likewise, the fraction II exhibited strong antimicrobial activity against *K. pneumoniae* (31.5 mm) followed by *S. aureus* (21.8 mm), *C. albicans* (20.3 mm) and *E. coli* (15.2 mm) (Table 4.12). On the other hand, the Fraction III inhibited the growth of *K. pneumoniae* (29.8 mm), *E. coli* (17.8 mm) and *S. aureus* (12.5 mm) only indicating the presence of antimicrobial activity against these three microorganisms, but failed to show activity against *B. subtilis* and *C. albicans* (Table 4.12). All four Fractions (I, II, III, and IV) exhibited the strongest antimicrobial activity against the pathogen *K. pneumoniae* as compared to the other pathogens. The results further indicated the presence of significant difference in antimicrobial activity against *S. aureus* between F-I and F-II, F-I and F-III, F-II and F-III and F-III and F-IV. The activity against *E. coli* was found to be significant between F-I and F-II, F-I and F-IV and F-III and F-IV. On the other hand, the significant difference was observed in the activity against *K. pneumoniae* between the F-I and F-II, F-I and F-III, F-I and F-IV, F-II and F-III, F-II and F-IV and F-III and F-IV. However, significant difference was observed in the activity against the fungal pathogen *C. albicans* between F-I and F-II, F-I and F-IV and F-II and F-IV. Somchit *et al.* (2003) evaluated the antimicrobial activity of ethanol and water extracts of leaves and barks of *Cassia alata* against *Aspergillus fumigeatus, Microspoum canis, Candida albicans, Staphylococcus aureus,* and *Escharichia coli. C. albicans* showed concentration-dependent susceptibility towards both ethanol and water extracts from the bark, but resistant towards the leaf extract. The water extract from barks showed larger inhibition zone than that of the ethanol extract (12 – 16 and 10 – 14 mm diameter, respectively).

The growth of *A. fumigatus* and *M. canis* was not affected by all types of plant extracts. Results were comparable to standard antifungal drug Tioconazole (18 mm diameter) at the equivalent concentration. The antibacterial activity of *C. alata* extracts on *S. aureus* was detected with the leaf extracts only using water and ethanol. The water extract exhibited higher antibacterial activity than the ethanol extract from leaves (inhibition zones of 11 – 14 and 9 – 11 mm, respectively). The bacteria *E. coli* exhibited resistance to all types of extracts.

5.3.5 Identification of TLC purified fraction

a) Gas Chromatography and Mass Spectroscopy (GC-MS) analysis

The result of the gas chromatography analysis on Fraction-I, purified and collected through TLC showed single peak at 4.8 min (Fig. 4.9), indicating the presence of a single compound in the fraction. Further, the Mass Spectroscopy analysis (Fig. 4.10) of the peak (molecular ion = 148) and subsequent comparison of results with the database of Saturn 2000 MS Library matched the compound with that of 1-methoxy-4-(1-propenyl)-benzene. This compound is commonly known as Anethole. The result of the analysis was reconfirmed by the reference data of the NIST (The National Institute of standard and Technology, USA). Based on the information generated through GC-MS and comparing with the Saturn 2000 MS Library and the NIST reference data, a probable chemical structure of the compound was generated (Fig. 4.11).

b) FTIR Spectroscopy

The FTIR spectral data (Fig. 4.12) obtained from the isolated compound were analyzed to reconfirm its identity and the structure of the probable compound: 1-methoxy-4-(1-propenyl)-benzene, which was elucidated through the GC-MS, Saturn 2000 MS Library Database and the NIST reference data. The spectra showed clear indication of the presence of aromatic ring displaying aromatic C=C stretching at 1461 cm^{-1} and 1607 cm^{-1} (Fig. 4.12). The band around 2853 cm^{-1} and 2925 cm^{-1} were assigned to the υ_{sym}C-H stretching and υ_{asym}C-H stretching, respectively. The strong absorption band at around 830 cm^{-1} was observed due to out-of-plane C-H banding. Absorption attributable to 1037 cm^{-1} was due to the presence of CH_3-O group. Existence of allylic C=C was evident from the band around 1700 cm^{-1}. Thus, the IR spectra support the structure of the compound 1-methoxy-4-(1-propenyl)-benzene (William, 1996). Lehman (1999) used IR spectroscopy to confirm the structure of the propenyl side chain of 1-methoxy-4-(1-propenyl)-benzene.

c) ^{1}H-NMR Spectra analysis

The ^{1}H-NMR spectra analysis also provided the clear indication in support of the proposed structure of the compound based on the GC-MS results and the NIST, USA reference data. The unsaturation number and the four aromatic protons, two at 6.82 and two at 7.24 ppm (Fig. 4.13), supported the conclusion that a para-disubstituted aromatic ring was present. The fact that two one-proton multiplets were present in the proton spectrum, one centered at 6.06 and the other at 6.32 ppm (Fig. 4.13), supported the presence of a disubstituted double bond. The δ=1.8 value evidenced the presence of the

methyl group attached to the double bond. Again the δ value at 3.8 also supported the existence of the methoxy group attached to the aromatic ring (William, 1996 and Silverstein and Webster, 1998). LeFevre (2000) utilized NMR spectroscopy to determine the structure of 1-methoxy-4-(1-propenyl)-benzene isolated from anise seed.

5.4 Molecular analysis

5.4.1 Genomic DNA isolation

In our earlier experiment with the protocol described by Khanuja *et al.*, (1999) for isolating genomic DNA, we encountered difficulties in obtaining high yield quality DNA (20.8 µg of DNA/g fresh young leaves of *Karphul*) with impurity (260/280=1.6, much below the normal range 1.8 – 2.0). The low yield of DNA with poor quality could be due to the presence of proteins and other secondary metabolites such as essential oils, polyphenols etc. The protocol we used was a modified CTAB-PVP method based on the one described by Kahnuja *et al.* (1999).

The protocol involves, the addition of high concentration of polyvinylpyrolidone (PVP) (100 mg/g leaf tissue) to purge polyphenols. A repeatation of chloroform : isoamyl alcohol extraction was done to clear the cloudy aqueous phase due to the presence of PVP. For precipitating the DNA, isopropanol was used and kept at room temperature for over night incubation. High concentration of NaCl (1.5 M) facilitated the removal of polysaccharides by increasing their solubility in isopropanol so as not to co-precipitate with the DNA. For better results, after the addition of RNase, the reaction mixture was incubated at 37°C for 2 h.

5.4.2 DNA quantification and purity test by UV spectroscopy

O.D. of the isolated genomic DNA at 260 nm = 0.042

Therefore,

the O.D. value 0.042 would indicate DNA yield = 50 µg/ml x 0.042

= 2.1 µg/ml

So, in 200 µl TE buffer, the amount of DNA would be $= \frac{2.1 \times 200}{5}$ µg

= 84.0 µg (3 g of fresh leaf)

= 28.0 µg per g of fresh leaf

The protocol invariably yielded a good amount of DNA from the fresh leaf tissue of *Karphul*. The total yield of DNA from 3 g of fresh leaves was found to be 84.0 µg. Therefore, the yield of DNA per g fresh leaves was determined to be 28.0 µg.

The purity of the isolated DNA was checked by finding out the optical density ratio at $\lambda_{260}/\lambda_{280}$. The OD at λ_{260} and λ_{280} was recorded with an UV spectroscopy.

λ_{260} = 0.042 and

λ_{280} = 0.021

Therefore, the ratio $\lambda_{260}/\lambda_{280}$ = 0.042/0.021

= 2.0

From the isolated DNA, the O.D. was calculated with $\lambda_{260}/\lambda_{280}$ as the indicator of purity of DNA (Sambrook *et al.*, 1989 and Henry, 1997). The results revealed that the estimated value of 2.0 refers to purity of the isolated DNA. The protocol was efficient

for isolating quality DNA from the herb, which was evident form the figure (Fig. 4.14) of the gel.

5.4.3 Restriction digestion

The isolated *Karphul* genomic DNA when subjected to restriction digestion with the restriction enzyme *Eco*R I and *Hind* III individually as well as in combination revealed effective digestion in all situations (Fig. 4.15). The enzyme *Eco*R I cut the DNA at fewer sites producing larger size fragments with higher molecular weight, which remained at the top part of the gel. During electrophoresis the fragments move slowly and occupy the top portion of the gel due to their larger size (Fig. 4.15, Lane 3). The DNA digested by the enzyme *Hind* III revealed that the endonuclease cut the DNA at more frequent sites as compared to the *Eco*R I, producing smaller fragments with low molecular weight. When electrophoresed, the fragments occupied the lower part of the gel due to their smaller size (Fig. 4.15, Lane 4). In the case of double digestion, both enzymes EcoR I and *Hind* III cut the DNA at more frequent sites producing smaller fragments with low molecular weight which occupied the lower portion of the gel on electrophoresis (Fig. 4.15, Lane 5). The purity of the isolated DNA was evident from the complete digestion by both enzymes. Khanuja *et al.* (1999) also reported the complete digestion of DNA by the restriction enzyme *Eco*R I isolated through the rapid isolation protocol standardized by them from tissues of diverse plant species producing large amount of secondary metabolites and essential oils.

5.4.4 Genome size (DNA C-value) determination

For the determination of the genome size (DNA C-value) of the herb, a new innovative, cost effective and simple method was employed. The method of genome size determination involves several steps including measurement of average volume of single cell of leaf tissue, measurement of the volume of intercellular space in the leaf tissue, cell number and DNA present per g of leaf tissue and finally DNA (approximate) present in a single cell of the leaf tissue of the plant. These steps could be worked out in a general laboratory having the facility of isolating plant DNA. The accuracy of results derived would depend on the correctness of the steps involved in the process.

The average volume of the cells present in the leaf tissue of *Karphul* was determined to be 90,249 μ^3 (Table 4.13). The intercellular space of the material was found to be 32% of the total volume, that is $3.0 \times 10^{10} \mu^3$ of the tissue section. Therefore, the actual volume of the tissue section devoid of intercellular space was found to be 2.04×10^{10} μ^3. The number of cells present in the tissue section with 2.04×10^{10} μ^3 volume was 2,26,041. The weight of the tissue section was determined to be 0.025 g which contained 2,26,041 cells. From this relationship, the number of cells present per g of tissue was determined to be 90,41,640. From our earlier experiment, we determined the amount of genomic DNA per g of fresh leaf tissue as 28.0 µg. Therefore, the amount of DNA content in a single cell was determined to be 3.10 pg as shown in the mathematical deductions. Since the plant *Karphul* is a tetraploid species (from karyotypic study), the DNA content (approximate) of the haploid set of chromosome or 2C DNA was found to be 1.55 pg having 1.52×10^9 bp. However, it needs further confirmation.

5.5 Tissue culture

5.5.1 Induction of callus from young rhizome

Young rhizome tissue explants of *Karphul* consisting of vegetative buds revealed swelling of tissues explants after 10 -15 days of culture in MS (Modified) medium supplemented with combinations and concentrations of 2,4-D, NAA, BAP and Kin. But none of the cultures could produce callus even after 50 days of culture (Table 4.14). Medium supplemented with combinations of 2,4-D (2.0 mg/l) and BAP (1.0 – 2.0 mg/l) produced green and friable calli (Table 4.14, Fig. 4.16 A). Medium supplemented with combinations of 2,4-D (1.5 - 2.0 mg/l), NAA (1.0 – 1.5 mg/l) and BAP (0.5 – 1.0 mg/l) produced compact nodular calli after 30 days of culture (Table 4.14). Martin *et al.* (2002) obtained greenish white and compact callus development from the young rhizome explant of *Alpinia calcarata* in media supplemented with combinations of 2,4-D (2.0 mg/l) and BAP (1.0 – 2.0 mg/l). Callus initiation was observed by Thiruvengadam and Narayanasamy (2001) tissue explant culture of *Vitex negundo* in medium containing 2,4-D (0.5 – 2.0 mg/l), IAA (0.5 – 2.0 mg/l) and NAA (0.5 – 2.0 mg/l) in combination with BAP (0.5 mg/l) after 14 days of culture and the highest frequency of green compact callus induction was in MS medium containing NAA (1.5 mg/l) and BAP (0.5 mg/l).

5.5.2 Organogenesis

Callus culture in BAP (1.5 mg/l) and 2,4-D (0.25 mg/l) supplemented MS (M) medium produced tiny protuberances after 7 – 10 days of subculture (Table 4.13). On callus subcultures in MS (M) medium supplemented with BAP (1.5 mg/l) and IAA (0.1 mg/l) produced 10-12 adventitious roots with 3-4 rudimentary shoots (Table 4.15, Fig.

4.16 B). The compact nodular calli in MS (M) medium supplemented with BAP (1.5– 2.0 mg/l) alone or in combination with 2,4-D (0.1 mg/l) produced 3 – 4 adventitious roots and 2 – 3 rudimentary shoots (Table 4.15, Fig. 4.16 C and Fig. 4.16 D). In all the cases, shoots and roots did not grow further. Martin *et al.* (2002) reported formation of meristematic protuberances on transferring of calli to MS medium containing BAP (0.5 mg/l) and NAA (1.0 mg/l) but did not show any development. On transfer of these protuberances to a medium having BAP (1.5 mg/l) and 2,4-D (0.25 mg/l) produced well developed single shoot with 2 – 5 roots along with 4 – 6 shoot primordia and numerous meristematic protuberances.

5.5.3 Direct regeneration of shoots and roots from rhizome explants

Young rhizome tissue-explants of *Karphul* consisting of vegetative buds while cultured in MS (M) medium supplemented with BAP (5 – 10 mg/l) and 2,4-D (0.25 mg/l); KIN (5 – 10 mg/l) and IAA (0.25 mg/l) and BAP (5 – 10 mg/l) and NAA (0.25 mg/l) produced shoot primordia after 30-40 days of culture. Among these, BAP (10.0 mg/l) with 2,4-D (0.25 mg/l) produced 4 – 6 shoots with 3-5 roots per explant (Table 4.17; Fig. 4.16 E). Explants cultured in MS (M) medium containing BAP/KIN (2.0 mg/l) alone failed to show any response. Borthakur *et al.* (1999) obtained on an average 8 shoots and roots from the emerging buds of *Alpinia galanga* Willd rhizomes when cultured in MS medium supplemented with Kin 3.0 mg/l within 8 weeks of culture. Rout *et al.* (1999) obtained multiple shoots from nodal explants of *Plumbago zeylanica* Linn. in MS medium supplemented with 0.5 – 1.0 mg/l 6-benzyladenine. The inclusion of IAA (0.01 mg/l) in the culture medium improved the production of multiple shoots. Sehrawat *et al.* (2002) obtained the best shoot proliferation from shoot apices when cultured on MS medium containing 2.0 mg/l BAP or 1.5 mg/l NAA.

Conclusions

An investigation was carried on the plant *Karphul* during 2002-2006. Based on the findings and discussion thereof the following conclusions were presented below:

1. The plant is closely allied to *E. linguiformis*. It is distinct from the later being taller (by 50-100 cm), hairy serrulate ligule, hairy petiole on inner side, bigger lamina size (by 2-12 x 4-6 cm), shorter peduncle (by 3.4 cm), outer bract white with light greenish tinge at tip, 8-14 florets/spike, calyx tube shorter than corolla tube with dentate tip and red in colour. Accordingly, a new name has been proposed as *E. assamica.*
2. Karyotype analysis revealed the plant to be a tetraploid with the chromosome number 2n = 48, where x = 12. However, this needs further confirmation.
3. The plant is rich in anethole (86%), which is a major additive of food items and medicines. Thus, the plant can be a better and alternative source for anethole against the present source of anise seed (82%) and funnel seed (75%).
4. Based on GC analysis, rhizome's essential oils comprise 2 major components viz., anethole (86%) and methyl chavicol (5.7%).
5. The essential oils and four crude fractions of rhizome of the plant possess differential levels of antimicrobial activity against test pathogens viz., *B. subtilis, S. aureus, M. flavus, E. coli, K. pneumoniae* and *C. albicans*.
6. Based on GC-MS, FTIR and ^{1}H-NMR analysis of the fragment 1 (F-I) of solvent extract confirmed to be the compound 1-methoxy-4-(1-propenyl)-benzene, commonly known as anethole.
7. Protocol was standardized for genomic DNA isolation with yield 28 μg/g of fresh leaf tissue and purity ratio 2. A new method for genome size determination (DNA c-value) was suggested, however it needs further standardization for its accuracy.
8. Tissue culture of the plant was standardized using young rhizome as explants.

APPENDIX

Appendix

1. Composition of Murashige-Skoog (MS) tissue culture medium (pH 5.7):

Components	(mg/l)
(A) INORGANICS	
Macro salts	
NH_4NO_3	1650.0
KNO_3	1900.0
$MgSO_4.7H_2O$	370.0
KH_2PO_4	170.0
$CaCl_2.2H_2O$	440.0
Minor salts	
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2EDTA	43.0
KI	0.83
(B) ORGANICS	
Vitamin	
Pyridoxin – HCl	0.5
Nicotinic acid	0.5
Thiamine – HCl	0.1
Glycine	2.0
Myo – inositol	100.0
Carbon source	
Sucrose	30,000.0
Agar	7,500.0
Distilled water	1,000.0 ml

2. Preparation of cleaning solution for glassware (sulphuric acid dichromate):

Sodium dichromate	25.0 g
Sulphuric acid (conc.)	1000.0 ml
Distilled water	50.0 ml

Sodium dichromate crystal 25.0 g was dissolved in 50 ml of warm distilled water. The solution was allowed to cool down to room temperature and slowly sulphuric acid was added to the preparation.

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