Cytotoxic, antitumor, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.

Thesis submitted to the University of Calicut for the award of the degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By

JOSE MARY DAS

GENETICS AND PLANT BREEDING DIVISION
DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA, INDIA

JUNE 2007

Cytotoxic, antitumor, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.

Thesis submitted to the University of Calicut
for the award of the degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By

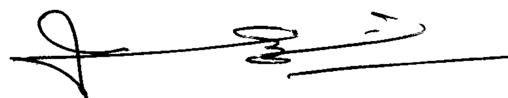
JOSE MARY DAS

GENETICS AND PLANT BREEDING DIVISION
DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA, INDIA

JUNE 2007

DECLARATION

I hereby declare that the thesis entitled "**Cytotoxic, antitumour, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.**" submitted for the degree of Doctor of Philosophy in Botany of Calicut University is a research work done by me under the guidance of **Dr. John E. Thoppil**, Reader, Genetics and Plant Breeding Division, Department of Botany, University of Calicut. This has not been submitted earlier for any other degree or diploma.



Calicut University,
30.06.2007.

Jose Mary Das

Cytotoxic, antitumor, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.

Thesis submitted to the University of Calicut for the award of the degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By

JOSE MARY DAS

GENETICS AND PLANT BREEDING DIVISION
DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA, INDIA

JUNE 2007

7

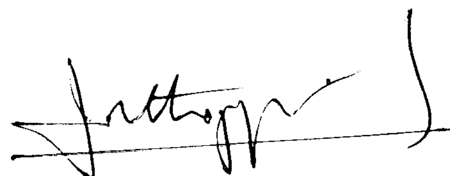
Dr. JOHN E. THOPPIL
READER
GENETICS & PLANT BREEDING DIVISION

DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA – 673 635

DATE: 30.06.2007

CERTIFICATE

This is to certify that the thesis entitled "**Cytotoxic, antitumour, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.**" is an authentic record of work carried out by **Jose Mary Das** during 2002-2007 under my supervision and guidance and that no part thereof has been presented earlier for any other degree or diploma.



Dr. John E. Thoppil

Dr. JOHN E. THOPPIL
Reader in Botany
Genetics & Plant Breeding
Division, Department of Botany
University of Calicut

Cytotoxic, antitumor, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.

Thesis submitted to the University of Calicut
for the award of the degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By

JOSE MARY DAS

GENETICS AND PLANT BREEDING DIVISION
DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA, INDIA

JUNE 2007

Acknowledgement

With proud respect and admiration, I express my gratitude to my guide Dr. John E. Thoppil, Reader, Genetics and Plant Breeding Division, Department of Botany, University of Calicut for having consented to supervise my research work. He has been of immense help and a source of inspiration at every step of my work.

I place on record my gratitude to Dr. P. V. Madhusoodanan, Head of the Department of Botany, University of Calicut for providing necessary facilities.

I wish to thank Dr. S. Nandakumar, former Head of the Department and Professor of Biochemistry, University of Calicut, for his sincere comments and valuable suggestions.

I owe a special debt of gratitude to Dr. Jose Paddikkala, Professor of Plant Science, Amala Cancer Research Centre, Trichur, for his constant help and suggestions during my research work.

I express my heartfelt thanks to Dr. Ramdasan Kuttan, Director, Amala Cancer Research Centre, for providing facilities to do a part of my research work.

I am happy to record my sincere thanks to Rev. Dr. Joseph Kappalumackel, Principal, St. Joseph's College, Devagiri, Calicut who has permitted me to do my Ph. D. work, and former Principals Rev. Dr. James Maruthukunnel and Rev. Dr. Joseph Vayalil, who has consented me to do my Ph.D. work.

I also extend a special thanks to UGC for financial assistance through the Faculty Improvement Programme.

I express my heartfelt thanks to Dr. Joseph Jose, Reader, Sacred Heart College, Thevara, for his sincere comments and valuable suggestions.

I express my sincere thanks to Dr. M. Sabu, Reader, Department of Botany, University of Calicut, for providing few specimens and identification of plants.

I would like to thank Mrs. Geetha Nair, Librarian, for her valuable help during this study.

I also express my affection to Mr. Fijesh P. Vijayan, Senior Research Fellow, Amala Cancer Research Centre for his constant help during my research work.

I express my thanks to Mr. Santhosh Kumar Dongre and Miss Jaimsha Rani for their valuable help.

I would like to express my heartfelt thanks to Mr. Jojo Joseph, my colleague who had been inspiring me during my research tenure.

I extent my special gratitude to Sr. Betty K.P. for her help during my work.

A special thanks to Mr. K.T. Thomachen for his valuable help in the statistical analysis of the data.

I also express my thanks to my labmates Mrs. Leeja Pramod, Asha Ramachandran and Sandhya Rani for their valuable help.

I also acknowledge the encouragement of Rev. Dr. Joseph Kappalumackel, Dr. C. J. Mani, Mr. Abis V. Cherussery, Dr. Sevichan P. J., Dr. Sibichen M. Thomas, Dr. Santhosh Nampy, Mr. Jojo Joseph, Dr. P.S. George, my colleagues, during the course of my work.

Jose Mary Das

Cytotoxic, antitumor, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.

Thesis submitted to the University of Calicut
for the award of the degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By

JOSE MARY DAS

GENETICS AND PLANT BREEDING DIVISION
DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA, INDIA

JUNE 2007

CONTENTS

		PAGES
1.	INTRODUCTION	1 - 8
2.	REVIEW OF LITERATURE	9 - 34
3.	MATERIALS AND METHODS	35 - 55
4.	RESULTS	56 - 68
5.	DISCUSSION	69 - 100
6.	SUMMARY	101 - 104
7.	REFERENCES	105 - 122

Cytotoxic, antitumor, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.

Thesis submitted to the University of Calicut
for the award of the degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By

JOSE MARY DAS

GENETICS AND PLANT BREEDING DIVISION
DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA, INDIA

JUNE 2007

INTRODUCTION

Plant derived products have been a prime source of a wide spectrum of successful drugs. Of the 520 new drugs approved between 1983 and 1994, 39% were either natural products or derived from natural products (Cragg *et al.*, 1997). The major advantage of natural products is the structural diversity provided by them, which is greater than that provided by synthetic heterocyclic compounds (Alan, 1999). The WHO estimates that about 80% of the world population residing in developing countries still rely almost entirely on plant products for their primary health care. The remaining 20% individuals living in other parts of the world use, pharmaceuticals which have been directly derived from plant products (Fransworth, 1984; Cox, 1994). Technical advances made the use of natural products easier than before. It is significant that over 60% of the currently used anticancer drugs are derived from natural sources including plants, marine organisms and microorganisms (Cragg *et al.*, 1997).

The search for anticancer agents from plant sources started in the 1950's with the discovery and development of vinca alkaloids, vinblastine and vincristine, isolated from *Catharanthus roseus* of Apocynaceae (Johnson *et al.*, 1963) and the isolation of podophyllotoxins derived from *Podophyllum peltatum* of Berberidaceae (Stahelin, 1973). Blemycin and cisplatin are currently used as very highly active drugs in testicular cancer ovarian carcinoma and etoposide against small cell lung carcinoma.

As a result, the United States National Cancer Institute (NCI) initiated plant collection programme in 1960's in the temperate region and in 1986 in tropical and subtropical regions. This led to the discovery of

many novel chemotypes showing a wide range of cytotoxic activities including the taxanes extracted from *Taxus brevifolia* of Taxaceae (Wani *et al.*, 1971) and the camptothecins derived from *Camptotheca accuminata* of the Nyssaceae. Taxol is a complex diterpene obtained from *Taxus brevifolia* of taxaceae (Ojima *et al.*, 1999). Paclitaxel is approved in many countries for the treatment of ovarian and breast carcinoma (McGuire *et al.*, 1996). Homoharringtonia, active against various leukemias (Kantarjian *et al.*, 1996) is isolated from the Chinese tree, *Cephalotaxus harringtonia* of Cephalotaxaceae (Powel *et al.*, 1970). It is interesting to note that no single plant derived clinical anticancer drug have yet been developed, but a number of agents are in the periclinical development.

The conventional anticancer therapy include surgery, radiation and chemotherapy, alone or in combination. The major drawback of the conventional treatment of cancer apart from its high cost is the immunosuppressive effects. Drugs that could reduce these side effects as well as stimulate immunity will be of great help in improving cancer treatment strategies (Leemol and Kuttan, 1998). Thus the continued task of searching new antineoplastic therapeutic agents remains critically important. Therefore, the current research efforts are more diverse than ever, being driven by explosive discoveries in molecular biology and related areas to fully elucidate the development of the malignant process. The hope for improvement in treatment for most patients with metastatic disease resides in continued research designed to discover novel therapeutic agents that exploits differences in molecular targets between normal and tumor cells and to use them in combination with biological agents and immune therapies to eradicate the systemic disease not curable by surgery or radiation. Chemoprevention is a concept defined as the prevention of cancer by the administration of natural or synthetic pure

chemicals or by daily food enriched with cancer preventive components (Wattenberg, 1985; Morse and Stoner, 1993). Primary cancer chemoprevention has two aspects in its methodology.

(1) Exclusion or avoidance of the environmental carcinogens or other chemical factors closely associated with carcinogenesis such as tumor promotors.

(2) The administration of inhibitory or suppressive agents against carcinogenesis (Murakami *et al.*, 1994).

Since, continuous exposure to environmental and food carcinogens is inevitable in daily life, direct inhibition or suppression at the tumor promoting stage could be an achievable strategy. Phytochemicals from edible plants have an advantage in their clinical application on account of low toxicity. In fact, a great number of epidemiological studies of the relationship between food and cancer, together with research in experimental animal models, have demonstrated, that daily ingestion of phytoproducts could undoubtedly contribute to cancer prevention (Rogers *et al.*, 1993; Doll, 1992; Wattenberg, 1992).

Several compounds have been discovered with inhibitory effects on the tumor – promoting stage and many of them were derived from plants. Kozhimizu *et al.* (1988) conducted screening tests of the methanol extracts from 121 species of plants for inhibitory activity towards Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-0-tetradecanoylphorbol-13 acetate (TPA) analogue. As a result 26% of the total showed significant inhibitory activity. These results show that antitumour promotors occur in a wide variety of plants.

Most of the plant derived products are classified as secondary metabolites or secondary products produced from primary metabolites in

target organisms. They are not believed to play any essential role in the basic life process but are ecologically important in the interaction between the organism and the environment. Chemically, secondary metabolites are relatively low molecular weight compounds having unique complex structures. Higher plants are able to synthesize a wide range of biologically active secondary metabolites used as pharmaceuticals (atropine, scopolamine, morphine, *etc.*). About 20% of all known natural products are classified as alkaloids. Many alkaloids are important as plant derived pharmaceuticals. Other major natural products used medicinally are terpenoids. The terpenoids or isoprenoids are the largest and most diverse family of natural products ranging in structure from linear to polycyclic molecules. They include mono (C_{10}), di (C_{20}), tri (C_{30}), tetra (C_{40}) and sesquiterpenoids (C_{15}). Terpenoids include a wide range of compounds like limonene, pinene, myrcene, linalool, camphene, fenchone, geraniol, ocimene, thujone, zerumbone, *etc.*

Many of these compounds are abundantly present in aromatic plants. These essential oil and oleoresin producing plants are present in Zingiberaceae, Apiaceae, Myrtaceae, Pinaceae, Piperaceae, Asteraceae, Verbenaceae, Lamiaceae, *etc.* Essential oils are valuable natural products used as raw materials in many fields including perfumes, cosmetics, aromatherapy, phytotherapy and pharmacology (Buchbauer, 2000). These oils are complex mixtures comprising the volatile principles contained in the plants (Brunetone, 1995). Terpenoids present in the essential oils and plant extracts are having extensive applications as flavouring agents, medicines, perfumes, insecticides, *etc.* They are also reported to have antimicrobial and anticarcinogenic properties (Crowel, 1999). Therefore, the intimate knowledge of essential oil composition allows for a better and specially directed application (Buchbauer, 2000). A detailed knowledge of

the constituents can only be obtained by means of carefully performed capillary GC experiments (Buchbauer, 2000).

In the new millennium, cancer remains the second leading cause of death in the world, among the most productive group *i.e.*, those aged between 45 and 64 years (Vay Liang *et al.*, 2001). American Cancer Society, in a survey reported about 1,368,030 newly diagnosed cases in 2004 (Carmia, 2004). Cancer causes the death of six million people globally. In India cancer registry data estimated that half a million cancer cases are reported per year in the country. As the plateau of cancer registry continues, the need for new approach to prevent this hazardous disease becomes imperative.

Cancer is a disease of the genome which is invariably altered at multiple sites in cancer cells. Cancer develops from an accumulation of mutation in a series of genes over a time. Multiple genetic defects are caused by exposure to environmental, dietary and infectious agents as well as other life style factors (Liang *et al.*, 2001). A panel of genes that are mutated in sequence, in cancer has been proposed (Kinzler *et al.*, 1991) such as p_{53}, that have been evaluated extensively in a wide range of tumor types. Yet to date no human cancer has been completely described with a clear picture of all the genes altered in that cancer.

Carcinogenesis is a multistep process that consists of at least three separate but closely linked processes, initiation, promotion and progression. Initiation is the first step of permanently modifying the target cell DNA. Promotion represents the expansion of the initially damaged or mutated cells. Promotion is a reversible process. In multistage carcinogenesis, promotion is closely linked to oxidative and inflammatory tissue damage. A substance with antioxidant and antiinflammatory effects is anticipated to act as antitumour promotor. A wide variety of

secondary metabolites derived from plants have been reported to possess marked antioxidant and antiinflammatory effects, which contribute to their chemopreventive potential (Surh, 1999). The tumor suppressor proteins which in response to DNA damage elicits either cell cycle arrest to allow DNA repair to take place or apoptosis (if DNA damage is excessive).

Progression is the final stage of carcinogenesis and is defined as the gain or loss of unit characters leading to the autonomous state. It involves chromosomal changes leading to invasion, metastasis and increased rate of growth that progresses to the higher degree of malignancy.

The traditional Indian system of natural medicine is centuries old and includes herbs and aromatic oils for the treatment of aches, pains and inflammations. The World Health Organization (WHO) has emphasized the need to ensure the quality control of herbs and herbal formulations by using modern techniques for herbal medicines so as to get accepted globally. The need of the hour is therefore to subject these herbs to rigorous modern scientific testing in order to regulate their quality with identity, potency, safety and efficacy. A correlation between biological activity and use in traditional medicine has been demonstrated in several cases and more recently in the field of anticancer activity.

Zingiberaceae is a well known plant family and many of its species are being used in traditional medicine which is found to be effective in the treatment of several diseases. It is one of the most evolved monocot families with the centre of distribution mainly in the Indo-Malayan region, but extending through tropical Africa to South America. They are perennial rhizomatous herbs, and are characterized by the possession of tuberous, and creeping rhizomes with an arial shoot often covered with sheathing leaf bases. This family includes about 47 genera and 1000

species. It includes conventional spices like *Elettaria cardamomum, Curcuma longa, C. aromatica, Amomum subulatum, Kaempferia galanga, Zingiber zerumbet, Zingiber officinale, Alpinia galanga, Alpinia calcarata etc.* In Malaysia, zingiberaceous rhizomes are widely used in the traditional treatment of ailments such as stomach problem, nausea, vomiting, cough, wounds, malignancies, *etc.* (Burkhill, 1966).

Alpinia is the largest, most widespread and most taxonomically complex genus in Zingiberaceae comprising about 230 species occurring throughout tropical and subtropical Asia. Species of *Alpinia* are often used as medicinals and ornamentals.

The rhizomes of Zingiberaceae possess diverse biological activities like antimicrobial (Yamada *et al.*, 1992; Burkhill, 1996 antiulcer (Matsuda *et al.*, 2003), antiinflammatory, antioxidant (Selvam *et al.*, 1995), cytotoxic and antitumor (Itokawa *et al.*, 1987; Pal *et al.*, 2001) activities. Although there have been many reports concerning chemical constituents of the essential oils and the biological activities of the various species, only a few reports are focussed on cytotoxic activity in plant systems, against cancer cell lines, anti tumor activity, and antioxidative activity against free radicals.

Hence a comparative study on the cytological effects of the polar and non polar fractions of seven selected species of *Alpinia* was undertaken using *Allium cepa* assay of Levan (1949). Claudio (2004) suggested that the *Allium* test is adequate for a preliminary screening of cytotoxicity of medicinal plants. The use of *Allium cepa* cells can be validated as an alernative model to mamalian test system (Chauhan, 1999). The present study also aims to compare the essential oil composition of different species, cytotoxic activity in cancer cell lines, *in*

vitro antioxidant activities and antitumour studies of cytotoxic species using animal model. An effort will also be made to find out the probable major chemical components/class of chemical compounds responsible for these activities.

Cytotoxic, antitumor, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.

Thesis submitted to the University of Calicut
for the award of the degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By

JOSE MARY DAS

GENETICS AND PLANT BREEDING DIVISION
DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA, INDIA

JUNE 2007

REVIEW OF LITERATURE

CYTOTOXIC ASSAYS

Cytotoxic studies of plant extracts on *Allium cepa*

Natural products are a source of therapeutic drugs and are used by physicians of indigenous systems of medicine for over hundreds of years (Houdret, 2000). The standard herbal preparations mostly consist of complex mixtures of one or more plants which are used in most countries (Calixio, 2000). In order to initiate the search for drugs from plants, the antimitotic activity of the extracts were tested by *Allium cepa* assay (Levan, 1949). The *Allium cepa* root meristem assay is considered widely as a practical and reliable system for the screening of environmental mutagens and carcinogens (Fiskesjo, 1985; Stich *et al.*, 1975).

As the patterns of divisions in onion cells and animal somatic cells are similar, an extract which is able to inhibit the cell division in *Allium cepa* root cells, will be effective in human/animal cells. Thus it is possible that chemicals that affect plant chromosomes will also affect the chromosomes of animals. Hence these meristematic cells of plants can be used for preliminary screening of antimitotic / anticancer activity of extracts / drugs (Williams, 1996). The onion root tip assay is used by many researchers to screen several plant extracts to evaluate their antimitotic activity.

Cytotoxic assays were conducted on a wide spectrum of plants by several earlier workers using different test materials. Shehab (1979) studied the cytological effect of water extract of *Pulicaria crispa* on *Allium cepa*. The extract affected the mitotic index and percentage of the mitotic

stages in treated roots. The percentage of anomalies increases with increase of concentration and duration of treatment. The abnormalities were spindle disturbances, stickiness, bridges and laggards.

In 1980, Shehab reported the antimitotic effect of water extract from *Teucrium pilosum* on *Allium cepa*. The induced cytotoxic effects include stickiness, C-mitosis, laggards, bridges, polyploidy and chromosomal breaks.

Cata edulis extract showed a decrease in the rate of cell division in *Allium cepa* root tip meristem (Kabarity and Mallalah, 1980). The leaves and buds of *Cata edulis* were said to contain alkaloids similar to caffeine. The mitodepressive effect increased as the time of exposure increased. In the short period of treatment with the same extract, the root tip showed a slight increase in the mitotic index after a period of recovery in water. The harmful effect of *Cata edulis* extract affected the rate of cell division of *Allium cepa* root tip cells and caused an imbalance in the frequency of mitotic phases.

The cytological effect of *Anastatica heirochuntica* extract on *Allium cepa* root meristem was studied by Shehab and Adams (1983). Mitotic depression occurred after direct and recovery treatments. The abnormalities observed were despiralization, spindle disturbances, chromosome stickiness, lagging, bridges, *etc.*

The antimitotic activity of the aqueous extract from *Erica undevalensis* on the root tip of *Allium cepa* was investigated. The role of the phenolic acid components in this activity was demonstrated (Pascual, 1987).

Ayuso (1988) reported the antimitotic activity of the aqueous extract of *Quercus rotundifolia* leaves on the root tips of *Allium cepa*.

Inhibition increased as a result of treatment duration. The importance of tannin content on inducing mitotic inhibition was demonstrated.

Mitotic effects of aqueous leaf extract of *Cymbopogon citratus* were demonstrated on *Allium cepa* root tips (Williams, 1996). A steroidal drug, sarsapogenin was tested for its cytotoxicity and antimitotic activity on root tip meristematic cells of *Allium cepa*. The drug was found to possess profound effect on mitotic spindle inhibition and chromosomal abnormalities during prolonged treatment (Sinha, 1996).

Minija *et al.* (1999) reported the mitoclastic properties of *Mentha rotundifolia* L. The abnormalities observed were clumped metaphase, scattered metaphase, polyploidy, diagonal anaphase, asynchronous movement of chromosomes, ball metaphase, *etc.*

Veronica *et al.* (2001) reported the effect of medicinal tea prepared from *Averrhoa carambola, Syzygium cuminum* and *Cissus sicyoides*. The results showed that tea did not alter the cell cycle of *Allium cepa*. But lower concentrations after 24 hr. treatment showed decrease in the mitotic index.

The genotoxic effect of an aqueous extract of neem was evaluated using *Allium cepa* chromosome aberration assay. Neem extracts suppressed the mitotic activity of *Allium* root meristems after 24-48 hr. treatment with all concentrations. The extracts caused different kinds of chromosome aberrations in dividing and non dividing cells of *Allium cepa* such as micronucleus, multinucleated cells in the interphase stage, bridges, stickiness, non-congression metaphase, laggards, polyploidy and disturbed anaphase (Soliman, 2001).

The cytotoxic potential of tomato fruit extract on onion root meristem was investigated by Yadav *et al.* (2001). The results showed that

tomato fruit extract induced various types of nuclear and chromosomal abnormalities such as chromosome breakage, scattered metaphase, disturbed polarity, lagging chromosomes, ring formation, extrusion, bridge formation and binucleate cells.

Cytotoxic effect of extract of castor seed was reported by Borah *et al.* (2002). The chromotoxic effects included fragments, ring chromosomes, c-mitosis and end to end attachment of chromosomes at metaphase, laggards, multipolar cells, *etc.* The most significant observations were the denaturation of chromatin fibre and somatic reduction that are encountered during treatment with 50 and 100% concentration of seed extract.

The cytotoxic potential of *Artemisia nilgirica* extract comprising both polar and non-polar fractions and plant extract having polar chemical compounds alone were evaluated on the meristematic root tip cells of *Allium cepa* (Leeja *et al.*, 2004). The abnormalities noticed were of both clastogenic and non-clastogenic types. Chromosome stickiness, bridges, binucleate cells, *etc.* were the most frequent abnormalities noticed. Mitotic index reduced considerably in a gradual manner as the concentration of the extract and duration of treatment increased.

Cytotoxicity of plant extracts on *in vitro* cell lines

Cytotoxic components of *Zingiber zerumbet, Curcuma zeodaria* and *C. domestica* were studied by Matthes *et al.* (1980). Root extracts of the three species of Zingiberaceae showed marked cytotoxicity against neoplastic cells. One new compound (3",4"-o-diacetyl afzelin) and five known compounds (zerumbone, zerumbone epoxide and the curcuminoids, diferuloyl methane, feruloyl-p-coumaryl methane and di-p-coumaryl-methane) were isolated and all these were found to be cytotoxic.

Toshihiko (1987) found that the growth of V-79 cells (lung fibroblast of Chinese hamster) were completely inhibited on treatment with camphor, present in *Bosenbergia pandurata* (Zingiberaceae) volatile oil at 0.3% for 24 - 48 hrs.

The sesquiterpene, β-sesquiphellandrene, one of the minor compounds detected in *Curcuma longa* volatile oil was reported to be cytotoxic against mouse lymphocytic leukaemia cells – L 1210 (Ahn and Lee, 1989).

Qureshi *et al.* (1994) studied the effects of *Alpinia galanga* (Zingiberaceae) on cytological and biochemical changes induced by cyclophosphamide in mice. The administration of the extract of *A. galanga* rhizomes caused mitodepression effects.

Zhao *et al.* (1995) have found the cytotoxic activity of *Hedychium forestii* (Zingiberaceae) rhizome extracts against KB cells. The diterpenoid hedychenone, T-hydroxyl hedychenone and coronarin were found to be the cytotoxic compounds.

The cytotoxic effect of eight synthetic curcuminoids on L929 cells were reported by John *et al.* (1996). All synthetic curcuminoids used in the study were found to be cytotoxic against L_{929} cancer cells.

Dubey *et al.* (1997) reported that citral, one of the major compounds of *Zingiber officinale* volatile oil was found to be cytotoxic against P_{388} mouse leukaemia cells.

The bioactive labdane diterpenoids isolated from *Renealmia alpinia* of Zingiberaceae collected from Surinam rain-forest was found to be cytotoxic against M_{109} cancer cells (Zhou-Bing Nan, 1997).

Ethanolic extracts of forty three Jordanian medicinal plants were examined for cytotoxicity. Among them, *Curcuma longa* and *Zingiber officinale* showed cytotoxicity against A_{549} cancer cells, MCF-7 female breast carcinoma and HT 29 colon adenocarcinoma cell lines (Alkofahi, 1997).

Cytotoxicity of curcuminoids and some novel compounds from *Curcuma zeodaria* were reported earlier by Syu *et al.* (1998). Extracts of roots of *C. zeodaria* led to the isolation of the curcuminoid, identified as dimethoxy curcumin. Cytotoxicity was exhibited against human ovarian cancer cells.

Curcuma domestica, C. xanthorrhiza, Kaempferia galanga, Zingiber casumnar, Z. officinale and *Z. zerumbet* exhibited cytotoxicity against 12-o-tetradecanoyl phorbol-13 acetate induced Epstein Barr virus early antigen. The results showed that Zingiberaceae rhizome extracts used in Malaysian traditional medicine contain naturally occurring non-toxic components that inhibits EBV activation (Vimala *et al.*, 1999).

The pharmacological effects of elemene isolated from the roots of *Curcuma zeodaria* were investigated in human leukaemia K_{562} cells (Yuan-Jing *et al.*, 1999). Inhibition of cell proliferation was measured using calorimetric MTT assay. Elemene induces apoptosis and regulates expression of bcl-2 protein in human leukaemia K_{562} cells.

Mackeen *et al.* (1999) reported the antimicrobial and cytotoxic properties of some Malaysian traditional vegetables (locally known as Ulam) belonging to 15 plant families. Among them *Kaempferia galanga* of Zingiberaceae was found to be cytotoxic against HeLa (Human Cervical Carcinoma) cell line. Cytotoxic, antioxidant and antiinflammatory activities of curcumin I-III from *Curcuma longa* were studied by Ramsewak

et al. (2000). The compounds showed cytotoxic activity against melanoma, renal and breast cancer cell lines.

Minor cytotoxic and antibacterial compounds were isolated from the rhizomes of *Amomum aculeatum* of Zingiberaceae. Aculeatin-D isolated from *A. aculeatum* showed high cytotoxicity against KB and other cell lines (Heilmann *et al.*, 2001).

Tezuka *et al.* (2001) reported eleven diarylheptanoids and two unusual diarylheptanoid derivatives from the seeds of *Alpinia blepharocalyx*. All compounds were examined for cytotoxicity against murine colon carcinoma 26-L5, human HT-1080 fibrosarcoma cells and showed cytotoxicity against both cell lines.

Moon *et al.* (2001) reported the antigenotoxicity of galangin as a cancer chemopreventive agent. Flavanoids extracted from *Alpinia officinarum* (galangin) was capable of modulating enzyme activities and suppressing cytotoxicity of chemicals.

Carnesecchi *et al.* (2001) studied the presence of geraniol as one of the minor compounds of *Bosenbergia pandurata* of Zingiberaceae and it was found to be cytotoxic against Caco-2 – human colon cancer cells.

Evaluation of cytotoxic potential of Indonesian medicinal plants in cultured human cancer cells was carried out by Gowooni *et al.* (2002). Out of the different plants studied *Zingiber casumunar* showed moderate toxicity to A_{549} cancer cells.

7,8-dihydroxy flavanone isolated from the seeds of *Alpinia kastumudai* was found to have an *in vitro* cytotoxic effect against A_{549} (human lung cancer cell line) and K_{562} (human leukemia cell line) cells (Ryeong *et al.*, 2003).

Han-Ahrenm (2003) reported the potential cytotoxic principle of chloroform extracts of *Zingiber casumunar* rhizomes. The cytotoxic compound curcumin was isolated, which showed significant cytotoxicity to human cancer cell lines like A_{549} cells.

Protective effects of *Alpinia oxyphylla* water extracts on neurons from ischemia damage and neuronal cell toxicity was studied by Koo-Bynug Soo *et al.* (2004). The results indicated that *Alpinia oxyphylla* protects neurons against ischemia induced cell death. *A. oxyphylla* may exert its neuroprotective effect by reducing the nitric oxide mediated formation of free radicals or antogonizing their toxicity.

Thippeswamy *et al.* (2006) reported that *Curcuma aromatica* extract induces apoptosis and inhibits angiogenesis in Ehrlich Ascites Carcinoma cells *in vivo.* The results showed that the ethanolic extract of *C. aromatica* has potent antiangiogenic and pro-apoptotic properties that can be further developed into potential anticancer drugs.

Twelve Thai medicinal plants including *Curcuma zeodaria* as the ingredients of a traditional formula for cancer treatment were selected to test cytotoxic activity against human cancer cell lines, *viz.*, large cell lung carcinoma and prostate cancer cell lines and one type of normal human cell line fibroblast cells. Ethanolic extracts of six plants including *Curcuma zeodaria* showed cytotoxicity against lung and prostate cancer cell lines. The water extract of these plants exhibited no activities against all types of human cells (Saetung *et al.*, 2005).

Toxicity of crude extract of *Kaempferia galanga* of Zingiberaceae was studied by Kanjana (2004). The ethanolic rhizome extract of *K. galanga* (Zingiberaceae) was studied by conventional pharmacological methods. In the acute toxicity test, oral administration of 5 gm/kg body weight of

the extract produced neither mortality nor significant differences in the body and organ weights between control and treated animals. Moreover, gross abnormalities and histopathological changes were not comparatively detectable. In subacute toxicity studies, no mortality was observed when varying doses of 25, 50, 100 mg/kg body weight of ethanolic extracts were administered orally.

Methanolic extracts, water extracts and volatile oils of fresh rhizomes of *Alpinia galanga, Bosenbergia pandurata, Curcuma longa* and *Zingiber officinale* have been assessed for cytotoxic activity against MCF-7 breast adeno-carcinoma and LS 174T – colon adeno-carcinoma cell lines by Zaeoung *et al.* (2005). The results showed that methanolic extract of *Curcuma longa* showed strong activity against MCF 7 and LS 174 T cell lines, whereas the water extracts of these plants exhibited slight cytotoxic activity. All volatile oils and methanol extracts were capable of inhibiting proliferation of the two cell lines. It is notable that volatile oils of these rhizomes were mainly composed of monoterpenes, sesquiterpenes and phenyl propanoids and could be responsible for the cytotoxic activity.

Lee *et al.* (2005) reviewed the cytotoxic activity of extracts of *Alpinia galanga, A. officinarum, Cayratia japonica, Physalis minima* and *Tabernaemontana divaricola* against human cancer cell lines, *viz.*, CoRL 23 – lung cancer cell line, MCF-7 breast cancer cell lines and a non cancer MCF-5 cell line. The results indicated that the extracts exhibited cytotoxicity. 1'-acetoxy chavicol acetate was isolated as the major cytotoxic component of both *Alpinia* species and physalin as the cytotoxic component of *Physalis minima*. The Malaysian *Alpinia galanga* showed weak activity when compared with Thai sample and this was due to the relatively high amounts of 1'-acetoxy chavicol acetate present in the Thai sample.

Biological effects of indigenous medicinal plants like *Curcuma longa* and *Alpinia galanga* were reported by Khattak *et al.* (2005).

The effect of turmeric extracts on inflammatory mediator production was reported by Lantz *et al.* (2005). Water soluble extracts were not cytotoxic and did not exhibit biological activity. Organic extracts of turmeric were found to be cytotoxic. Crude organic extract of turmeric were found to be capable of inhibiting lipopolysaccharide induced tumor necrosis factor (TNF-x) production. Purified curcumin was found to be more active. Fractions and subfractions of turmeric extracts obtained via preparative HPLC resulted in a loss of activity, indicating interactions of the compounds within the fraction to produce antiinflammatory effects.

Photo-induced cytotoxicity of curcumin in selected aqueous preparations on the salivary gland acinar cells and difference in photo-toxic effects of natural and synthetic curcumin was investigated by Bruzell *et al.* (2005). The results indicated that photo-toxic effect on cells was dependent on curcumin concentration, the light dose and the type of preparation.

ANTITUMOR ASSAYS

Cancer research is developing into a logical science, where the complexities of the disease, described in the laboratory and clinic, will become understandable in terms of a small number of underlying principles. Several lines of evidences indicate that tumorigenesis is a multistep process and these steps reflect genetic alterations that drive the progressive transformation of normal cells to the highly malignant derivatives.

Cancer development is now commonly recognized as a micro-evolutionary process that requires the cumulative action of multiple events. These events may occur in a single cell clone and can be explained by a simplified three-stage model. These stages include (a) induction of DNA mutation in a somatic cell, known as initiation (b) stimulation of the initiated cell and its clonal expansion, referred to as promotion, and (c) malignant conversion of the benign tumor into cancer, termed as progression. Oxygen free radicals have been shown to stimulate cancer development by playing a role at all the three stages namely, initiation, promotion and progression.

Plants have always been a common ingredient in the traditional medicinal preparations. So plants are invaluable in the generation of new drugs. Some examples of plant derived drugs are taxol, camptothecin, vincristin and vinblastin (DeVita *et al.*, 1993). Some of the indigenous plants have cytotoxic and antitumour property in experimental animal models (Shylesh and Padikala, 2000).

A survey of literature cited below depicts the antitumour effect of members of Zingiberaceae, spices and medicinal plants.

Anticancer activity of the rhizome of turmeric (*Curcuma longa*) was evaluated *in vitro* using tissue culture methods and *in vivo* in mice using Daltons lymphoma cells grown as ascites form. Turmeric extract inhibited the cell growth in Chinese hamster ovary (CHO) cells at a concentration of 0.4 mg/ml and was toxic to lymphocytes and Daltons lymphoma cells at the same concentration. Cytotoxic effect was found within 30 minutes at room temperature (30°C). The active constituent was found to be curcumin, which showed cytotoxicity to lymphocytes and Daltons lymphoma cells. Initial experiments indicated that turmeric extract and

curcumin reduced the development of animal tumours (Kuttan *et al.*, 1985).

In the course of search for antitumor agents, Sang Hyun *et al.* (1996) found that the extract of *Curcuma longa* was effective in inducing apoptosis or programmed cell death (PCD) in human myeloid leukaemia cells. Active compounds for PCD were isolated and identified as ar-turmerone and β-atlantone. These findings suggest that isolated compounds may exert their antitumor activity through induction of apoptosis.

Antitumour activity of synthetic curcuminoids were studied by John *et al.* (1996). Eight synthetic curcuminoids were investigated for their cytotoxic and tumoricidal activities. All curcuminoids were found to be cytotoxic to cultured L_{929} cells. As antitumor agents, veratryl curcuminoids and salicyl curcuminoids increased life span of animals by 100% and 86.9% respectively.

Inhibition of tumor promotion in SENCAR mouse skin by ethanolic extract of *Zingiber officinale* rhizome was reported by Katiyar *et al.* (1996). The results showed that pre-application of ginger extract to mouse skin *i.e.*, 30 min. prior to that of each 12-0-tetradecanoyl phorbol-13-acetate (TPA) application could reduce edema by 56% and hyperplasia by 44%.

Mehta *et al.* (1997) studied the antiproliferative effect of curcumin against human breast tumor cell lines. The results indicated that the growth inhibitory effects of curcumin was time and dose dependent and curcumin is a potent antiproliferative agent for breast tumor cells and may act as a potential anticancer agent.

Chemopreventive substances are capable of inhibiting, retarding or reversing the multistage carcinogenesis. A wide range of phenolics present in dietary and medicinal plants have been reported to possess substantial anticarcinogenic and antimutagenic activities. Curcumin, a yellow ingredient from *Curcuma longa (*Zingiberaceae) has been extensively investigated for its chemopreventive potential. Yakuchinone A and Yakuchinone D present in *Alpinia oxyphylla* (Zingiberaceae) have inhibitory effects on phorbol ester-induced inflammation and skin carcinogenesis in mice and oxidative stress *in vitro*. The results suggests that these compounds have the ability to suppress proliferation of human cancer cells via induction of apoptosis (Surh, 1999).

Zingiberaceae rhizomes commonly used in Malasian traditional medicine were screened by Vimala *et al.* (1999) for antitumor promotor activity. Using the short term assay of inhibition of 12-0-tetradecanoyl phorbol-13-acetate (TPA), they induced Epstein Barr virus early antigen (EBV-EA) in Raji cells. The rhizomes of *Curcuma domestica, Curcuma xanthorrhiza, Kaempferia galanga, Zingiber casumunar, Zingiber officinale, Zingiber zerumbet* and *Zingiber officinale* (red variety) were found to possess inhibitory activity towards EBV activation. The study suggests that the naturally occurring non-toxic compounds that inhibit the EBV activation, if further investigated could contribute in the development of cancer prevention methods at the tumoral promoting stage.

Inhibitory effects of curcumin and catechin on lung metastasis induced by B16 F-10 melanoma cells were studied in male mice by Menon (1999). Curcumin and catechin inhibited lung tumor formation 89.3% and 82.9% respectively and significantly increased life span 143.9% and 80.8% respectively. Curcumin and catechin treatment significantly inhibited the invasion of B16 F-10 melanoma cells. These findings suggest

that curcumin and catechin inhibit the invasion of B16 F-10 melanoma cells by inhibition of metallo proteinase, thereby inhibiting lung metastasis.

Curcumin, a dietary pigment in turmeric, possess anticarcinogenic and antimetastatic properties. The study conducted by Kim *et al.* (2001) revealed the *in vitro* chemopreventive effect of curcumin in transformed breast cells. The results indicate that curcumin inhibits invasion and induces apoptosis, proving the chemopreventive potential of curcumin.

Tanaka *et al.* (2001) revealed that dietary administration of zerumbone caused reduction in the frequency of azoxymethane-induced colonic abberant crypt foci (ACF) in rats. Further studies revealed that zerumbone feeding, significantly lowered the number of silver stained nucleolar organizer regions (AgNORs) in colonic cryptal cell nuclei and it reduced expression of cyclo oxygenase (COX) in colonic mucosa. These findings might suggest the possible chemopreventive ability of zerumbone.

Murakami *et al.* (2002) reported that zerumbone suppresses proinflammatory protein production and cancer cell proliferation accompanied by apoptosis. The results indicated that zerumbone, the food phytochemical has distinct potentials for use in antiinflammation, chemoprevention and chemotherapy strategies.

Redox regulated mechanism by which the zerumbone suppresses cancer cell proliferation was studied by Hoffman *et al.* (2002). The results showed that an appropriate dose of zerumbone can be made high enough to stop the proliferation of cancer cells, but not high enough to stop proliferation of normal cells.

Murakami *et al.* (2003) studied the suppression of dextran sodium sulfate induced colitis in mice by zerumbone, a subtropical ginger

sesquiterpene and nimesulide, separately and in combination. Ulcerative colitis and Crohn's disease are inflammatory disorders of unknown cause and difficult to treat, though some synthetic chemicals including ligands for peroxisome proliferator – activated receptors are anticipated to be useful drugs. A few food phytochemicals have been reported to suppress colitis in animal models. The results indicates that a food chemical, zerumbone has a marked suppression effect on dextran sodium sulfate induced colitis in mice.

Antitumor activity of the extract of *Zingiber aromaticum* and its bioactive sesquiterpenoid, zerumbone was studied by Kirana *et al.* (2003). The results indicated that zerumbone is effective as an anticancer agent, possibly by its apoptosis-inducing and antiproliferative activities.

A diarylheptanoid from *Alpinia officinarum* (Lesser galangal) inhibits proinflammatory mediator (via) inhibition of mitogen activated protein kinase and transcription factor, nuclear factor KB was reported by Yadav *et al.* (2003). The study also suggest the mode of action of dietary biologically active compounds in preventing inflammation, which may be helpful in the development of therapeutic treatment of chronic/acute inflammatory diseases.

Zerumbone, a sesquiterpenoid in subtropical ginger, suppresses skin tumor initiation and promotion stages in mice (Murakami *et al.*, 2004). The results indicate that zerumbone acts as a promising agent for the prevention of both tumor initiating and promoting processes, through induction of antioxidative and phase II drug metabolizing enzymes as well as attenuation of proinflammatory signalling pathways.

Murakami *et al.* (2004) revealed that the antiinflammatory phytochemical, zerumbone induces expression of proinflammatory genes in human adenocarcinoma cell lines.

The effect of turmeric extract on inflammatory mediator production was investigated by Lantz *et al.* (2005). The results indicated that the water soluble extracts were not cytotoxic and did not exhibit biological activity. Organic extracts of turmeric were cytotoxic only at concentrations above 50 μg/ml. Crude organic extracts were capable of inhibiting lipopolysaccharide induced necrosis factor. Purified curcumin was more active than either dimethoxy or bisdimethoxy curcumin.

Takada *et al.* (2005) reported that zerumbone found in subtropical ginger *Zingiber zerumbet* abolishes nuclear factor kappa B and kinase activation leading to suppression of antiapoptotic and metastatic gene expression, upregulation of apoptosis and down regulation of invasion. The results indicated that zerumbone inhibits activation of nuclear factor Kappa B and NP Kappa B regulated gene expression induced by carcinogens and this inhibition may provide a molecular basis for the prevention and treatment of cancer by zerumbone.

Formation of new blood vessels is critical for the growth of the tumor. Angiogenic factor secreted by the tumor cells induces neo angiogenesis. Inhibition of tumor angiogenesis and activation of tumor cell apoptosis will inhibit the growth of tumor. Thippeswamy *et al.* (2006) reported that *Curcuma aromatica* extract induces apoptosis and inhibits angiogenesis *in vivo*. The growth of Ehrlich ascites tumor cells and formation of ascites in the peritoneum of Ehrlich ascites tumor bearing mice was inhibited by *Curcuma aromatica* ethanolic extract. The decrease

in the peritoneal angiogenesis and microvessel density shows the antiangiogenic potential of *Curcuma aromatica* extract *in vivo.*

ANTIOXIDANT ASSAYS

Free radicals are unstable atoms or molecules with an unpaired electron that include hydrogen atoms, nitric oxide and molecular oxygen (O_2). These are naturally occurring in the body as a result of chemical reactions during normal cellular processes. Reactive oxygen species (ROS) are various forms of activated oxygen, which include free radicals such as super oxide ions, hydroxyl radicals and non free radical species such as hydrogen peroxide. In living organisms various forms of ROSs can form in different ways, including normal aerobic respiration, via stimulated polymorphonuclear leukocytes, macrophages and peroxisomes. These appear to be the main endogenous sources of most of the oxidants produced by cells. In an attempt for free radicals to stabilize, they attack other molecules in the body potentially leading to cell damage and triggering the formation of other free radicals resulting in a chain of reactions (Odukoya *et al.*, 2005).

Excess production of reactive oxygen species generated from isolated leukocytes, under chronic inflammation may have an important role in tumor initiation and promotion. All aerobic organisms, including human beings have antioxidant defences that protect excess production of reactive oxygen species, generated from isolated leukocytes under chronic inflammation and may have an important role in checking tumor initiation and promotion.

Oxidative stress has been implicated in numerous pathological conditions including cancer due to genetic variations (Forsberg, 2001). The complex process of carcinogenesis involves oxidative stress.

Oxidative stress can come from both endogenous and exogenous sources and is ubiquitous to all aerobic organisms. It was demonstrated that oxidative stress could cause chromosomal damage or aberrations, induce many types of DNA-protein cross-links, depurination and depyramidination (Mates, 2000). Oxidative stress also damages mechanisms which include defence enzymes like catalase, superoxide dismutase, and cell cycle check point systems (Shackelford, 2000). Thus free radicals often attack DNA, protein molecules, enzymes and cells leading to alterations in genetic material and cell proliferation (tumor masses).

Antioxidants are compounds that help to inhibit the many oxidation reactions caused by free radicals such as singlet oxygen, superoxide, peroxyl radical, hydroxyl radicals and peroxy nitrite, thereby preventing or delaying damage to the cells and tissues. Their mechanism of action include scavenging reactive oxygen and nitrogen free radical species, decreasing the localized O_2 concentration thereby reducing molecular oxygen's oxidation potential, metabolizing lipid peroxides to non-radical products to prevent generation of free radicals. In this way antioxidants limit the free radical damage from oxidizing low density lipoprotein (LDL) cholesterol, which may increase the risk of various diseases (Lakenbrink, 2000).

However, this natural antioxidant mechanism can be inefficient and hence dietary intake of antioxidant compound is important. Recent reports indicated that there is an inverse relationship between dietary intake of antioxidant rich food and the incidence of human diseases (Ali *et al.*, 2001) *i.e.*, inhibition that block reactive oxygen and nitrogen species may inhibit or delay carcinogenic processes (Cross *et al.*, 1987).

The strongest antioxidant and anticancer activity has been demonstrated by natural compounds having multifunctional ability. Suppressive effects of curcumin isolated from *Curcuma longa* of Zingiberaceae on lipid peroxidation induced in rats by carbon tetra chloride was reported by Ikuonishigaki (1992). The results indicated that administration of curcumin once a day for three successive days before the administration of carbon tetra chloride suppressed this increase in lipid peroxide level in the liver.

Free radical scavenging activity of synthetic curcuminoids were studied by John *et al.* (1996). Eight synthetic curcuminoids were screened for their free radical scavenging activity and found to inhibit *in vitro* lipid peroxidation and scavenged superoxides and hydroxyl radicals. The results showed that synthetic curcuminoids, like natural curcumin are potent antioxidants.

Sreejayan *et al.* (1996) reported the nitric oxide scavenging activity of curcuminoids. Curcumin being a compound with antiinflamatory and anticancer activity, inhibits induction of nitric oxide synthase in activated macrophages and has been shown to be a potent scavenger of free radicals. Curcumin reduced the amount of nitrite formed by the reaction between oxygen and nitric oxide generated from sodium nitroprusside. The result indicated curcumin to be a scavenger of nitric oxide.

Novel sesquiterpenes, *viz.,* oxyphyllenoidiol A and B, and tri noreudesmane type sesquiterpenes were isolated from the methanolic extract of *Alpinia oxyphylla* kernels and were found to inhibit the nitric oxide production in lipopolysaccharide activated macrophages (Muraoka *et al.*, 2001).

Antioxidant activity of extracts of *Alpinia kastumudai* seed was reported by Lee *et al.* (2003). The results indicated that high levels of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity were detected in the seed extract. The total seed extract of *A. kastumudai* shows dose dependently enhanced activities of superoxide dismutase, catalase and glutathione peroxidase in V79-4 cells *i.e.*, taken together, the findings showed that *A. kastumudai* contain significant antioxidant activity.

Nakamura *et al.* (2004) implicated zerumbone, a sesquiterpene compound from *Zingiber zerumbet* as one of the promising chemopreventive agents against colon and skin cancer. The result provide biological evidence that zerumbone has a significant ability to suppress oxidative stress possibly through induction of the phase II xenobiotic metabolizing enzymes. Considering the importance of oxidative damage in carcinogenesis, the antioxidant effect of zerumbone can be explored as a cancer chemopreventive agent targeted towards inflammation related carcinogenesis.

Kalpana *et al.* (2004) reported the modulating effects of curcumin on lipid peroxidation and antioxidant status during nicotine induced toxicity. The results indicated that administration of curcumin reversed the changes induced by nicotine, supporting the hypothesis that plant products are effective antioxidant agents. Curcumin significantly enhanced the antioxidant status in the liver, lung and kidney of nicotine treated rats. Thus curcumin exerts its protective effects against nicotine induced lung toxicity by modulating the extent of lipid peroxidation and augmenting antioxidant defence system.

Suppressive effects of Mioga ginger (*Zingiber mioga*) and ginger constituents on reactive oxygen and nitrogen species generation and the

expression of inducible proinflammatory genes in macrophages was studied by Kim *et al.* (2005). The results indicated that the constituents of *Zingiber mioga* have antioxidative and antiinflammatory potentials and may be considered as a promising candidate in prevention and or therapy for chronic inflammation associated carcinogenesis.

Antioxidant activity of Nigerian dietary plants including *Aframomum danielli* (Zingiberaceae) was reported by Odukoya *et al.* (2005). The results suggest that reducing power does not fully characterize the antioxidant activity. Moreover, spices containing high phenolics provide a source of dietary antioxidant in addition to imparting flavours to the food. They possess potential benefits by inhibiting lipid peroxidation, justifies their traditional use in pepper soup as a cure all medicine for the sick and potential use as a value added ingredient for stabilizing food matrixes against lipid peroxidation reactions.

Antioxidant activities of rhizome extracts of six plants of Zingiberaceae family (*Zingiber casumunar, Alpinia galanga, A. allughas, Hedychium corancium, H. coccinum, Kaempferia galanga*) was measured by Padma *et al.* (2006). The results showed that the extracts from six species of Zingiberaceae family showed moderate to good antioxidant properties. *Alpinia galanga, A. allughas* and *Zingiber casumunar* were found to be more effective. Methanolic extracts of the plants show better results for DPPH analysis than dichloromethane extract.

Matsuda *et al.* (2006) suggested that 80% aqueous extract from the rhizome of *Alpinia officinarum,* a Chineese medicinal herb, were found to inhibit nitric oxide production in lipid polysaccharide activated mouse peritoneal macrophages.

PHYTOCHEMICAL ASSAYS

GC-MS analysis of essential oils

Essential oils are valuable natural products used as raw materials in perfumes, cosmetics, aromatherapy, phytotherapy, spices and nutraceuticals (Buchbauer, 2000). The volatile oils are complex mixtures comprising several compounds. Each of these constituents contribute to the beneficial or potential effects of these oils. Thus the intimate knowledge of essential oil composition allows for a better and specially directed application (Buchbauer, 2000). Considering all the above mentioned difference in essential oil composition it is clear that only a detailed knowledge of the constituents of the essential oil will lead to a proper use in cosmetics by perfumers and cosmetic chemists. Such a detailed knowledge can be obtained by means of carefully performed capillary – GC experiments (Buchbauer, 2000).

Panizzi (1993) reported that variation in the chemical composition of essential oil may be due to climatic, seasonal, geographic conditions, harvest period and distillation technique followed. The effect of plant maturity at the time of oil production and the existence of chemotype difference can also drastically affect the composition of essential oils (Lahlou and Berrada, 2003).

The previous works on the essential oil composition of different species of *Alpinia* is reviewed in the following table.

Table 1. Previous reports on the essential oil composition of different species of *Alpinia*

Name of the plant	Parts used	Components	Authority	Year
Alpinia breviligulata	Seed	(E,E)-farnesol 65.0%, α-humulene 6.1%	Nguyen *et al.*	1994
A. breviligulata	Fruit peel	β-pinene 22.9%, α-terpinene 7.3%, caryophyllene oxide 11.2%	Nguyen *et al.*	1994
A. breviligulata	Leaf	Caryophyllene oxide 23.1%, α-pinene 17.7%	Nguyen *et al.*	1994
A. chinensis	Flower	α-farnesene 26.5%, α-humulene 22.3%, β-bisabolene 17%, β-caryophyllene 13.1%	Nguyen *et al.*	1994
A. chinensis	Leaf	β-bisabolene 47.9%	Nguyen *et al.*	1994
A. conchigera	Rhizome	β-sesquiphellandrene 20.5%, β-bisabolene 12.1%, 1,8-cineole 11.6%	Sirat *et al.*	1995
A. conchigera	Rhizome	β-bisabolene 28.9%, 1,8-cineole 15.3%, β-caryophyllene 10%	Wong *et al.*	2005
A. galanga	Rhizome	Limonene 3.7%, 1,8-cineole 33%, camphor 5%, α-terpineol 9.3%, α-fenchyl acetate 12.7%, (E) methyl cinnamate 5.3%	Mallavarapu *et al.*	2002
A. galanga	Leaf	α-pinene 6.6%, camphene 5%, β-pinene 21.5%, 1,8-cineole 34.4%, camphor 7.8%	Mallavarapu *et al.*	2002

Name of the plant	Parts used	Components	Authority	Year
A. galanga	Rhizome	1,8-cineole 39.4%, β-pinene 11.9%	Raina *et al.*	2002
A. galanga	Leaf	1,8-cineole 32.5%, β-pinene 22.7%, camphor 12.8%	Raina *et al.*	2002
A. galanga	Leaf	1,8-cineole 28.3%, camphor 15.6%, β-pinene 5% (E) methyl cinnamate 4.6%, bornyl acetate 4.3%, guaicol 3.5%	Leopold *et al.*	2003
A. galanga	Stem	1,8-cineole 31.1%, camphor 11%, (E) methyl cinnamate 7.4%, guaicol 4.9%, bornyl acetate 3.6%, β-pinene 3.3%, α-terpineol 3.3%	Leopold *et al.*	2003
A. galanga	Rhizome	1,8-cineole 28.4%, α-fenchyl acetate 18.4%, camphor 7.7%, (E) methyl cinnamate 4.2%, guaicol 3.3%	Leopold *et al.*	2003
A. galanga	Root	α-fenchyl acetate 40.9%, 1,8-cineole 9.4%, borneol 6.3%, boryl acetate 5.4%, elemol 3.1%	Leopold *et al.*	2003
A. hainanensis	Leaf	Ocimene 27.4%, β-pinene 10%, 9-octadecanoic acid 6.5%, n-hexa decanoic acid 5.8%, 9-12-octa decadienoic acid 5.4%, terpinene 4.3%	Nan Peng *et al.*	2004

Name of the plant	Parts used	Components	Authority	Year
A. hainanensis	Flower	Ocimene 39.8%, β-pinene 17.7%, terpinene 5.5%, p-menth-1-en-ol 4.9%, phellandrene 4.4%	Nan Peng *et al.*	2004
A. kastumudai	Leaf	p-menth-1-en-ol 22%, terpinene 19%, 4-carene 9.1%, 1,8-cineole 8.3%, camphor 5.6%	Nan Peng *et al.*	2004
A. kastumudai	Flower	p-menth-1-enol-21.3%, 1,8-cineole 20.2%, terpinene 12.6%, phellandrene 7%, 4-carene 6.4%, β-pinene 5.2%	Nan Peng *et al.*	2004
A. latilabris	Rhizome	Methyl cinnamate 89.5%	Wong *et al.*	2005
A. purpurata	Rhizome	α-pinene 24.9%, β-pinene 65.8%	Sadaquat Ali *et al.*	2002
A. purpurata	Leaf	α-pinene 79.6%, β-pinene 29.4%	Sadaquat Ali *et al.*	2002
A. purpurata	Flower	α-pinene 81%, β-pinene 43%, β-caryophyllene 24.2%	Sadaquat Ali *et al.*	2002
A. smithiae	Leaf	1,8-cineole 14.68%, β-caryophyllene 27.22%, geraniol 1.37%, camphor 6.3%	Roy *et al.*	2001
A. speciosa	Flower	β-pinene 34%, α-pinene 14.8%, β-caryophyllene 10.8%	Nguyen *et al.*	1994

Name of the plant	Parts used	Components	Authority	Year
A. speciosa	Rhizome	terpinen-4-ol 20.2%, 1,8-cineole 15.9%, sabinene 9.8%, γ-terpinene 9.3%	Pooter *et al.*	1995
A. zerumbet	Rhizome	β-pinene 4.0%, 1,8-cineole 28%, terpinen-4-ol 41.9%	Sadaquat Ali *et al.*	2002

Cytotoxic, antitumor, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.

Thesis submitted to the University of Calicut
for the award of the degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By

JOSE MARY DAS

GENETICS AND PLANT BREEDING DIVISION
DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA, INDIA

JUNE 2007

MATERIALS AND METHODS

PLANT MATERIALS

Seven species of *Alpinia* Roxb. (Zingiberaceae) collected from the wild as well as from the collections maintained in the Calicut University Botanic Garden were used for the study (Plate I & II). Voucher specimens were deposited at the Herbarium of Botany Department, University of Calicut. Voucher numbers allotted to each plant is given in brackets.

Experiments of the present study were conducted mainly in the Cell and Molecular Biology Laboratory of Genetics and Plant Breeding Division, Department of Botany, University of Calicut and the remaining portion in the Laboratory of Amala Cancer Research Centre, Trichur.

Alpinia calcarata Roscoe (CALI-37331)

Leafy stem 1-1.5 m tall. Leaves sessile; ligule shortly bifid. Inflorescence terminal, 10-15 cm long, paniculate. Bracts minute, 1 mm long, deciduous. Bracteoles splitting to the base, 1-1.5 cm long, deciduous. Flowers shortly pedicellate. Calyx tubular, 0.8-1 cm long. Corolla tube almost equal to the calyx; corolla lobes 1.5-1.8 cm long. Labellum, 3 cm long, obovate, variegated with dark purple and yellow, glabrous. Lateral staminodes small, subulate, at the base of the labellum. Stamen 2 cm long; anther 8 mm long, ecrestate. Epigynous glands two, 3 mm long. Ovary 4x3 mm, densely pubescent. Fruit globose, 2-2.5 cm long orange-red.

Alpinia galanga **(L.) Sw. (CALI-39164)**

Leafy stem over 2 m high. Leaves upto 60-70 cm; petiole short, 5 mm long; ligule 7-8 mm long, entire. Inflorescence terminal, panicled, 25-30 cm long. Bracts 2-2.5 cm long, deciduous, each subtending a cincinnus of 4-5 flowers. Bracteoles smaller and enclosing buds. Flowers 4 cm long, shortly pedicellate. Calyx 1 cm long. Corolla tube slightly longer than the calyx, corolla lobes 1.7-2 cm long. Labellum 2 cm long, white with a few lilacinus at the center. Lateral staminodes 4-5 mm long, subulate. Filament 1.3-1.6 cm long; anther thecae 6-8 mm long. Epigynous glands two, 3 mm long. Ovary 3 mm long, glabrous. Fruit orange – red, 1 cm across.

Alpinia malaccensis **(Burm. f.) Roscoe (CALI-95677)**

Leafy stem up to 3m tall. Leaves long petioled; petiole 3-3.5 cm; lamina 50-60 cm long; ligule 0.5-1 cm long, entire. Inflorescence terminal, erect or slightly curved. Bracts absent. Cincinni of 1-2 flowers. Bracteoles white, 1.5-2 cm long, deciduous. Calyx white, 1.8-2 cm long. Corolla tube 1 cm long, shorter than the calyx; corolla lobes white, 0.3 cm long. Labellum yellow, 3-4 cm long. Lateral staminodes subulate, 5 mm long. Filament 1 cm long; anther-thecae 1.2 cm. Epigynous glands 3 mm long. Style long, filiform, stigma funnel-shaped. Ovary 5 mm long, pubescent. Capsule turning red at maturity, globose.

Alpinia purpurata **(Vieill.) K. Schum. (CALI-78811)**

Leafy stem 1.5-2 m long. Leaves 25-35 cm long; petiole 1-1.15 cm long; ligulate. Inflorescence terminal, young spike compact, becoming lax when mature, red, 10-20 cm long. Bracts 2-3 cm long, deep purple, showy. Bulbils present in the axil of bract. Flower 4-5 cm long, white. Calyx 2-2.5 cm long, pale red, glabrous. Corolla tube 3 cm long, corolla

36.17

Plate I. Habit 1-*Alpinia calcarata*, 3-*Alpinia galanga*, 2-*Alpinia malaccensis*.

lobes 1.2-1.5 cm long. Labellum 1.2-1.5 cm, white, apex orbicular, base narrowed. Lateral staminodes absent. Anther connective prolonged into a flat hood. Epigynous 2 mm long. Ovary glabrous.

***Alpinia smithiae* M. Sabu *et* Mangaly (CALI-17563)**

Leafy stem 2-3 m tall. Leafy petioles 3-4 cm long; lamina 50-60 cm long; ligule entire. Inflorescence terminal, 15-25 cm long. Bracts only towards the tip, small. Flowers pedicellate. Bracteoles white, clasping the bud, deciduous. Calyx 1.8-2 cm long. Corolla tube 1 cm long, glabrous; corolla lobes 2.5 cm long. Labellum 3.5-4 x 3.5 cm, yellow, striped with purple-red, margin dark yellow. Lateral staminodes 5 mm long. Filament 1 cm long, densely hairy; anther 1.2 cm long, minutely crested. Epigynous glands two, united on one side. Ovary 7 mm long, densely pubescent. Fruit globose, 2.5 x 2.5 cm yellow-orange when mature.

***Alpinia vittata* W. Bull (CALI-78810)**

Leafy stem 1.5-2 m tall. Leaves 25-25 cm long; petiole 0.4-0.5 cm; ligule 3mm long; leaves glabrous, variegated with white lines on both surfaces. Inflorescence in terminal racemes. Bracteate, subtends two flowers. Flowers white and showy. Bracteole 0.8 – 1 cm long, hyaline. Calyx 1.5-1.6 cm long, Corolla dull-white, longer than calyx tube, corolla lobes 1.2-1.4 cm long. Labellum oblong-lanceolate, white, thick, 1.2-1.4 cm, apex slightly fringed. Lateral staminodes absent. Stamen 0.7 cm long. Ovary pale yellow, 0.4 cm diameter, glabrous; style filiform; stigma cup-shaped. Epigynous glands 2-3 mm long.

***Alpinia zerumbet* (Pers.) B. L. Burtt and R. M. Sm. (CALI-39146)**

Leafy shoot up to 3 m tall. Leaves large, lamina 60-80 cm long; petiole 1 cm long; ligule slightly bifid. Inflorescence terminal, pendulous.

Plate II. Habit 4-*Alpinia purpurata*, 5-*Alpinia smithiae*, 6-*Alpinia vittata*, 7-*Alpinia zerumbet*.

Bracts absent. Bracteoles large, encloses the bud, white with a pink tip. Corolla shorter than the calyx, corolla lobes 2.5-3.5 cm long. Labellum 4.5-5.5 cm long, yellow, heavily lined with red. Lateral staminodes 3-5 mm long. Filament 0.8-1 cm long. Epigynous glands two, 2 mm long, Ovary 6-7 x 2 mm, densely hairy. Capsule large, orange red, 2.5 x 2 cm, hairy.

Test Plant Material

The test plant material selected for the cytotoxic assay is *Allium cepa* 2n = 16.

Allium cepa bulb is a classical test material because

1. They can be easily propagated.
2. They are easy to handle.
3. They produce large number of roots with in a short period.
4. They posses large sized cells with small number of chromosomes and chromosomes are large in size for easy cytological observations.
5. The chromosomes show less variation in size and shape.
6. Root tip meristem posses a large number of actively dividing cells.
7. It can express the symptoms of clastogenicity and non-clastogenicity very easily.
8. Feasibility to squash.

CYTOTOXIC ASSAYS

Cytotoxicity of *Alpinia* extracts in *Allium cepa* root tip meristem

The healthy and uniform sized bulbs of *Allium cepa* were purchased from the local market, selected and washed in distilled water. These

selected bulbs were planted in pure sandy soil without manure to prevent other chemically induced chromosomal alterations. After 2 days the roots sprouted out.

Cytotoxic activity of the various *Alpinia* spp. extracts comprising both polar and non-polar compounds and extracts having polar compounds alone were analysed.

Fresh extract having both the polar and non-polar compounds was prepared from the rhizome of the plants with the help of mortar and pestile. Different concentrations of the extracts 0.125%, 0.25%, 0.5% and 1% (w/v) were prepared in distilled water. Fresh extract of rhizome having polar compounds alone was also prepared in different concentrations 0.125%, 0.25%, 0.5% and 1% (w/v) by the selective removal of non-polar compounds using diethyl ether as non-polar solvent in a separating funnel.

Determination of period of peak mitotic activity

In order to find out the time of peak mitotic activity, the untreated root tips of *Allium cepa* were fixed in acetic alcohol (1:3) mixture at different times from 8.30 to 11 am. After many trials, it was found that maximum dividing cells (peak mitotic activity) occurred between 9 am and 10 am under normal sunshine conditions.

Determination of concentration of *Alpinia* rhizome extracts for cytotoxic assays

Various concentrations were prepared with distilled water and after preliminary analysis it was confirmed that higher concentrations were found to be more toxic and then they were eliminated. So, the lower

concentrations such as 0.125%, 0.25%, 0.5% and 1% were selected for the present study.

Mode of treatment of *Alpinia* extracts

Germinated *Allium cepa* bulbs with healthy roots (1-2 cm) were selected and washed thoroughly with distilled water. The onion bulbs were kept at the rim of the bottle in which extract was taken in such a manner, that only the roots remain completely immersed in the solution. A few root tips were cut from each sample after the treatment, washed thoroughly with distilled water and immediately fixed in modified Carnoy's fluid (1 acetic acid : 2 alcohol) for one hour.

After fixing, the root tips were transferred to 70% ethyl alcohol and kept under refrigeration. Mitotic squash experiments were conducted with the help of improved techniques of Sharma and Sharma (1980).

The root tips were washed in distilled water and treated with 1N HCl for 5 minutes to separate the cells during squashing. The root tips were then washed thoroughly in distilled water and stained with 2% aceto orcein for 3 hours. After staining the roots were destained with 45% acetic acid, squashed and mounted on clean glass micro slides.

All the slides were scanned under Olympus microscope CX21 and the photographs were taken with Olympus Camedia C-4000 Zoom digital compact camera attached to the microscope.

Drug Preparation - Crude Extracts

Aqueous Extract

Rhizomes of seven species of *Alpinia* used in the study were chopped into small pieces and dried in an oven at 45°C and powdered.

The powdered rhizome was extracted twice in 250 ml of distilled water and stirred overnight using a magnetic stirrer. The supernatant collected was centrifuged at 10,000 rpm at 4°C, concentrated and evaporated to dryness in a water bath and stored at 4°C (to avoid fungal attack) in a refrigerator.

Methanolic extract

Rhizomes of seven species of *Alpinia* used in the study were chopped into small pieces and dried in an oven at 45°C and powdered. Powdered rhizome was extracted twice in 250 ml of 100% methanol and stirred overnight using a magnetic stirrer. The supernatant collected after centrifugation at 10,000 rpm at 4°C was concentrated and evaporated to dryness in a water bath for complete removal of methanol and stored at 4°C in a refrigerator.

Drug Preparation for *in vitro* studies

Stock solutions of the drug was prepared by dissolving 10 mg of methanolic extract of different species of *Alpinia* in 1 ml dimethyl sulphoxide. The stock solution for aqueous extract was prepared by dissolving 5 mg of aqueous extract in 1 ml of distilled water. Various dilutions of the both stock solutions of the seven plant extracts were prepared in distilled water. Concentrations of the methanolic extract ranging from 10 µg to 100 µg were used for the *in vitro* antioxidant assay.

Drug Preparation for *in vivo* Studies

The stock solutions of the drug for *in vivo* studies was prepared by suspending 500 mg of methanol extracts of the seven species of *Alpinia* in 10 ml of 1% gum acacia. It was done by dissolving 500 mg of the extracts in a minimum volume of methanol and was poured into the beaker

containing 5 ml of 1% gum acacia dissolved in phosphate buffered saline by boiling. The stock solutions were further diluted to 25 mg/ml and 125 mg/ml in PBS.

Cytotoxicity of *Alpinia* extracts on *in vitro* cell lines

In vitro cell lines

Daltons Lymphoma Ascites (DLA) cells and Ehrlich Ascites Carcinoma (EAC) cells were obtained from Amala Cancer Research Institute, Thrissur. L_{929} (Mouse lung fibroblast cells) and A_{549} (Human small cell lung carcinoma cells) were obtained from National Faculty for Animal Tissue and Cell Culture, Pune.

Propagation of tumor cell lines

The Daltons Lymphoma Ascites and Ehrlich Ascites Carcinoma cells were maintained in the intraperitoneal cavity of mice. At first 1 x 10^6 cells (100 µl) were injected into the intraperitoneal cavity of mice. After 15 days the cells were aspirated using a 1 ml syringe and PBS. The cells were washed three times in PBS to remove impurities. The number of cells were counted using a haemocytometer and made up to 100 µl PBS containing 1 x 10^6 cells. Then the cells were injected into the intraperitoneal cavity of another mice and it continued in every 15 days intervals. The L_{929} and A_{549} cells were maintained in *in vitro* culture using animal cell culture medium and maintained in 3% CO_2 atmosphere.

Cytotoxic assays on *in vitro* cell lines

Determination of 3 hr. time cytotoxicity of the *Alpinia* spp. extracts

Cytotoxic studies were carried out using DLA and EAC tumor cells. Different concentrations of the extracts 20 µg/ml, 40 µg/ml, 60

μg/ml, 80 μg/ml and 100 μg/ml were incubated at 37°C with tumor cells (1×10^6) suspended in PBS (pH 7.2) and cytotoxicity was determined after 3 hr. using Trypan blue exclusion method (Babu *et al.*, 2002).

Determination of 72 hr. time cytotoxicity of the *Alpinia* spp. extracts

Cytotoxicity of the extract in culture was determined using A549 cells and L-929 cells (Anis and Kuttan, 1999). The cells (10^4) were plated in a 96 well flat-bottomed titreplates and maintained with MEM (Minimum Essential Medium) containing 10% goat serum. Various concentrations of the extract 10-100 μg/ml were added into the wells and incubated for 72 h. 20 μl MTT (3-4,5-Dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (5 mg/ml) was added to the wells 4h before the end of incubation. Medium and reagents were aspirated, 98% dimethyl sulphoxide (DMSO) was added and after shaking for 15 minutes the absorbance was measured at 545 nm with reference wave length of 630 nm. The percentage of cytotoxicity was calculated using the formula given below:

$$\% \text{ of growth inhibition} = \left[\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}}\right] \times 100$$

Chemicals used

PBS - Phosphate Buffered Saline (pH 7.2).

Composition

NaCl	-	8 gm
KI	-	0.2 gm
Na_2HPO_4	-	1.44 gm
KH_2PO_4	-	0.2 gm

Dissolved in 1 litre double distilled water and pH adjusted to 7.2.

Trypan Blue

Dissolve 1% of Trypan blue in Saline and filter.

Minimum Essential Medium (MEM)	Himedia Laboratories Pvt. Ltd. India
Nitro Blue Tetrazolium	Sigma Chemical Co. St. Luis, USA.
[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)	Sigma Chemical Co. St. Luis, USA
Cisplatin	Dabur India Ltd.
Dimethyl Sulphoxide, DMSO	E Merck India Ltd.
Deoxy ribose	E Merck India Ltd.
5, 5'- dithio – bis-2-nitro benzoic acid (DTNB)	Sisco Research Laboratories, India.
Thiobarbituric Acid	Sisco Research Laboratories India.

Experimental Animals

Swiss albino mice of either sexes belonging to the age group of 8-10 weeks, which weighs 20-25 gm were purchased from the Small Animal Breeding Station, College of Veterinary and Animal Science Mannuthy, Thrissur. The animals were maintained under sterilized environmental conditions (22-28°C, 60-70% relative humidity, 12 hr dark/light cycle) and fed with standard rat feed (Sai Feeds, India) and water *ad libitum*. All animal experiments were conducted during the present study after getting

prior permission, from the Institutional Animal Ethics Committee (IAEC) (Lic No. 149/1999) and followed the guidelines of IAEC.

Drug administration

The drug was administered orally to Swiss albino mice (6-8 week old and weighing 20-25 gm) for 10 consecutive days (Babu *et al.*, 2002). The standard group was administered with cisplatin intraperitoneally.

ANTITUMOR ASSAYS

Acute toxicity studies of *Alpinia* species

The animals (Swiss albino mice) were divided into 3 groups of 8 animals each. The treatment schedule is as follows:

Group I	-	Normal without any treatment.
Group II	-	*Alpinia* spp. 1 gm/kg (Oral administration)
Group III	-	*Alpinia* spp. 2 gm/kg (Oral administration)

The animals were sacrificed after 48 hours of *Alpinia* spp. extract administration and the SGOT, SGPT, ALP and creatinine levels were estimated in serum.

Determination of serum glutamate oxaloacetate transaminase (SGOT) activity

SGOT activity was determined according to the method of Reitman and Frankle (1957).

Principle

Serum containing glutamate oxaloacetate transaminase catalyses the reaction between L-aspartate and α-ketoglutarate, to form oxaloacetate and glutamate. The unstable oxaloacetate is converted to

pyruvate and reacts with 2,4-dinitrophenyl hydrazine. The absorbance of the resultant brown colored phenylhydrazone is measured at 505 nm under alkaline conditions.

Procedure

Reagents used were from (Span diagnostics Commercial kit). 0.1 ml of serum was added to 0.5 ml of the buffered substrate (2 mM of α-ketoglutarate and 100 mM L-aspartate in 100 ml phosphate buffer 0.1 M, pH 7.4) at 37°C and incubated for 60 min. After the incubation, 0.5 ml of dinitrophenyl hydrazine (19.8 mg/dl 1 N HCl) was added, mixed well and kept at room temperature for 20 min. 0.4 ml of NaOH was added and read the absorbance after 10 min at 505 nm using the reagent blank. A control tube containing buffered substrate was treated with serum after the incubation at 37°C in the same manner. The enzyme activity was calculated from the standard (sodium pyruvate, 2 mM) calibration curve. The enzyme activity (U/ml) is converted to IU/l by multiplying with 0.483.

Determination of serum glutamate pyruvate transaminase (SGPT) activity

SGPT activity was determined according to the method of Reitman and Frankle (1957).

Principle

Serum containing glutamate pyruvate transaminase catalyses the reaction between L-alanine and α-ketoglutarate, to form pyruvate and glutamate. The pyruvate thus formed was treated with 2, 4-dinitro-phenylhydrazine. The absorbance of the resultant brown colored phenylhydrazone is measured at 505 nm under alkaline condition.

Procedure

Reagents used were from Span diagnostics (commercial kit). 0.1 ml of serum was added to 0.5 ml of the buffered substrate (2 mM of α-ketoglutarate and 100 mM L-alanine in 100 ml phosphate buffer 0.1 M, pH 7.4) at 37°C and incubated for 30 min. After the incubation, 0.5 ml of dinitrophenylhydrazine (19.8 mg/dl 1 N HCl) was added, mixed well and kept at room temperature for 20 min. 0.4 ml of NaOH was added and read the absorbance after 10 min. at 505 nm using the reagent blank. A control tube containing buffered substrate was treated with serum after incubation at 37°C in the same manner. The enzyme activity was calculated from the standard (sodium pyruvate 2 mM) calibration curve. The enzyme activity (U/ml) is converted to IU/l by multiplying with 0.483.

Determination of serum alkaline phosphatase (ALP) activity

Serum ALP activity was determined according to the method of Kind and King (1954).

Principle

ALP in the serum reacts with disodium phenyl phosphate under alkaline pH 10 and release phenol. Phenol reacts with 4-aminoantipyrene in the presence of alkaline oxidizing agent to give a red colored complex, which is measured at 510 nm against reagent blank.

Procedure

Reagents used were from Span diagnostics (Commercial kit). 0.05 ml of serum was incubated with 0.5 ml of the buffered substrate (1 ml of 0.254 g of disodium phenyl phosphate dihydrate/dl water mixed with 1 ml of the carbonate buffer pH 10) and 1.54 ml of distilled water at 37°C

for 15 min. After the incubation, 2 ml chromogen (1 ml of 0.6 g 4-aminoantipyrene/dl water and 1 ml of potassium ferricyanide 2.4g/dl water) reagent was added and measured at 510 nm. Phenol (10 mg %) was used as the standard for the calibration curve. The activity (KA/dl) is converted to IU/l by multiplying with 7.1.

Determination of Serum Creatinine Activity

Serum creatinine was determined according to method of Brod and Sirota (1980).

Principle

Creatinine forms a yellow-orange compound in alkaline medium with picric acid. The intensity of the color is measured at 500 nm. The concentration of the dyestuff formed over a certain reaction time is a measure of the creatinine concentration.

Procedure

Reagents used were from Span diagnostics (commercial kit). 0.2 ml of serum was mixed with 0.5 ml of buffer (313 mM NaOH and 12.5 mM phosphate, pH 8) and 0.5 ml of 8.73 mM picric acid. The absorbance is measured immediately after 1 min at 500 nm. A standard creatinine solution (1 mg/dl) was treated in the same way.

$$\text{Creatinine concentration (mg/dl)} = \frac{\text{OD of Test - OD of Blank}}{\text{OD of Standard - OD of Blank}}$$

Determination of tumor reducing activity of *Alpinia* species

Solid tumor model

DLA cells aspirated from Swiss albino mice (1 x 10^6 cells) were washed 3 times with PBS. The animals (Swiss albino mice) were divided

into four groups of 6 animals in each group. The solid tumor was induced in all the four groups of animals by intramuscular injection of 1 x 10^6 Daltons Lymphoma Ascites cells in the hind limb of mice. Oral drug administration was started simultaneously (24 hrs. after tumor induction) for 10 consecutive days.

Treatment schedule in Swiss albino mice

Group I was kept as the untreated vehicle control which received the vehicle of 1% gum acacia during the same period.

Group II was treated with standard drug cisplatin 10 mg/kg, administered intraperitoneally for 10 days.

Group III and IV were treated with *Alpinia* species extracts in 1% gum acacia in PBS, 100 mg/kg and 500 mg/kg respectively for 10 consecutive days. The development of tumor on each animal in each group was measured using Vernier calipers on every 5th day. The tumor volume was calculated using the formula.

$$V = \frac{4}{3}\pi r_1^2 r_2$$

where r_1 and r_2 are the radii of tumor along two directions (Kuttan *et al.*, 1985). This was compared with untreated control.

Synergistic action of *Alpinia* species extract along with radiation

The animals (Swiss albino mice) were divided into 4 groups of 6 animals each. The solid tumor was induced in all the four groups of animals by intramuscular injection of 1 x 10^6 DLA cells in the hind limb of mice.

Group I was kept as the untreated vehicle control which received 1% gum acacia in PBS during the same period for 10 days orally.

Group II was subjected to gamma radiation 100 rad/animal [single dose was given on the 7th day after tumor induction] and treated as standard using ^{60}Co gamma source [Theratron 780 Tele Cobalt Unit, Atomic Energy Canada Ltd.).

Group III was treated with *Alpinia* species extract in 1% gum acacia 100 mg/kg orally for 10 consecutive days.

Group IV was treated with *Alpinia* species extract in 1% gum acacia 100mg/kg orally for 10 days simultaneously with one time exposure to gamma radiation using ^{60}Co gamma source (100 rad/animal) on the 7th day after tumor induction.

The radii of the developing tumor was measured using Vernier calipers on every 5th day. The tumor volume was calculated using the formula

$$V = \frac{4}{3}\pi r_1^2 r_2$$

Synergistic action of *Alpinia* species extract along with cisplatin

The animals (Swiss albino mice) were divided into 4 groups of 6 animals each. The solid tumor was induced in all the four groups of animals by intramuscular injection of 1×10^6 DLA cells in the hind limb of mice.

Group I was kept as the untreated vehicle control which received 1% gum acacia for 10 days orally.

Group II was treated with standard drug Cisplatin 10 mg/kg administered intraperitoneally for 10 consecutive days.

Group III was treated with *Alpinia* species extract 100 mg/kg for 10 days orally.

Group IV was treated simultaneously with *Alpinia* species extract 100 mg/kg orally and cisplatin 10 mg/kg administered intraperitoneally for 10 days.

Radii of the tumors were measured on every 5th day using Vernier calipers and the tumor volume was calculated using the formula.

$$V = \frac{4}{3}\pi r_1^2 r_2$$

Ascites Tumor Model

Ehrlich Ascites Carcinoma cells were aspirated, washed and suspended in PBS. Four groups (6 each of Swiss albino mice) of test material were induced with ascites tumor by injecting 1 x 10^6 cells into the peritoneal cavity. Drug administration was started 24 hrs after tumor inoculation. Drug was given orally and continued for 10 consecutive days.

The treatment schedule is as follows: Group 1 was kept as untreated vehicle control and received 1% gum acacia in PBS for 10 days orally.

Group II was treated with standard drug cisplatin 100 mg/kg for the same period intraperitoneally.

Group III and IV were treated with *Alpinia* species extracts in 1% gum acacia in PBS 100 mg/kg and 500 mg/kg respectively for 10 days orally.

Animals were observed for the development of Ascites tumor and death due to tumor burden was recorded periodically. The increase in the life span of treated group was calculated using the formula.

$$\text{Increase in Life span} = \frac{T-C}{C} \times 100$$

where T and C are the mean survival of treated and control mice, respectively (Babu *et al.*, 2002).

Statistical analysis

To compare the effectiveness of *Alpinia* species extract on DLA induced solid tumor for 30 days in Swiss albino mice, two way analysis of variance (two way ANOVA) has been performed for each treatment category. The results are furnished in the table 26. The significance of the treatment effect for all groups are shown by P values ($P < 0.05$).

ANTIOXIDANT ASSAYS

Superoxide scavenging activity of *Alpinia* extracts

It was determined by the light induced superoxide generation by riboflavin and subsequent reduction of nitroblue tetrazolium as described by McCord and Fridovich (1969). The reaction mixture contained EDTA (6μM) containing 3 μg NaCN, riboflavin (2μM), NBT (50 μM), various concentrations of the test material and phosphate buffer in a final volume of 3 ml. The tubes containing the reaction mixture were uniformly illuminated with an incandescent lamp for 15 min and the optical density

was measured at 530 nm before and after illumination. The percentage inhibition was evaluated by comparing the absorbency value of the control tubes and experimental tubes.

$$\% \text{ of inhibition} = \frac{\text{OD of the control - OD of the treated}}{\text{OD of the control}} \times 100$$

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the test material was measured by studying the competition between deoxyribose and test compounds for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system (Fenton reaction). The hydroxyl radicals attack deoxyribose which eventually results in the formation of thiobarbituric acid reacting substances (Elizabeth and Rao, 1990). The reaction mixture containing deoxyribose (2.8 mM), ferric chloride (0.1mM), EDTA (0.1 mM), H_2O_2 (1mM), ascorbate (0.1mM), KH_2PO_4 - KOH buffer (20 mM, pH7.4) and various concentrations of the sample in a final volume of 1 ml was incubated for 1 h at 37°C. Deoxyribose degradation was measured as thiobarbituric acid reactive substances by the method of Ohkawa *et al.* (1979). The inhibition produced by different concentrations of the sample as well as the concentration required for 50% inhibition was calculated.

$$\% \text{ of inhibition} = \frac{\text{OD of the control - OD of the treated}}{\text{OD of the control}} \times 100$$

Lipid peroxidation assay

Lipid peroxidation was induced in rat liver homogenate by the method described by Bishayee and Balasubramanian (1971) in the presence of different concentrations of the test material and estimated by thiobarbituric acid reactive substances by the method of Ohkawa *et al.*

(1979). Different concentrations of the test material was incubated with 0.1 ml of rat liver homogenate (25%) containing 30 mM KCl, Tris-HCl buffer (40 mM, pH 7.0), ascorbic acid (0.06 mM) and ferrous ion (0.16 mM) in a total volume of 0.5 ml was incubated at 37°C for 1 h. At the end of the incubation period, 0.4 ml of the reaction mixture was treated with 0.2 ml SDS (8.1%), 1.5 ml thiobarbituric acid (0.8%) and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4 ml by adding distilled water and kept in a water bath at 95°C for 1h. After cooling, 1 ml distilled water and 5 ml butanol-pyridine mixture (15:1 v/v) was added. After vigorous shaking, the tubes were centrifuged and the upper layer containing the chromophore was read at 532 nm. The percentage of inhibition was calculated using the formula

$$\% \text{ of inhibition} = \frac{\text{OD of the control - OD of the treated}}{\text{OD of the control}} \times 100$$

Nitric oxide radical scavenging activity

Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess reaction (Green *et al.*, 1982). The reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate buffered saline (PBS) and different concentrations of the extract (from 1 to 100 μg/ml) was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture was removed and 0.5 ml of Griess reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% naphthyl ethylene diamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm.

$$\% \text{ of inhibition} = \frac{\text{OD of the control - OD of the treated}}{\text{OD of the control}} \times 100$$

PHYTOCHEMICAL ASSAYS

Extraction of Essential Oil

The shade dried plants were hydro distilled in a Clevenger apparatus (Clevenger, 1928) at 100°C for 4 hours. The aromatic essential oil was collected and dried over anhydrous sodium sulphate. The pure oil was transferred into a small amber coloured bottle and stored at 4-6°C.

Gas chromatography – Mass spectrometry (GC-MS)

GC-MS of the oil was performed on HP6890/5973-GC-MSD-D5 at 75 eV and 250°C. The GC column used were: HP-5 (DB5), fused silica capillary – 0.32 mm x 30 m with film thickness 0.25 μ. Carrier gas – helium. flow rate 1.4 ml/min. Temperature programme: initial temperature 60°C for 1 min and then heated at the rate of 3°C/min to 246°C. Run time is 62 min. The components were analysed and various components were ascertained with the help of Wiley library 275 combined with the analyser.

Supplementary Test for terpenoids

Liebermann-Burchardt test was done as a supplementary phytochemical assay to confirm the probable existence of terpenoids in the methanolic extracts of those species of *Alpinia* that were proved to be cytotoxic.

Liebermann-Burchardt test

To 1 ml of the methanolic extract of the drug, 1 ml of chloroform, 2-3 ml of acetic unhydride and 1-2 drops of conc. sulphuric acid were added (Ravishankar *et al.*, 2002).

Cytotoxic, antitumor, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.

Thesis submitted to the University of Calicut for the award of the degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By

JOSE MARY DAS

GENETICS AND PLANT BREEDING DIVISION
DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA, INDIA

JUNE 2007

RESULTS

CYTOTOXIC ASSAYS

Cytotoxicity of *Alpinia* extracts in *Allium cepa* root tip meristem

The effects of different treatments with the extracts of seven species of *Alpinia* containing polar and non-polar components and that with only polar fractions on mitotic divisions in the root tip meristems of *Allium cepa* in three different time durations (½, 1 and 2 hrs) are given in tables 2-15. The species studied were *Alpinia calcarata, A. galanga, A. malaccensis, A. purpurata, A. smithiae, A. vittata* and *A. zerumbet*. In the present investigation, *Allium cepa* root tip cells showed normal mitotic divisions when treated with distilled water as control (Plate III). Mitotic inhibition and cytotoxicity induced by the various extracts containing polar and non-polar fractions were found to be severe. Normal mitotic divisions were found to be more in lower concentration treatments than in higher. Normal mitotic stages were found to be fewer in treatments with extracts containing polar and non-polar fractions than in controls. Treatments with extracts containing polar fractions alone showed more divisional stages than treatments with extracts having polar and non-polar fractions.

A wide spectrum of cytological aberrations were found to be induced by treatments with both types of extracts (Plate IV – IX). Many clastogenic and non-clastogenic abnormalities were detected during the different treatments. The major clastogenic abnormalities observed were nuclear lesions, chromosome stickiness, chromosome bridges and ring chromosomes whereas the non clastogenic abnormalities observed were chromosome clumping, binucleate cell, polyploidy, early movement of chromosomes, non-synchronous movement of chromosomes, diagonal

metaphase, anaphase and telophase. The most frequent abnormalities observed were nuclear lesions, binucleate cells, stickiness, ball metaphase, diagonal metaphase and diagonal anaphase.

Mitotic indices in the various treatments with the plant extracts (Tables 2-15) were found to be less than that of control. Mitotic index showed an inverse relationship with the increase in the concentration of the extract. There is a positive correlation between concentration of extract and the frequency of aberrations. The frequency of abnormalities were found to increase with the increasing concentration of the extract and time durations.

The percentage of cytotoxicity and mitotic inhibition was found to be different in plant extracts comprising both polar and non-polar components and the plant extracts having polar constituents alone. The various treatments on *Allium cepa* root meristems with plant extracts of different species of *Alpinia* having both polar and non-polar components showed severe reduction of mitotic indices when compared to the plant extracts having polar components alone. The mitotic indices were found to be almost near to the control when treated with plant extracts having polar components alone. The frequency of abnormalities observed were found to be much higher in plant extracts having both polar and non-polar components than in the plant extract having polar compounds alone.

Alpinia calcarata

A wide spectrum of cytological aberrations were scored with the extracts containing polar and non-polar fractions and that with polar fraction alone. Nuclear lesions and binucleate cells were very common in the treatments with extracts having polar and non-polar components. Ball metaphase, diagonal metaphase and anaphase, clumping at metaphase,

early movement at anaphase and multipolarity were of moderate occurrence in the extract with polar and non-polar principles. The mitotic index ranges from 13.27 to 6.82 and the percentage of abnormality from 13.26 to 22.51 during different treatments with polar and non-polar fraction containing extract of *A. calcarata* (Table 2).

In the various treatments with extracts containing polar fraction alone the frequency of aberrations were comparatively lesser. These extracts induce moderate amounts of diagonal metaphase, misorientation, metaphase clumping and bridges. Mitotic index ranges from 27.19 to 18.89 and frequency of aberrations from 1.37% to 5.05% (Table 3).

Alpinia galanga

Large number of cytological aberrations were observed with the extracts containing polar and non-polar fractions and that with polar fraction alone. Nuclear lesions and binucleate cells were predominant in the treatments with extracts having polar and non-polar components. Diagonal metaphase, ball metaphase, metaphase clumping, chromosome agglutination, diagonal anaphase, multipolarity, equatorial separation were of moderate occurrence in the extract with polar and non-polar fractions. The mitotic index ranges from 12.95 to 7.09 and the percentage of abnormality from 20.08% to 24.87% during different treatments with polar and non-polar fraction containing extract of *A. galanga* (Table 4).

In the treatment with extracts containing polar fraction alone the frequency of aberrations were comparatively lesser. The treatments with these extracts showed nuclear lesions moderate amounts of diagonal metaphase, clumping, misorientation with bridges and chromosome fragments. Mitotic index ranges from 27.91 to 19.22 and frequency of aberrations from 1.16% to 5.43% (Table 5).

Alpinia malaccensis

Treatments with extracts of polar and non-polar fractions induce large number of cytological aberrations than polar fraction alone. Nuclear lesion and binucleate cells were dominant in the treatments with extracts having polar and non-polar components. Diagonal metaphase, ball metaphase – sticky, contorted chromosomes, diagonal non-synchronous movement in anaphase, bridges and broken bridges and micronuclei were of moderate occurrence in the treatments with extracts containing polar and non-polar principles. The mitotic index ranges from 8.59 to 4.94 and the percentage of abnormality from 19.93% to 24.43% during various treatments with polar and non-polar fractions of *A. malaccensis* (Table 6).

The frequency of aberrations were comparatively lesser in the various treatments with extracts containing polar fractions alone. The treatments with these extracts induced giant polyploid cell with chromosome fragments at metaphase, diagonal metaphase, sticky anaphase, equatorial separation of chromosomes, binucleate cells and nuclear lesions at interphase. Mitotic index ranges from 25.45% to 15.72% and frequency of aberrations from 2.00% to 6.54% (Table 7).

Alpinia purpurata

The results obtained from the treatments with extracts containing polar and non-polar fractions showed large number of cytological aberrations than the treatments with extracts containing polar fractions alone. Nuclear lesions and binucleate cells were observed in almost all concentrations of polar and non-polar fractions. Ball metaphase, scattering of chromosomes, diagonal metaphase, diagonal anaphase, single bridges, tripolarity with connecting bridges were of moderate occurrence in the treatments with polar and non-polar fractions. The

mitotic index ranges from 20.29 to 13.94 and the frequency of abnormalities from 14.87% to 17.78% during different treatments with polar and non-polar fractions containing extracts of *A. purpurata* (Table 8).

In the treatments with extracts containing polar fraction alone the percentage of aberrations were comparatively lesser. These extracts induce nuclear lesions, binucleate cells, moderate amounts of diagonal metaphase, stickiness at metaphase, non synchronous movement, early movement, diagonal stellate anaphase with bridges and non synchronous chromosome groups at telophase. Mitotic index ranges from 26.97 to 18.82 and percentage of abnormalities from 3.22% to 8.06% (Table 9).

Alpinia smithiae

A wide spectrum of chromosomal aberrations were observed in the treatments with extracts of polar and non-polar fractions than that with polar fractions alone. Nuclear lesions and binucleate cells were most common in the treatments with extracts having polar and non-polar components. Diagonal metaphase, ball metaphase, giant cell with isochromatid breaks, chromosome agglutination, ring chromosome, diagonal anaphase, multipolarity, giant cell with giant nucleus were of moderate occurrence in the extract with polar and non-polar principles. The mitotic index ranges from 15.59 to 8.38 and the frequency of abnormality from 21.43% to 30.45% during different treatments with polar and non-polar fractions containing extract of *A. smithiae* (Table 10).

In the various treatments with extracts containing polar fractions alone the percentage of aberrations were comparatively lesser. These extracts induce moderate amounts of misorientation, diagonal metaphase, anaphase bridges and nuclear lesions at interphase. Mitotic index ranges

from 27.35 to 20.20 and percentage of abnormalities from 1.09% to 4.42% (Table 11).

Alpinia vittata

Various drastic chromosomal aberrations were noticed in the treatment with polar and non-polar fractions than with polar fractions alone. Nuclear lesions and binucleate cells were the most frequent abnormalities in the treatments with extracts having polar and non-polar components. Diagonal metaphase, ball metaphase, polyploidy, stickiness at metaphase, metaphase clumping, giant polyploid cell with chromosome fragments, early movement, diagonal anaphase, anaphase bridges, multipolarity in giant cell and stellate anaphase were of moderate occurrence in the extracts with polar and non polar principles. The mitotic index ranges from 14.19 to 10.13 and the percentage of abnormality from 24.12% to 27.47% during different treatments with polar and non-polar fractions containing extracts of *A. vittata* (Table 12).

The frequency of aberrations were comparitively lesser in the treatments with extracts containing polar fractions alone. These extracts induce moderate amounts of diagonal metaphase, pulverization at metaphase, tripolarity and anaphase bridges. Mitotic index ranges from 26.90 to 20.45 and frequency of aberrations from 1.08% to 4.99% (Table 13).

Alpinia zerumbet

A wide range of chromosomal aberrations were observed with the extracts containing polar and non-polar fractions than polar fractions alone. The most prevalent abnormalities were nuclear lesions and binucleate cells in the treatments with extracts of polar and non-polar fractions. Moderate occurrence of diagonal metaphase, ball metaphase,

clumping at metaphase, hypercondensed chromosomes, early movement at anaphase, diagonal non-synchronized movement and nuclear budding were observed in the extracts with polar and non-polar principles. The mitotic index ranges from 18.22 to 10.14 and percentage of abnormality from 18.88% to 24.65% during different treatments with polar and non-polar fraction containing extracts of *A. zerumbet* (Table 14).

In the various treatments with extracts containing polar fraction alone the frequency of abnormalities were comparatively lesser. These extracts induce moderate amounts of stickiness at metaphase, diagonal metaphase, misorientation at metaphase, multiple bridges and diagonal anaphase. Mitotic index ranges from 28.05 to 20.64 and percentage of aberrations from 1.22% to 4.93% (Table 15).

Cytotoxicity of *Alpinia* extracts on *in vitro* cell lines

The results of the short term cytotoxic studies using DLA and EAC cell lines with the water extracts of *A. calcarata, A. galanga, A. malaccensis, A. purpurata, A. smithiae, A. vittata* and *A. zerumbet,* and showed weak activity. But the cytotoxic studies using methanolic extracts of different species of *Alpinia* showed more cytotoxicity in *A. vittata* and *A. malaccensis*, whereas the other five species showed weaker cytotoxicity. The cytotoxic studies using methanolic extracts of *A. malaccensis* with DLA cell line showed 50% inhibition at a concentration of 460 μg/ml and *A. vittata* extracts showed 50% inhibition at 350 μg/ml. The 50% cytotoxic inhibition was noticed with EAC cell line in *A. malaccensis* methanolic extract at a concentration of 530 μg/ml and *A. vittata* extracts at a concentration of 350 μg/ml respectively (Table 16). Hence those plants showing weak activity towards DLA and EAC cell lines were eliminated and the antitumor work was continued on *A. vittata* and *A. malaccensis.*

The methanolic extracts of *A. vittata* and *A. malaccensis* were further studied by using long term cell lines A_{549} and L_{929}. The results showed that the methanolic extracts of *A. vittata* at a concentration of 50 µg/ml showed more than 50% cytotoxicity and *A. malaccensis* showed more than 50% cytotoxicity at 28 µg/ml concentration with L_{929} cell line. Cytotoxic studies using A_{549} cell lines with *A. vittata* extracts showed 50% cytotoxicity at a concentration of 375 µg/ml and *A. malaccensis* extract showed 50% cytotoxicity at a concentration of 160 µg/ml (Table 17).

ANTITUMOR ASSAYS

Acute toxicity studies of *Alpinia malaccensis* and *Alpinia vittata*

The acute toxicity studies of the methanolic extracts of *A. malaccensis* and *A. vittata* showed no considerable change in the level of SGOT, SGPT, ALP and creatinine when compared to the normal group (Table18).

Antitumor activity of extracts of *Alpinia malaccensis* and *Alpinia vittata* on DLA induced solid tumor

The oral administration of the methanolic extracts of *A. malaccensis* and *A. vittata* significantly inhibited the formation of solid tumor in a dose dependent manner (Plates X and XI; Tables 19-24).

Effect of *Alpinia malaccensis* extract

Solid tumor was induced in all animal groups with DLA cell line. In the control groups solid tumor was found to increase gradually from 1st day to 30th day. The application of the standard drug cisplatin showed an increase of tumor volume from the 1st day to 15th day and then gradually decreased to the 30th day. The oral application of 100 mg/kg body weight

of methanolic extract of *A. malaccensis* results in the gradual increase in the solid tumor volume from the 1st day to 25th day and then reduced to the 30th day. The administration of the same drug at a concentration of 500 mg/kg body weight increased the tumor volume from the 1st day to 15th day and then reduces gradually to the 30th day (Fig. 1; Table 19).

The synergistic application of 100 mg/kg body weight of methanolic extract of *A. malaccensis* with the standard drug cisplatin increased the tumor volume from 1st day to the 15th day and then reduced to the 30th day (Fig. 2; Table 20).

On exposure to a standard dose of 100 rad radiation, the tumor volume remains almost the same on the 1st and 5th day, then gradually increases to the 15th day and then reduces gradually to the 30th day. The simultaneous application of 100 mg/kg body weight of the plant extract of *A. malaccensis* along with one time exposure to 100 rad radiation increased the tumor volume from 1st day to 15th day and then reduced to the 30th day (Fig. 3; Table 21).

Effect of *Alpinia vittata* extract

When 100 mg/kg body weight of methanolic extract of *A. vittata* was administered the tumor volume was increased upto 25th day and then decreased gradually to 30th day. The application of 500 mg/kg body weight of the *A. vittata* extract gradually increased the tumor volume from the 1st day to the 20th day and then reduced the tumor volume from the 20th day onwards (Fig. 4; Table 22).

The synergistic administration of 100 mg/kg body weight *A. vittata* extracts and the standard drug cisplatin results in the gradual increase in the solid tumor volume from the 1st day to 15th day and then decreases upto the 30th day in a gradual manner. The synergistic application of the

drug along with plant extract was more effective in reducing the tumor volume when compared with the standard drug alone (Fig. 5; Table 23).

When the animals were given single exposure to 100 rad radiation, the tumor volume was increased upto the 15th day and then significantly reduced to the 30th day (Fig. 6; Table 24). The simultaneous administration of 100 mg/kg body weight of the extract and one time exposure to 100 rad radiation, the tumor volume was increased from 1st day to 10th day and then gradually reduced to the 30th day.

Ascites tumor activity induced by EAC cell lines

Ascites tumor harbouring mice after treatment with methanolic extracts of *A. malaccensis* and *A. vittata* were found to exhibit an increase in the life span in a dose dependent manner. Percentage of increase in the life span using *Alpinia malaccensis* extract (100 mg/kg body weight and 500 mg/kg body weight) was found to be 26.29% and 41.31% respectively. *Alpinia vittata* treated groups of animals showed an increase in the life span of 23.27% and 30.80% at doses 100 mg/kg body weight and 500 mg/kg body weight respectively (Table 25). All the antitumor experiments except the antitumor activity on EAC cell lines were found to be statistically significant (Table 26).

ANTIOXIDANT ASSAYS

Antioxidant activities of *Alpinia* plant extracts in different antioxidant systems

The methanolic extracts of seven species of *Alpinia* was assessed for their free radical scavenging activities against superoxide radical, hydroxyl radical, nitric oxide radical and inhibition of lipid peroxidation. The

methanolic extracts showed significant antioxidant ability *in vitro* at concentrations ranging from 10-100 μg/ml.

Superoxide scavenging activity

Methanolic extract of five of the seven species of *Alpinia* were found to scavenge the superoxide generated by riboflavin photoreduction method (Table 27). *A. calcarata, A. galanga, A. malaccensis, A. vittata* and *A. smithiae* were found to scavenge superoxide. The concentrations of the sample causing 50% inhibition of superoxide radicals, *i.e.*, IC50 values were shown in Table 28. Among these five plants the percentage of inhibition (IC_{50}) ranges from 24 μg/ml to 85 μg/ml with the lowest values shown by *A. calcarata* and the highest by *A. malaccensis* (Fig. 7).

Hydroxyl radical scavenging activity

Degradation of deoxyribose by hydroxyl radical generated by Fe^3/ ascorbate/EDTA/H_2O_2 was found to be inhibited by extracts of *A. galanga, A. malaccensis, A. purpurata, A. smithiae* and *A. vittata* (Table 27). IC_{50} values shown in Fig. 8 and Table 28 reveals that the maximum value (IC_{50}) was shown by *A. malaccensis* (136 μg/ml) and the minimum by *A. purpurata* (37 μg/ml).

Nitric oxide scavenging activity

Nitric oxide radicals generated from sodium nitroprusside was found to be inhibited by methanolic extracts of *A. galanga, A. smithiae* and *A. vittata* only (Table 27). IC_{50} values (Fig. 9; Table 28) shows that maximum value was exhibited by *A. galanga* (70 μg/ml) and minimum value by *A. smithiae* (57 μg/ml).

Inhibition of lipid peroxidation

The extracts of *A. galanga*, *A. malaccensis*, *A. purpurata*, and *A. smithiae* were found to inhibit lipid peroxidation generated by the induction of Fe^2 / ascorbate on rat liver homogenate (Table 27). The IC_{50} values (Table 28) ranges from 58 μg/ml to 114 μg/ml, with the lowest value exhibited by *A. smithiae* and the highest value by *A. malaccensis* (Fig. 10).

PHYTOCHEMICAL ASSAYS

GC-MS analysis of essential oil

The major essential oil components identified in the present investigation broadly belongs to monoterpenoids, sesquiterpenoids and few phenols (Figs. 18(i) to 18(x)). Various aromatic species of *Alpinia* possess oils that are rich in odoriferous monoterpenoids, *viz.*, camphene, 1-8 cineole, β-pinene, myrcene, p-cymene, limonene, γ-terpinene, α-thujone, L-camphor, terpinene-4-ol, α-fenchyl alcohol, α-pinene, β-fenchyl acetate, methyl cinnamate, sabinene, α-terpinene, geranyl acetate, β-fenchyl alcohol, α-phellandrene, fenchol, α-terpineol, α-terpinolene, fenchane, borneol, 1,4-terpineol, citral, *etc.* Phenols such as methyl eugenol and acetyl eugenol also contribute towards the peculiar aroma and flavour of various taxa studied. Fragrant monoterpenes, oxygenated monoterpenes, monoterpene derivatives and phenols are probably responsible for the characteristic odour of the essential oils. However, some taxa are poor in these odoriferous compounds instead they contain high amounts of sesquiterpene hydrocarbons like β-caryophyllene, α-humulene, epizonaren, valencene, azulenol, α-selinene, α-farnesene, germacrene-D, β-bisabolene, β-sesquiphellandrene, nerolidol, trans-

caryophyllene, zerumbone, caryophyllene oxide, γ-cadinene, β-elemene, alloaromadendrene, germacrene-A, eudesmol, neo-intermediol, azulene, alliodorin and zerumbone. A wide range of chemical compounds were detected in the GC/MS analysis of essential oils of seven species of *Alpinia* in the present study. Details of essential oils of different species of *Alpinia* are given in Figs. 11-17 and Table 29.

Supplementary test for terpenoids in the cytotoxic species of *Alpinia*

Liebermann-Burchardt test

The supplementary test for terpenoids, *viz.,* Liebermann-Burchardt test gives a pink colouration which indicates the presence of terpenoids in the methanolic extracts of the two cytotoxic species, *viz.*, *A. malaccensis* and *A. vittata.*

TABLE 2 : MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR AND NON-POLAR FRACTIONS OF *ALPINIA CALCARATA* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase								Anaphase						Telophase		Normal cytokinesis	Interphase			% of Abnormality	Mitotic index (%)
			Normal	Nuclear vacuolization	Normal	Misorientation	Diagonal	Ball Metaphase	Polyploid	Clumping	Diagonal and stickiness	Stickiness	Normal	Early movement	Diagonal	Bridges	Multipolarity	Stellate anaphase	Normal	Diagonal		Binucleate with a connection	Nuclear lesion	Binculeate cell		
½	Control	927	120	--	59	--	--	--	--	--	--	--	41	--	--	--	--	--	34	--	11	--	--	--	--	28.59
½	0.125	618	10	--	6	4	8	7	--	5	--	6	10	--	10	--	3	--	4	--	2	3	31	13	14.56	12.14
½	0.25	596	8	--	4	--	10	10	--	--	--	5	8	5	8	3	--	1	4	4	--	--	41	13	16.78	11.74
½	0.5	699	6	--	7	--	5	10	2	8	6	12	--	5	5	--	5	2	--	--	2	1	51	20	18.88	10.73
½	1	588	3	2	3	--	8	6	--	8	--	8	--	4	5	6	--	--	--	--	--	--	54	19	20.41	9.01
1	Control	836	113	--	53	--	--	--	--	--	--	--	39	--	--	--	--	--	30	--	16	--	--	--	--	30.02
1	0.125	829	8	--	4	--	12	6	--	8	5	7	13	7	18	--	--	--	15	7	--	--	39	11	14.48	13.27
1	0.25	670	7	--	8	--	6	12	--	6	8	5	--	--	5	--	6	5	--	5	--	--	41	15	17.01	10.90
1	0.5	933	8	1	8	--	5	8	3	8	--	7	--	6	8	3	10	5	--	6	1	2	59	49	19.29	9.32
1	1	733	4	--	3	--	9	6	--	7	--	11	--	8	2	--	--	--	--	--	--	--	83	34	21.83	6.82
2	Control	871	132	--	58	--	--	--	--	--	--	--	38	--	--	--	--	--	29	--	9	--	--	--	--	30.54
2	0.125	719	9	--	3	--	11	8	6	8	--	9	3	--	9	4	4	--	3	--	--	--	48	10	16.27	10.71
2	0.25	839	9	--	8	--	9	12	--	2	--	10	5	10	5	--	5	--	5	--	--	--	70	27	17.88	9.54
2	0.5	819	4	--	6	--	7	13	--	9	--	11	4	5	5	--	5	--	2	--	--	--	79	26	19.54	8.67
2	1	844	3	4	4	--	9	10	--	8	6	6	--	7	--	--	6	5	--	4	--	--	89	36	22.51	8.53

TABLE 3 : MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR FRACTION OF *ALPINIA CALCARATA* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase							Anaphase				Telophase		Normal cytokinesis	Interphase			% of Abnormality	Mitotic index (%)
			Normal	Sticky chromatin	Normal	Diagonal	Misorientation	Stickiness	Contorted chromosomes	Ball metaphase - sticky	Clumping	Normal	Disturbed anaphase	Bridges	Early movement	Normal	Diagonal		Misorientation	Binucleate cell	Nuclear lesions		
½	Control	920	110	--	64	--	--	--	--	--	--	40	--	--	--	31	--	15	--	--	--	--	28.26
½	0.125	732	58	--	48	5	--	--	--	--	--	34	2	2	--	50	--	--	1	--	--	1.37	27.19
½	0.25	886	86	--	53	--	--	--	--	--	--	38	--	5	--	32	6	--	--	4	5	2.26	24.83
½	0.5	810	68	--	42	9	6	--	--	--	--	35	--	6	4	20	--	--	--	--	--	3.09	23.46
½	1	926	67	1	13	9	8	--	5	--	6	30	--	--	--	40	5	--	6	--	--	4.32	19.87
1	Control	960	115	--	68	--	--	--	--	--	--	45	--	--	--	34	--	25	--	--	--	--	29.90
1	0.125	866	66	--	29	10	--	--	--	--	--	55	--	5	--	65	--	--	--	--	--	1.73	26.56
1	0.25	1012	85	3	43	--	--	8	--	3	6	48	--	8	--	22	--	12	2	--	--	2.96	23.52
1	0.5	835	65	--	35	6	8	--	4	--	2	38	--	7	--	32	--	--	--	3	--	3.59	23.95
1	1	919	35	--	20	10	8	5	--	--	7	40	--	--	--	45	7	--	8	--	--	4.9	19.26
2	Control	1020	125	--	78	--	--	--	--	--	--	47	--	--	--	36	--	27	--	--	--	--	30.69
2	0.125	944	90	--	61	8	7	--	--	--	--	42	--	--	5	27	--	--	--	--	--	2.12	25.42
2	0.25	1020	80	--	50	--	9	--	--	3	9	41	--	8	--	30	2	8	--	--	--	3.04	23.53
2	0.5	889	60	--	45	7	--	--	--	--	8	32	--	--	--	33	9	--	8	3	--	3.94	21.82
2	1	831	42	2	33	8	6	7	--	4	--	28	--	8	7	12	--	--	--	--	--	5.05	18.89

TABLE 4 : MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR AND NON-POLAR FRACTIONS OF *ALPINIA GALANGA* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase							Anaphase						Telophase				Normal cytokinesis	Interphase			% of Abnormality	Mitotic index (%)
			Normal	Sticky chromatin	Normal	Diagonal	Ball Metaphase	Hypercondensed chromosomes	Clumping	Stickiness	Chromosome agglutination	Normal	Early Movement	Diagonal	Bridges	Multipolarity	Stickiness	Normal	Single bridge	Equatorial separation	Stickiness		Bizzare nucleus	Nuclear lesion	Binucleate cell		
½	Control	899	108	--	54	--	--	--	--	--	--	40	--	--	--	--	--	33	--	--	--	17	--	--	--	--	28.03
½	0.125	772	16	--	12	8	7	--	9	--	9	10	--	11	--	6	--	12	--	--	--	--	--	80	25	20.08	12.95
½	0.25	944	9	2	11	6	11	4	--	--	9	10	8	10	--	3	7	5	--	5	--	--	--	85	45	20.66	10.59
½	0.5	898	15	6	5	6	8	--	6	8	9	--	--	8	--	2	--	--	2	7	--	--	5	95	45	23.05	9.13
½	1	563	8	--	7	4	10	--	--	--	8	--	--	10	--	--	--	3	--	--	--	--	--	78	30	24.87	8.88
1	Control	869	115	--	58	--	--	--	--	--		49	--	--	--	--	--	33	--	--	--	13	--	--	--	--	30.84
1	0.125	811	12	1	13	--	6	6	--	2	--	8	--	9	--	10	--	17	--	8	8	--	--	78	42	20.96	12.33
1	0.25	872	10	3	8	10	8	--	8	--	10	8	--	14	7	10	4	6	--	--	7	--	5	70	42	22.70	12.04
1	0.5	779	8	--	12	9	11	--	--	10	5	--	--	10	--	5	--	--	--	--	--	--	--	90	40	23.11	8.99
1	1	849	14	--	12	6	8	--	--	8	12	--	--	--	8	--	--	2	--	--	--	--	--	98	62	23.78	8.24
2	Control	828	110	--	59	--	--	--	--	--	--	48	--	--	--	--	--	36	--	--	--	15	--	--	--	--	32.37
2	0.125	699	11	--	11	--	8	--	8	--	4	8	--	6	7	--	2	10	--	--	--	--	--	68	47	21.46	10.73
2	0.25	955	8	--	8	8	6	--	4	--	15	15	--	8	--	2	--	5	6	--	5	--	--	108	48	21.99	9.42
2	0.5	877	13	--	7	8	7	5	--	--	14	--	--	--	12	9	--	--	--	--	--	--	--	110	45	23.95	8.55
2	1	846	16	1	8	7	--	--	--	--	8	--	--	--	8	--	--	7	--	5	--	--	4	106	71	24.82	7.09

TABLE 5 : MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR FRACTION OF *ALPINIA GALANGA* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase					Anaphase							Telophase		Normal cytokinesis	Interphase		% of Abnormality	Mitotic index (%)
			Normal	Sticky chromatin	Normal	Diagonal	Chromosome fragment	Clumping	Stickiness	Normal	Early movement	Diagonal	Diagonal equatorial separation	Misorientation with bridges	Multipolarity	Scattering	Normal	Diagonal with bridge		Nuclear budding - early	Nuclear lesions		
½	Control	953	120	--	52	--	--	--	--	46	--	--	--	--	--	--	36	--	16	--	--	--	28.33
½	0.125	860	102	--	68	6	--	2	--	28	2	--	--	--	--	--	32	--	--	--	--	1.16	27.9
½	0.25	839	98	--	32	9	--	8	--	38	--	--	3	--	5	--	27	--	--	--	--	2.98	26.22
½	0.5	739	68	--	35	7	5	8	--	30	--	7	--	3	--	--	17	--	--	--	--	4.06	24.35
½	1	902	105	3	10	8	--	7	--	22	--	--	8	--	--	4	23	3	--	4	5	5.43	21.18
1	Control	1080	140	--	65	--	--	--	--	58	--	--	--	--	--	--	38	--	15	--	--	--	29.26
1	0.125	920	108	--	72	8	--	4	3	25	--	5	--	--	--	--	20	--	--	--	--	2.17	26.63
1	0.25	771	92	--	28	8	--	6	--	30	--	4	--	5	--	2	25	--	--	--	--	3.24	25.94
1	0.5	842	101	2	49	5	6	8	--	10	--	--	--	5	4	--	5	3	--	2	--	4.15	23.52
1	1	771	80	3	20	4	--	8	--	18	--	--	4	7	4	--	12	--	--	4	2	4.67	20.23
2	Control	1110	140	--	85	--	--	--	--	55	--	--	--	--	--	--	40	--	20	--	--	--	30.63
2	0.125	832	90	--	60	4	4	--	2	20	5	3	--	--	2	--	15	--	15	--	--	2.40	26.44
2	0.25	728	60	--	25	6	--	5	--	48	--	5	--	--	5	--	27	--	--	--	4	3.43	24.86
2	0.5	908	65	1	40	3	--	4	5	25	--	6	5	4	5	2	20	--	--	--	--	3.85	22.37
2	1	692	25	--	35	7	--	6	--	30	--	4	--	3	3	--	20	--	--	--	7	4.34	19.22

TABLE 6: MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR AND NON-POLAR FRACTIONS OF *ALPINIA MALACCENSIS* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase									Anaphase						Telophase		Normal cytokinesis	Interphase				% of Abnormality	Mitotic index (%)
			Normal	Nuclear vacuolization	Normal	Diagonal	Ball Metaphase - sticky	Ring chromosome & isochromatid gap	Polyploidy	Pulverization	Clumping	Stickiness	Contorted chromosomes	Normal	Early movement	Diagonal non synchronous movement	Bridges and broken bridges	Multipolarity	Diagonal stellate with bridges	Normal	Diagonal with bridges		Hyperchromasia	Nuclear lesion	Micronucleus	Binucleate cell		
½	Control	963	80	--	66	--	--	--	--	--	--	--	--	45	--	--	--	--	--	34	--	18	--	--	--	--	--	25.23
½	0.125	602	12	--	8	--	5	--	5	--	--	--	--	6	--	8	--	--	--	--	--	--	--	50	2	50	19.93	7.31
½	0.25	670	8	--	7	6	9	--	--	--	--	11	--	--	--	--	--	--	--	6	--	--	--	70	--	57	22.83	7.01
½	0.5	715	14	--	5	7	--	--	--	--	5	6	--	--	4	3	--	--	5	--	--	--	--	87	6	41	22.93	6.85
½	1	918	10	1	5	8	7	5	4	--	5	--	--	--	--	7	--	--	--	--	5	5	4	78	13	80	23.64	6.75
1	Control	956	110	--	68	--	--	--	--	--	--	--	--	48	--	--	--	--	--	37	--	21	--	--	--	--	--	29.71
1	0.125	568	9	--	11	--	8	--	--	--	--	7	5	--	--	4	--	--	4	--	--	--	--	60	--	32	21.13	8.45
1	0.25	710	15	--	14	8	6	--	--	--	--	--	--	2	--	5	5	--	--	2	4	--	--	80	2	48	22.25	8.59
1	0.5	812	8	3	6	6	8	4	6	--	4	--	7	--	5	10	--	--	--	--	--	--	5	74	3	50	22.78	8.25
1	1	830	8	1	6	--	6	--	--	3	--	--	--	--	--	--	8	9	--	--	--	--	--	94	10	69	24.10	4.94
2	Control	892	105	--	66	--	--	--	--	--	--	--	--	48	--	--	--	--	--	35	--	23	--	--	--	--	--	31.05
2	0.125	640	10	--	8	4	5	2	--	4	--	--	--	--	--	5	5	--	3	4	--	--	--	66	4	44	22.19	7.8
2	0.25	680	9	--	8	--	5	--	--	5	--	--	8	--	--	8	5	5	--	--	--	--	--	85	--	34	22.79	7.79
2	0.5	776	9	2	8	5	9	--	--	3	--	--	1	--	5	7	--	--	--	--	--	--	--	88	--	60	23.20	6.31
2	1	880	9	7	8	--	6	--	--	--	5	--	--	--	--	5	--	5	--	--	8	--	5	87	8	79	24.43	6.02

TABLE 7 : MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR FRACTION OF *ALPINIA MALACCENSIS* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase				Anaphase						Telophase			Normal cytokinesis	Interphase			% of Abnormality	Mitotic index (%)
			Normal	Sticky chromatin	Normal	Sticky	Giant polyploid cell with chromosome fragments	Diagonal	Normal	Multiple bridge - sticky	Sticky	Early movement	Equatorial separation of chromosomes	Multipolarity	Normal	Diagonal	Misorientation		Tetranucleate cell	Binucleate cell	Nuclear lesion		
½	Control	911	105	--	59	--	--	--	43	--	--	--	--	--	32	--	--	5	--	--	--	--	26.78
½	0.125	998	75	--	50	--	5	6	70	--	--	3	--	--	30	--	--	15	--	2	4	2.00	25.45
½	0.25	799	50	--	60	--	5	5	30	--	--	--	--	--	15	--	5	--	--	7	8	3.75	21.28
½	0.5	722	40	--	20	5	--	6	20	--	--	--	--	5	30	10	--	--	4	3	7	5.54	18.84
½	1	820	48	--	12	--	2	7	18	3	15	3	2	--	22	2	8	--	--	2	8	6.34	17.32
1	Control	985	120	--	66	--	--	--	47	--	--	--	--	--	34	--	--	20	--	--	--	--	29.14
1	0.125	878	70	--	60	5	--	--	30	--	--	--	5	--	30	--	--	--	5	2	7	2.73	22.78
1	0.25	865	45	-	51	5	--	5	58	5	--	5	--	3	10	--	--	--	--	5	6	3.93	21.62
1	0.5	882	50	--	15	--	--	15	48	--	--	--	--	--	11	5	5	--	--	2	10	4.20	16.89
1	1	738	38	2	--	--	--	8	32	--	--	--	6	--	28	--	2	--	4	5	13	5.42	15.72
2	Control	1030	130	--	69	--	--	--	49	--	--	--	--	--	37	--	--	30	--	--	--	--	30.58
2	0.125	908	60	--	40	--	--	5	60	--	5	--	--	5	30	--	--	--	2	5	8	3.30	22.58
2	0.25	840	70	--	30	5	5	--	40	5	--	5	--	--	10	--	--	--	--	6	9	4.17	20.24
2	0.5	788	40	--	8	7	2	8	42	7	--	8	--	--	20	--	3	--	--	3	7	5.71	18.40
2	1	765	46	1	--	8	--	7	34	--	4	8	7	--	15	2	--	--	3	4	6	6.54	17.25

TABLE 8 : MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR AND NON-POLAR FRACTIONS OF *ALPINIA PURPURATA* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase						Anaphase						Telophase				Normal cytokinesis	Interphase				% of Abnormality	Mitotic index (%)
			Normal	Sticky chromatin	Normal	Diagonal	Ball Metaphase	Clumping	Stickiness	Scattering	Normal	Misorientation in giant cell	Diagonal	Single Bridge	Chromosome fragment	Tripolarity with connecting bridges	Normal	Sticky telophase with micronuclei	Diagonal	Stickiness		Fragmentation leading to chromatin globules	Nuclear lesion	Nuclear fragmentation	Binucleate cell		
½	Control	810	80	–	48	–	–	–	–	–	39	–	–	–	–	–	28	–	–	–	25	–	–	–	–	–	27.16
½	0.125	616	18	–	12	15	15	10	–	8	10	–	7	10	–	–	10	5	5	–	–	2	20	3	–	16.23	20.29
½	0.25	600	20	–	10	12	13	–	11	–	7	–	14	5	–	8	8	6	–	–	–	3	15	5	8	16.66	19.00
½	0.5	733	15	3	15	4	15	–	–	15	12	–	8	9	2	12	8	3	3	8	–	-	31	2	8	16.78	18.01
½	1	580	17	4	13	5	10	5	–	–	6	–	5	5	6	5	14	–	–	–	–	–	46	2	10	17.41	16.38
1	Control	868	95	–	54	–	–	–	–	–	46	–	–	–	–	–	31	–	–	–	26	–	–	–	–	–	29.03
1	0.125	638	10	–	15	10	8	7	10	10	8	–	8	7	–	–	7	–	10	10	–	–	20	–	–	15.67	18.81
1	0.25	710	10	–	12	18	18	–	–	14	10	–	10	–	–	–	10	7	–	–	8	–	34	3	8	15.77	16.48
1	0.5	576	12	6	18	10	12	10	1	–	10	–	–	–	–	–	8	–	–	–	–	6	39	3	8	16.49	15.10
1	1	662	12	–	13	10	18	7	–	10	10	2	–	–	–	–	10	–	–	–	–	–	54	2	13	17.52	13.90
2	Control	890	105	–	68	–	–	–	–	–	46	–	–	–	–	–	37	–	–	–	27	–	–	–	–	–	31.80
2	0.125	538	18	1	25	8	–	5	–	7	23	–	5	5	–	–	10	–	–	–	–	–	39	–	10	14.87	19.89
2	0.25	688	22	2	18	12	18	–	5	10	10	5	8	–	3	–	7	–	–	–	–	5	25	5	10	15.84	17.44
2	0.5	595	12	1	17	–	15	–	–	5	11	4	–	10	5	5	10	–	–	–	–	–	36	2	15	16.47	15.97
2	1	748	10	–	10	7	18	8	–	14	8	6	–	10	–	–	10	6	–	2	–	2	42	–	18	17.78	14.57

TABLE 9 : MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR FRACTION OF *ALPINIA PURPURATA* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase					Anaphase					Telophase			Normal cytokinesis	Interphase		% of Abnormality	Mitotic index (%)
			Normal	Sticky chromatin	Normal	Diagonal	Misorientation	Stickiness	Clumping	Normal	Non-synchronous movement	Bridges	Early movement	Diagonal stellate with bridges	Normal	Diagonal	Non-synchronous chromosome group		Binucleate cell	Nuclear lesions		
½	Control	892	90	--	69	--	--	--	--	40	--	--	--	--	31	--	--	30	--	--	--	29.15
½	0.125	866	60	--	48	9	3	--	--	42	10	--	7	--	20	--	--	20	--	3	3.70	25.29
½	0.25	833	72	--	52	8	--	8	--	25	--	9	6	--	10	--	--	--	--	9	4.80	22.81
½	0.5	978	61	2	49	16	--	12	--	42	--	8	--	5	10	--	--	--	--	7	5.11	20.96
½	1	977	68	--	32	--	3	12	--	28	8	--	9	3	12	7	8	--	7	8	6.65	19.45
1	Control	878	98	--	72	--	--	--	--	43	--	--	--	--	32	--	--	33	--	--	--	31.66
1	0.125	812	68	1	48	3	--	5	--	50	4	--	4	2	24	5	--	--	--	5	3.57	26.97
1	0.25	911	58	--	63	8	--	--	--	46	8	--	--	--	28	--	--	--	5	18	4.28	23.16
1	0.5	828	57	--	20	--	--	10	--	43	13	12	8	2	16	5	--	--	--	--	6.04	22.46
1	1	882	30	--	40	--	3	12	8	32	--	12	15	--	18	6	9	--	--	--	7.37	20.98
2	Control	918	115	--	76	--	--	--	--	47	--	--	--	--	35	--	--	28	--	--	--	32.79
2	0.125	932	60	--	53	5	--	--	--	68	--	10	5	--	12	--	--	17	2	8	3.22	24.68
2	0.25	855	42	1	38	12	--	12	6	43	--	8	--	--	18	--	--	--	--	6	5.26	21.05
2	0.5	938	24	2	38	12	--	11	--	32	--	8	12	5	38	--	--	--	2	8	6.4	19.40
2	1	744	23	1	17	--	--	12	8	25	--	10	14	--	17	7	6	--	--	2	8.06	18.82

TABLE 10 : MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR AND NON-POLAR FRACTIONS OF *ALPINIA SMITHIAE* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase								Anaphase							Telophase			Normal cytokinesis	Interphase			% of Abnormality	Mitotic index (%)
			Normal	Giant cell – nuclear dissolution	Normal	Diagonal	Ball metaphase	Polyploid	Clumping	Stickiness	Giant cell with isochromatid gaps	Chromosome agglutination	Normal	Ring chromosome	Early movement	Diagonal	Bridges	Multipolarity	Stellate anaphase	Normal	Diagonal	Non-synchronised chromosomal groups		Nuclear lesion	Giant cell with giant nucleus	Binucleate cell		
½	Control	829	110	--	52	--	--	--	--	--	--	--	33	--	--	--	--	--	--	28	--	--	13	--	--	--	--	28.47
½	0.125	965	20	1	18	12	11	6	13	--	4	8	12	3	15	17	--	--	--	10	--	--	--	86	2	34	21.97	15.59
½	0.25	680	19	--	18	12	--	5	12	--	--	--	10	--	--	5	--	2	--	4	--	--	2	88	--	28	22.35	13.09
½	0.5	690	10	--	11	11	15	4	--	11	6	--	4	5	--	6	--	2	--	4	--	--	--	84	3	30	25.65	12.90
½	1	712	8	--	7	16	11	--	--	12	5	--	4	7	--	5	--	--	--	--	3	--	--	98	8	33	27.81	10.96
1	Control	898	120	--	58	--	--	--	--	--	--	--	39	--	--	--	--	--	--	32	--	--	18	--	--	--	--	29.73
1	0.125	930	18	1	12	10	10	--	16	--	6	9	18	8	12	--	8	--	--	14	--	--	--	80	3	42	22.04	15.27
1	0.25	788	17	--	14	--	16	--	12	--	--	6	6	--	--	6	--	7	4	8	--	12	--	86	--	38	23.73	13.71
1	0.5%	748	13	2	11	12	16	--	8	--	--	--	3	6	1	7	--	--	--	7	3	8	--	85	--	46	25.94	12.97
1	1	776	6	--	8	12	15	--	--	8	7	--	--	--	--	--	4	--	--	--	5	--	--	109	--	44	26.29	8.38
2	Control	918	120	--	62	--	--	--	--	--	--	--	41	--	--	--	--	--	--	38	--	--	26	--	--	--	--	31.26
2	0.125	868	16	--	12	7	8	--	5	--	--	15	14	--	--	6	--	4	5	8	--	--	--	83	--	53	21.43	11.52
2	0.25	967	19	--	12	14	15	--	--	--	--	--	8	2	12	--	12	--	6	--	4	--	--	97	6	61	23.68	10.75
2	0.5	849	12	--	10	9	--	--	--	12	--	--	7	--	12	10	--	8	--	--	2	5	--	104	--	73	27.68	10.25
2	1	693	6	3	9	9	5	--	7	--	8	--	--	--	8	5	--	3	--	--	2	--	--	112	--	49	30.45	9.38

TABLE 11 : MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR FRACTION OF *ALPINIA SMITHIAE* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase					Anaphase						Telophase		Normal cytokinesis	Inter-phase		% of Abnormality	Mitotic index (%)
			Normal	Sticky chromatin	Normal	Ball metaphase	Stickiness	Diagonal	Misorientation	Normal	Chromosomal associations	Stathmo - anaphase	Bridges	Early movement	Multipolarity	Normal	Diagonal		Nuclear lesion	Nuclear leakage		
½	Control	998	108	--	76	--	--			51	--	--	--	--	--	42	--	12	--	--	--	28.96
½	0.125	914	140	--	30	--	--	4	--	50	--	--	6	--	--	20	--	--	--	--	1.09	27.35
½	0.25	819	110	--	30	--	5	--	7	48	3	2	--	--	--	10	--	--	--	3	2.44	26.25
½	0.5	689	80	--	20	--	7	6	5	25	--	--	5	--	--	15	--	--	2	--	3.63	23.66
½	1	949	100	1	25	3	--	7	--	15	--	--	7	6	--	35	7	7	2	3	3.79	22.94
1	Control	1116	120	--	72	--	--	--	--	58	--	--	--	--	--	42	--	38	--	--	--	29.57
1	0.125	889	118	--	62	--	5	--	4	30	--	--	--	--	--	15	--	--	1	--	1.12	26.32
1	0.25	885	120	--	30	--	--	--	5	26	5	--	--	--	--	19	--	--	2	--	1.36	23.16
1	0.5	832	60	--	40	--	--	12	8	30	2	3	4	--	--	30	--	--	--	1	3.61	22.72
1	1	889	90	2	35	--	--	--	5	20	--	--	5	7	3	5	8	--	5	--	3.94	20.25
2	Control	1032	120	--	70	--	--	--	--	51	--	--	--	--	--	48	--	40	--	--	--	31.88
2	0.125	788	105	--	60	--	7	--	8	30	--	--	--	--	--	--	--	--	--	--	1.90	26.65
2	0.25	918	101	--	39	2	6	3	12	19	5	--	--	--	--	31	--	--	2	--	3.27	23.75
2	0.5	978	105	--	38	--	9	8	5	20	3	--	--	--	--	19	5	--	5	3	3.89	21.68
2	1	792	70	1	20	--	--	5	--	10	3	4	7	5	5	30	--	--	3	2	4.42	20.20

TABLE 12 : MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR AND NON-POLAR FRACTIONS OF *ALPINIA VITTATA* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase										Anaphase								Telophase			Normal cytokinesis	Interphase				% of Abnormality	Mitotic index
			Normal	Giant cell - nuclear dissolution	Normal	Diagonal	Ball metaphase	Polyploidy	Giant cell with isochromatid gaps	Ball metaphase-sticky	Clumping	Stickiness	Chromosomal associations	Giant polyploid cell with chromosome fragments	Normal	Early movement	Hemistellate condition in a giant cell	Non synchronization in giant cell	Diagonal	Bridges	Multipolarity in giant cell	Stellate anaphase	Normal	Diagonal	Stickiness		Nuclear diminution	Nuclear lesion	Nuclear disintegration	Binucleate cell		
½	Control	840	90	--	57	--	--	--	--	--	--	--	--	--	40	--	--	--	--	--	--	--	31	--	--	15	--	--	--	--	--	27.74
½	0.125	860	16	--	8	12	10	13	--	--	5	--	--	-	15	12	--	--	15	8	--	--	8	--	--	--	--	86	--	58	25.47	14.19
½	0.25	936	17	1	12	11	8	4	3	--	8	--	2	12	3	--	1	--	6	6	6	--	4	6	--	--	--	91	--	76	25.75	11.75
½	0.5	926	9	2	11	16	14	--	--	--	8	8	--	12	2	--	--	--	--	--	12	--	--	--	--	--	--	98	--	80	27.00	10.15
½	1	1058	7	1	10	--	--	6	--	--	--	10	--	18	5	--	--	12	--	12	-	--	4	5	14	--	2	112	--	98	27.41	9.83
1	Control	966	110	--	68	--	--	--	--	--	--	--	--	--	48	--	--	--	--	--	--	--	37	--	--	21	--	--	--	--	--	29.40
1	0.125	1060	14	--	11	8	12	4	--	7	6	6	--	9	11	--	--	9	--	--	4	5	12	8	4	--	4	82	--	88	24.15	12.26
1	0.25	992	15	1	12	11	12	--	--	5	8	--	--	11	6	6	--	--	4	5	6	5	9	--	--	--	--	90	--	93	25.91	11.69
1	0.5	1032	8	--	12	14	16	5	--	--	6	9	--	18	--	4	--	--	6	--	--	6	--	8	--	-	--	92	--	91	26.65	10.85
1	1	987	7	3	9	--	--	--	5	--	--	--	8	16	--	11	--	--	13	8	--	10	--	--	10	--	2	96	--	86	27.15	10.13
2	Control	820	98	--	60	--	--	--	--	--	--	--	--	--	43	--	--	--	--	--	--	--	33	--	--	17	--	--	--	--	--	30.61
2	0.125	1028	12	1	13	6	5	--	--	5	5	8	--	--	9	10	6	--	15	10	--	2	18	--	--	--	--	96	7	72	24.12	12.16
2	0.25	998	16	--	12	10	--	--	4	5	5	--	6	18	--	10	8	--	12	--	6	--	--	2	--	--	--	82	7	87	26.25	11.42
2	0.5	978	10	--	16	8	--	--	8	--	--	10	7	12	--	--	6	--	--	10	9	6	--	--	--	--	--	98	2	81	26.28	10.43
2	1	932	16	6	16	--	12	--	5	--	--	--	4	16	--	8	3	--	--	9	--	--	--	--	--	--	--	120	--	73	27.47	10.19

68 K

TABLE 13 : MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR FRACTION OF *ALPINIA VITTATA* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase				Anaphase							Telophase		Normal cytokinesis	Interphase		% of Abnormality	Mitotic Index (%)
			Normal	Sticky chromatin	Normal	Diagonal	Sticky	Pulverization	Normal	Multipolar movement of chromosomes	Early movement	Bridge	Pulverization	Tripolarity	Multipolarity	Normal	Non synchronized chromosome groups		Nuclear lesions	Binucleate cell		
½	Control	998	105	--	76	--	--		51	--	--	--	--	--	--	42	--	15	--	--	--	28.96
½	0.125	922	140	--	45	3	--	2	40	--	--	3	--	--	--	15	--	--	1	1	1.08	26.90
½	0.25	768	105	--	45	5	--	4	18	--	--	--	2	4	--	17	--	--	--	--	1.95	26.04
½	0.5	812	102	--	38	6	--	6	16	--	7	--	--	--	--	14	--	--	--	1	2.46	23.28
½	1	832	80	1	40	5	8	--	20	--	4	--	--	5	7	5	3	--	2	--	4.21	21.39
1	Control	1116	158	--	50	--	--	--	58	--	--	--	--	--	--	42	--	22	--	--	--	29.57
1	0.125	817	118	--	42	3	--	4	25	3	-	3	--	--	3	12	--	-	--	--	1.96	26.56
1	0.25	941	125	--	45	6	--	7	30	--	--	--	--	5	--	20	--	--	1	--	2.02	25.50
1	0.5	922	106	1	27	4	5	4	40	--	--	5	--	--	--	12	--	--	2	--	2.28	22.13
1	1	788	85	--	25	8	8	6	19	--	--	--	--	7	5	11	--	--	--	1	4.44	22.08
2	Control	1032	160	--	48	--	--	--	51	--	--	--	-	--	--	48	--	22	--	--	--	31.88
2	0.125	877	118	--	42	6	--	6	25	--	--	3	--	--	--	30	--	--	--	--	1.71	26.23
2	0.25	861	95	--	35	9	--	5	35	--	--	6	--	--	--	25	--	--	--	--	2.32	24.39
2	0.5	819	90	--	30	6	8	--	16	--	5	6	--	3	2	14	--	--	--	--	3.66	21.98
2	1	802	70	--	20	8	6	4	30	--	--	6	--	6	--	10	4	--	2	4	4.99	20.45

TABLE 14 : MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR AND NON-POLAR FRACTIONS OF *ALPINIA ZERUMBET* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase							Anaphase						Telophase				Normal cytokinesis	Interphase				% of Abnormality	Mitotic index (%)
			Normal	Nuclear vacuolization	Normal	Diagonal	Ball metaphase	Polyploid stellate metaphase	Clumping	Stickiness	Hypercondensed chromosomes	Normal	Early movement	Hemistellate condition in a giant cell	Diagonal non - synchronized movement	Double bridge - sticky	Multipolarity	Normal	Pulverization	Diagonal	Non-synchronous movement		Nuclear leakage	Nuclear lesion	Nuclear budding	Binucleate cell		
½	Control	831	99	--	48	--	--	--	--	--	--	38	--	--	--	--	--	27	--	--	--	25	--	--	--	--	--	28.51
½	0.125	688	16	--	15	8	14	--	12	6	--	17	9	--	--	--	--	12	--	3	--	--	--	75	--	15	20.64	16.28
½	0.25	846	12	1	18	--	18	4	15	--	5	11	--	8	15	9	--	7	8	--	--	--	6	50	9	40	22.22	15.48
½	0.5	840	14	--	13	11	17	4	11	--	--	--	12	--	10	--	10	--	--	--	--	--	--	65	2	46	22.38	12.14
½	1	845	10	2	14	13	15	--	12	--	2	--	--	6	14	--	--	--	9	--	--	--	3	70	5	49	23.67	11.48
1	Control	760	98	--	45	--	--	--	--	--	--	36	--	--	--	-	--	24	--	--	--	12	--	--	--	--	--	28.29
1	0.125	890	18	--	17	5	18	--	7	12	--	16	19	5	18	--	--	8	6	2	--	--	4	67	5	--	18.88	16.97
1	0.25	670	14	--	16	--	26	--	14	--	--	--	--	--	6	--	--	8	--	--	--	--	--	68	--	36	22.39	12.54
1	0.5	676	11	--	14	8	12	--	--	--	--	--	12	--	8	--	8	4	--	--	--	--	--	66	--	40	22.78	11.39
1	1	660	11	--	14	8	--	--	--	--	8	--	--	8	--	--	16	--	7	--	--	--	--	70	--	43	24.24	10.91
2	Control	688	80	--	48	--	--	--	--	--	--	39	--	--	--	--	--	28	--	--	--	23	--	--	--	--	--	31.69
2	0.125	796	18	--	19	12	16	16	--	13	--	15	12	--	--	--	15	1	--	--	9	--	--	59	6	17	21.98	18.22
2	0.25	700	17	--	18	15	23	--	8	--	5	--	--	--	--	6	--	--	--	--	--	--	--	73	--	30	22.86	13.14
2	0.5	725	14	--	12	10	18	--	--	--	3	--	--	--	10	3	4	--	--	--	8	--	4	63	8	39	23.45	11.31
2	1	730	9	1	11	6	--	--	--	--	--	--	--	8	12	6	11	--	--	--	10	--	5	75	5	41	24.65	10.14

TABLE 15 : MITOTIC INDEX, TYPES AND FREQUENCY OF MITOTIC ABNORMALITIES INDUCED BY DIFFERENT CONCENTRATIONS OF EXTRACTS CONTAINING POLAR FRACTION OF *ALPINIA ZERUMBET* IN *ALLIUM CEPA* ROOT TIPS AFTER DIFFERENT TREATMENTS

Duration of treatment (hrs)	Concentration of extract (%)	No. of cells observed	Prophase		Metaphase					Anaphase					Telo-phase	Normal cytokinesis	Interphase		% of Abnormality	Mitotic index (%)
			Normal	Sticky chromatin	Normal	Stickiness	Diagonal	Misorientation	Coalesced ends of chromosomes	Normal	Diagonal	Multiple bridges	Disturbed anaphase	Multipolarity	Normal		Nuclear lesion	Binucleate cell		
½	Control	922	88	--	68	--	--	--	--	48	--	--	--	--	35	37	--	--	--	29.93
½	0.125	820	118	--	62	--	4	--	--	20	--	6	--	--	20	--	--	--	1.22	28.05
½	0.25	982	138	--	42	8	6	--	--	35	4	--	--	--	25	--	1	1	2.04	26.37
½	0.5	816	115	--	30	5	7	5	--	18	--	8	--	--	12	--	--	--	3.06	24.51
½	1	843	108	1	22	7	6	6	4	5	5	--	--	5	5	--	1	--	4.15	20.64
1	Control	978	112	--	73	--	--	--	--	51	--	--	--	--	37	20	--	--	--	29.96
1	0.125	901	126	--	54	6	--	--	4	18	--	5	3	--	17	16	-	--	2.0	27.64
1	0.25	922	118	--	62	--	6	--	--	20	4	8	--	2	20	--	--	--	2.17	26.03
1	0.5	843	70	2	31	8	8	5	2	12	--	5	--	--	15	49	--	--	3.56	24.56
1	1	732	90	--	25	8	6	8	2	19	5	--	--	--	11	--	2	1	4.37	23.77
2	Control	890	164	--	28	--	--	--	--	48	--	--	--	--	34	14	--	--	--	32.36
2	0.125	833	114	--	26	--	6	--	--	40	--	6	--	3	35	--	--	--	1.80	27.61
2	0.25	791	105	--	25	4	4	--	--	15	8	4	--	--	35	--	--	--	2.53	25.28
2	0.5	900	122	2	33	8	7	--	--	12	--	--	6	2	18	--	--	--	2.78	23.33
2	1	912	86	--	24	--	9	8	2	30	7	6	5	3	25	--	2	3	4.93	22.48

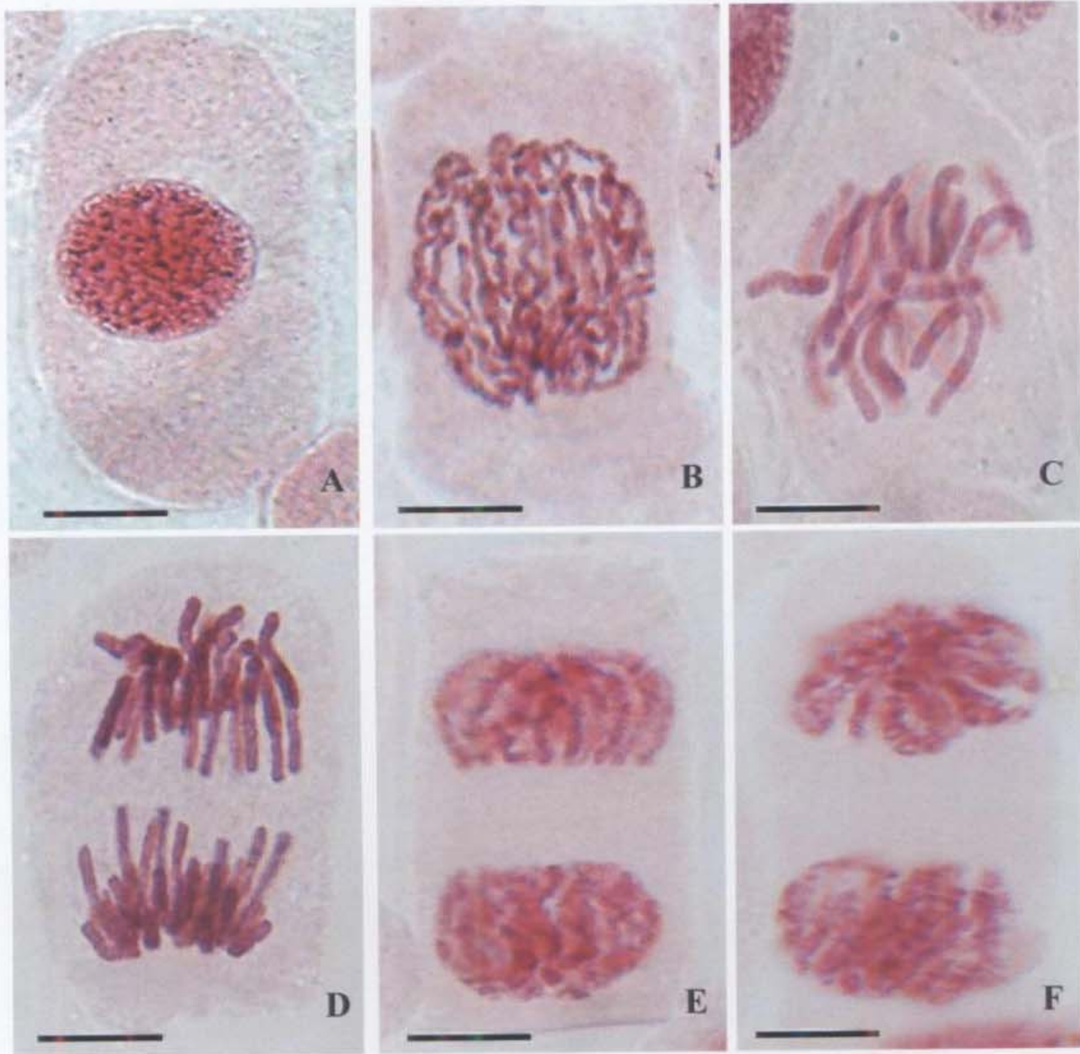


Plate III. Normal mitotic stages in *Allium cepa*

A. Interphase
B. Prophase
C. Metaphase
Bar-10μm

D. Anaphase
E. Telophase
F. Cytokinesis

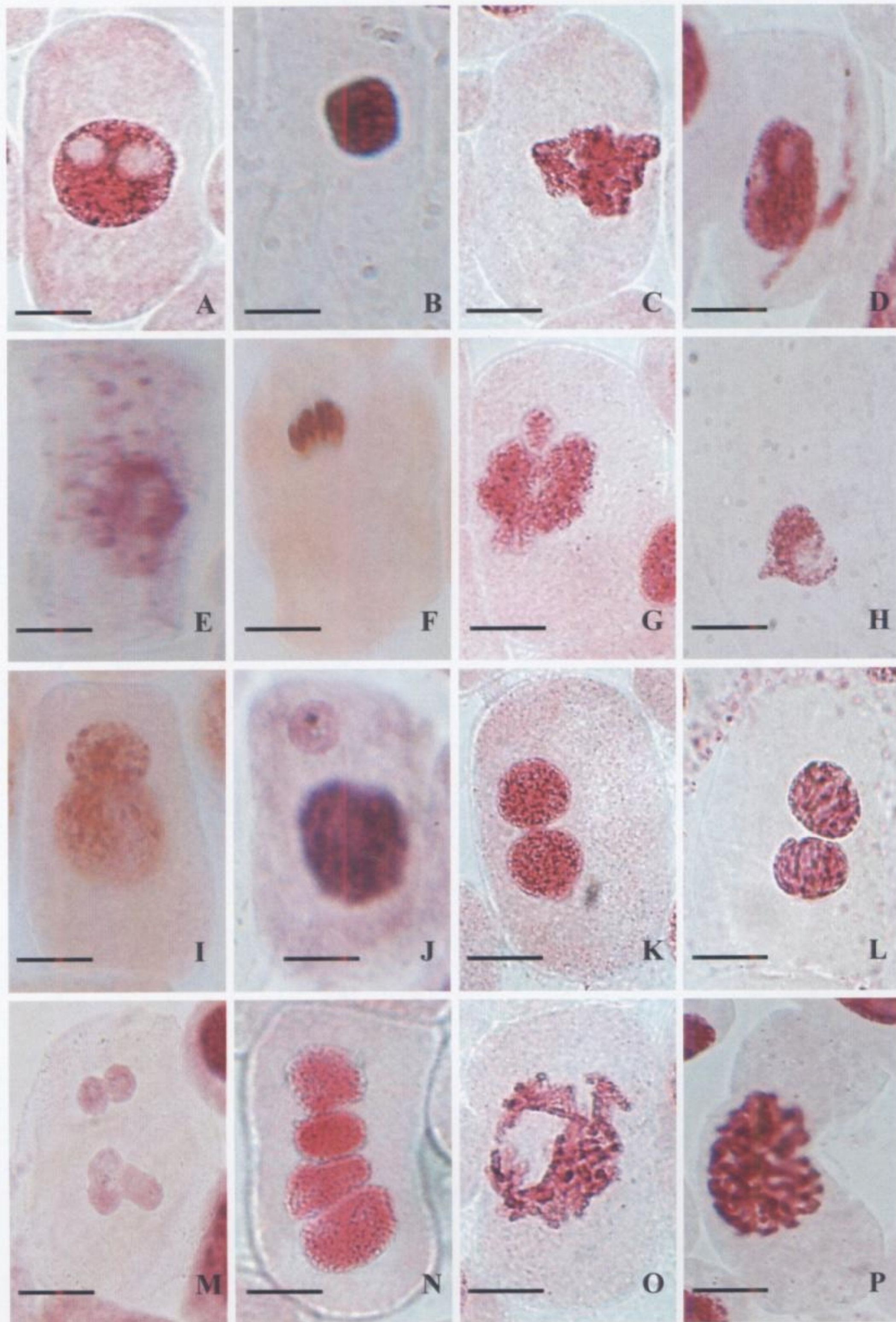


Plate IV. Cytological aberrations observed in *Allium cepa* root meristem treated with extracts of seven species of *Alpinia*. A-N : Interphase, O&P : Prophase; A-Nuclear lesion, B-Hyperchromasia, C-Bizzare nucleus, D-Nuclear leakage, E-Nuclear disintegration, F-Nuclear diminution, G-Nuclear fragmentation, H-Nuclear budding-early, I-Nuclear budding-late, J-Micro-nucleus, K-Binucleate with a connection, L-Binucleate cell, M-Fragmentation leading to chromatin globules , N-Tetra nucleate cell, O-Nuclear vacuolization, P-Sticky chromatin. Bar-10μm

68.a

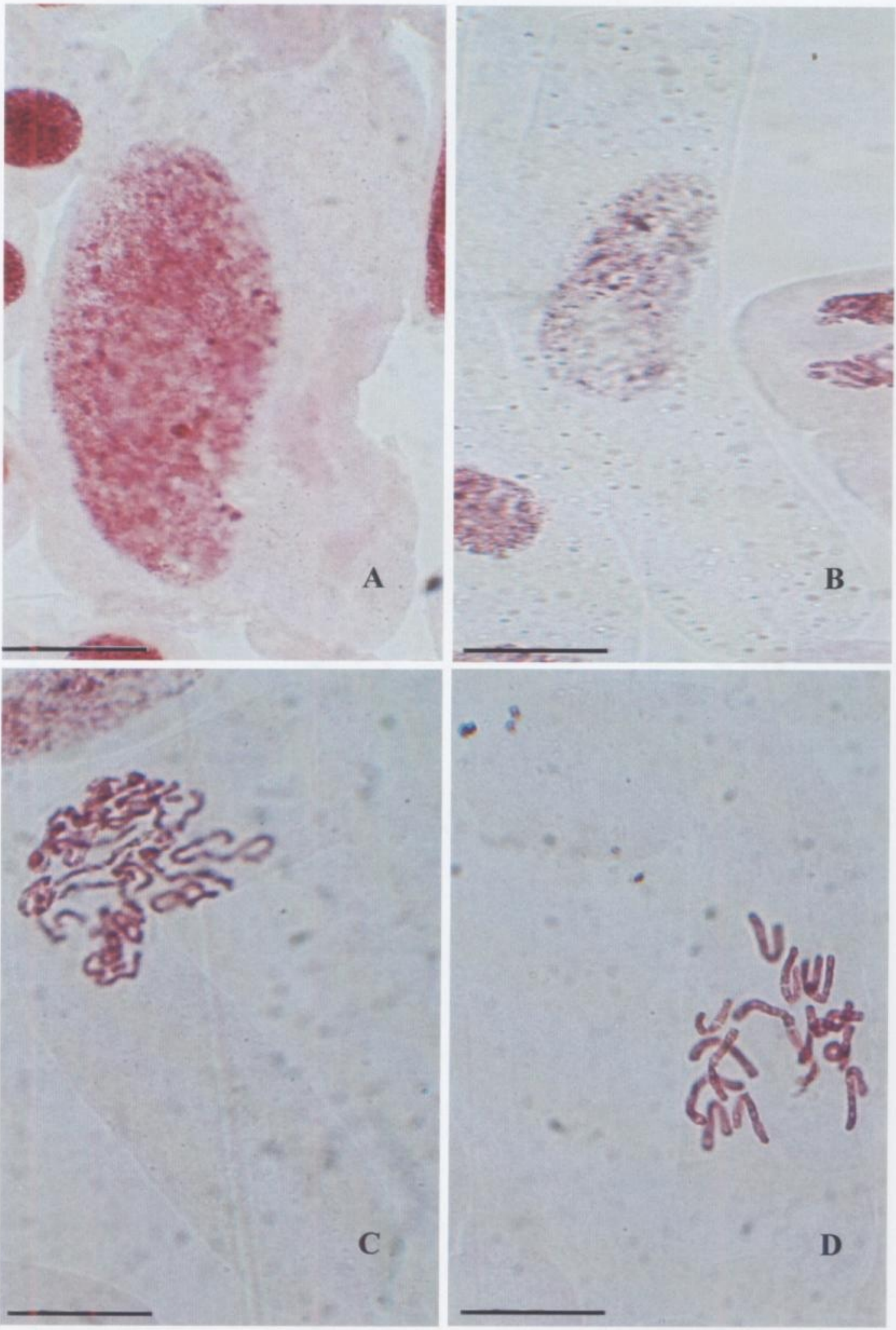


Plate V. Cytological aberrations observed in *Allium cepa* root meristem treated with extracts of seven species of *Alpinia*. A-Giant cell with a giant nucleus at interphase, B-Nuclear dissolution in a giant cell at prophase, C-Giant polyploid cell with chromosome fragments at metaphase, D-Giant cell with isochromatid gaps at metaphase. Bar-10μm

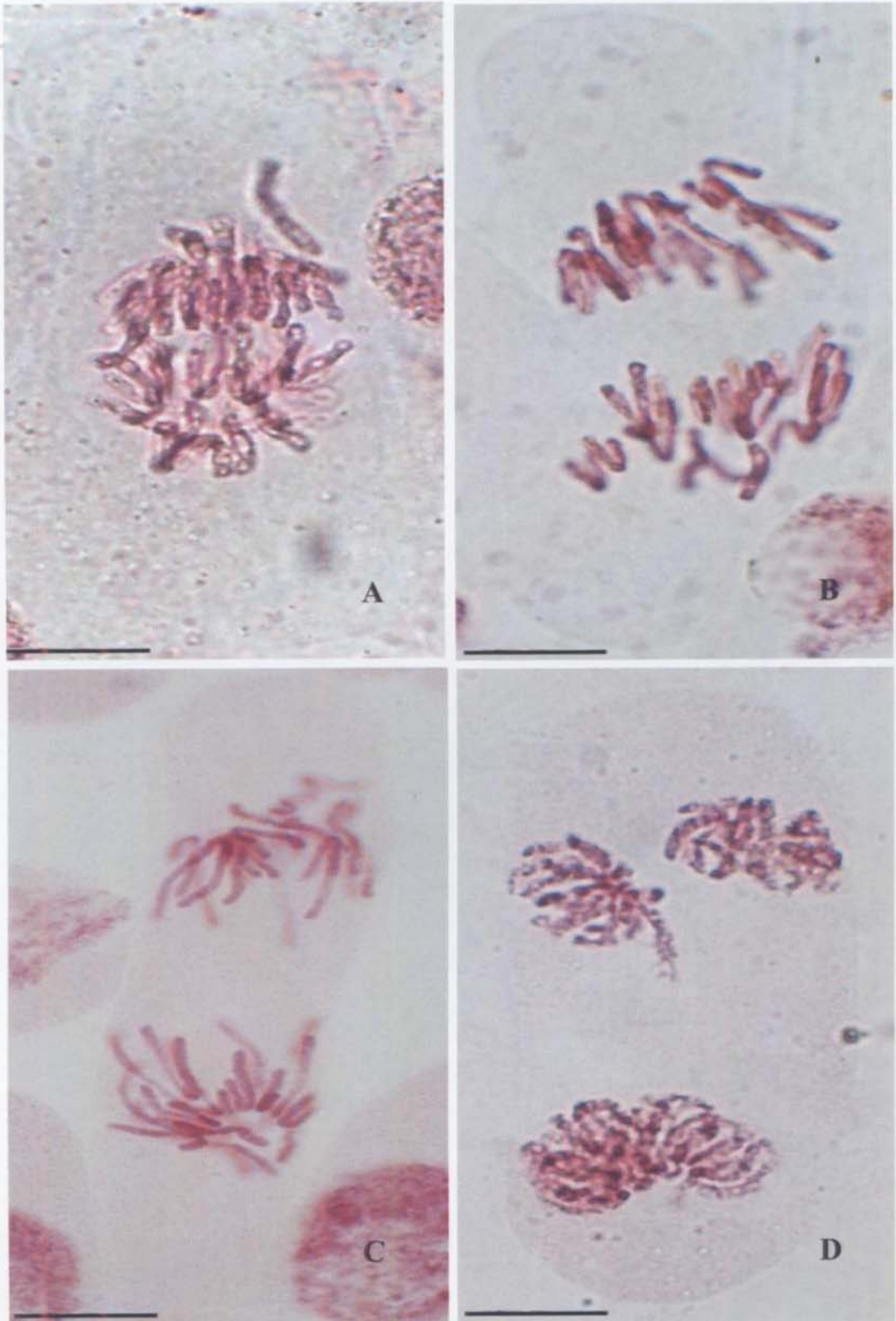


Plate VI. Cytological aberrations observed in *Allium cepa* root meristem treated with extracts of seven species of *Alpinia*. A-Hemi-stellate anaphase in a giant cell, B-Anaphase non-synchronization in a giant cell, C-Anaphase misorientation in a giant cell, D-Anaphase multipolarity in a giant cell. Bar-10μm

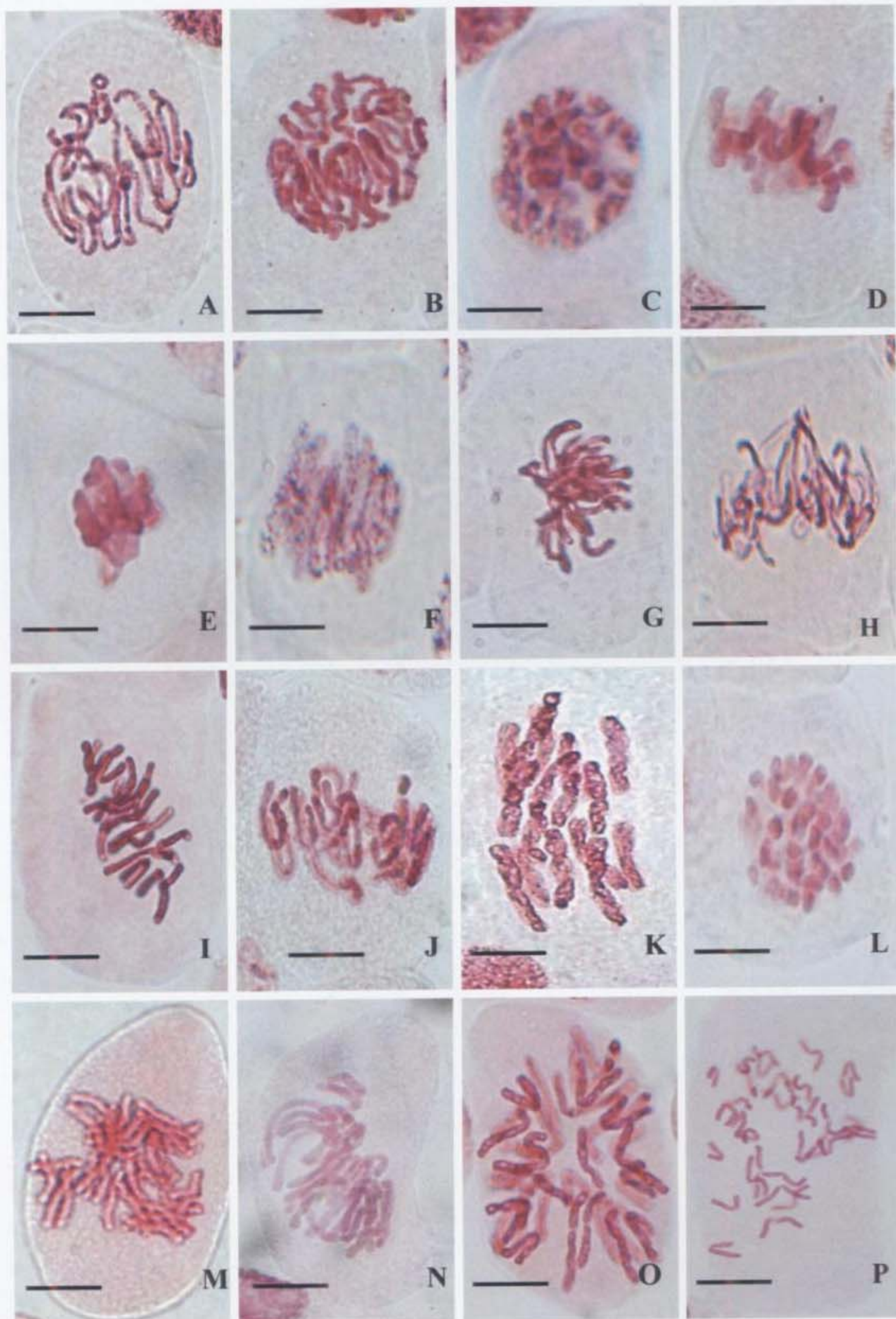


Plate VII. Cytological aberrations observed in *Allium cepa* root meristem treated with extracts of seven species of *Alpinia*. A-P: Metaphase; A-Ring chromosome and isochromatid gaps, B-Ball metaphase, C-Ball metaphase - sticky, D-Stickiness, E-Chromosome agglutination, F-Pulverization, G-Chromosome clumping, H-Coalesced ends of chromosomes, I-Diagonal metaphase, J-Chromosome fragment, K-Contorted chromosomes, L-Hypercondensed chromosomes, M-Abnormal chromosomal associations, N-Misorientation, O-Polyploid stellate metaphase, P-Polyploidy. Bar-10μm

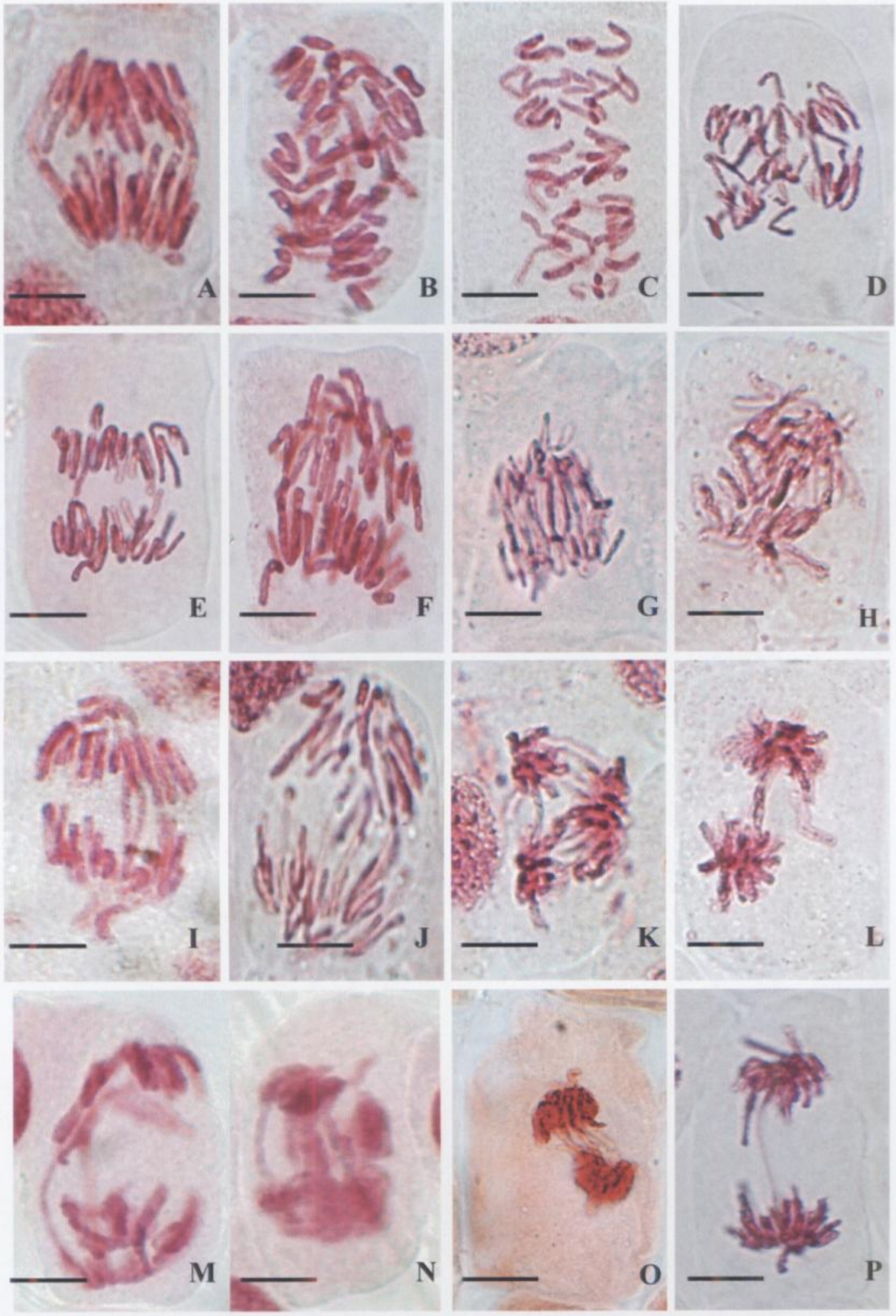


Plate VIII. Cytological aberrations observed in *Allium cepa* root meristem treated with extracts of seven species of *Alpinia*. A-P:Anaphase; A-Chromosome fragment, B-Scattering, C-Equatorial separation of chromosomes, D-Disturbed anaphase, E-Early movement, F-Diagonal non-synchronized movement, G-Stathmo anaphase, H-Diagonal stellate with bridges, I-Double bridge, J-Multiple bridges, K-Tripolarity with connecting bridges, L-Bridge and a broken bridge, M-Double bridge-sticky, N-Multiple bridges-sticky, O-Misorientation with bridges, P-Single bridge. Bar-10µm

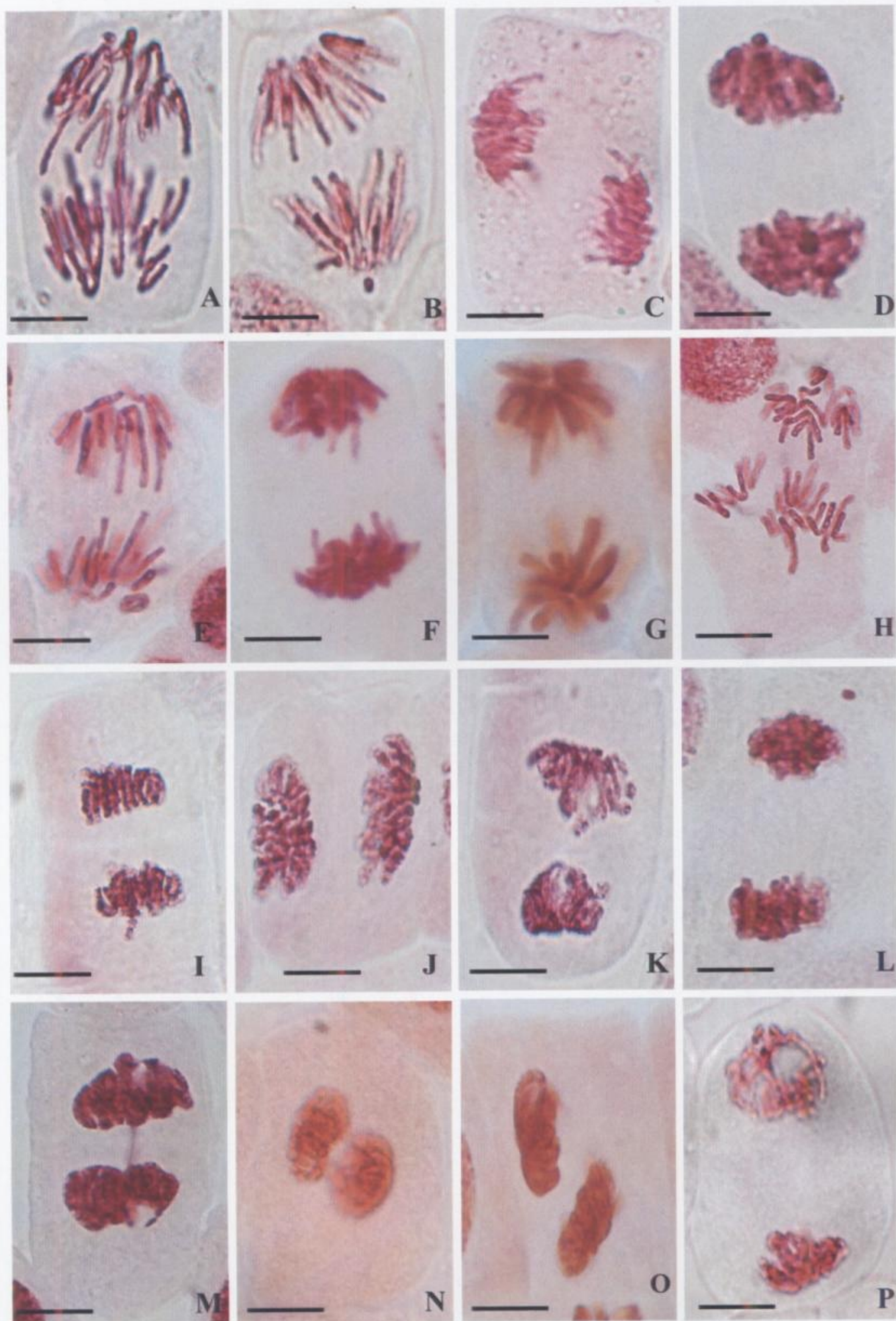


Plate IX. Cytological aberrations observed in *Allium cepa* root meristem treated with extracts of seven species of *Alpinia*. A-I: Anaphase, J-P: Telophase; A-Non-synchronous movement, B-Diagonal anaphase, C-Diagonal equatorial separation, D-Hypercondensed chromosomes, E-Ring chromosome, F-Stickiness, G-Stellate anaphase, H-Multipolar movement of chromosomes, I-Pulverization, J-Equatorial orientation, K-Pulverization, L-Sticky telophase with micro nucleus formation, M- Single bridge, N-Diagonal telophase with two bridges, O-Misorientation, P-Non-synchronized stages in chromosome groups. Bar-10μm

TABLE 16

Cytotoxic activities of methanolic extracts of *Alpinia* species on EAC and DLA cell lines

Short duration assay on EAC cell line	
Plants	IC_{50}* (μg/ml)
A. malaccensis	530 μg/ml
A. vittata	350 μg/ml
A. calcarata	No toxicity
A. galanga	No toxicity
A. purpurata	No toxicity
A. smithiae	No toxicity
A. zerumbet	No toxicity
Short duration assay on DLA cell line	
Plants	IC_{50}* (μg/ml)
A. malaccensis	460 μg/ml
A. vittata	350 μg/ml
A. calcarata	No toxicity
A. galanga	No toxicity
A. purpurata	No toxicity
A. smithiae	No toxicity
A. zerumbet	No toxicity

*The concentration of samples causing 50% inhibition is referred to as their IC_{50} values.

TABLE 17

Cytotoxic activities of methanolic extracts of *Alpinia malaccensis* and *Alpinia vittata* on L_{929} cells and A_{549} cells

Long duration assay on L_{929} cells	
Plants	IC_{50}* (µg/ml)
A. malaccensis	28 µg/ml
A. vittata	50 µg/ml
Long duration assay on A_{549} cells	
Plants	IC_{50}* (µg/ml)
A. malaccensis	160 µg/ml
A. vittata	375 µg/ml

*The concentration of the samples causing 50% inhibition is referred to as their IC_{50} values.

68 X

Table 18. Acute toxicity studies of *Alpinia malaccensis* and *Alpinia vittata*

Groups of extracts	SGOT (IU/l)	SGPT (IU/l)	ALP (IU/l)	Creatine (IU/l)
Normal	45.71 ± 2.72	50.70 ± 4.01	35.20 ± 3.00	0.92 ± 0.100
A. malaccensis 2 g/kg body wt.	46.31 ± 4.00	53.24 ± 3.86	34.27 ± 3.54	0.95 ± 1.00
A. malaccensis 1 g/kg body wt.	44.21 ± 3.84	52.23 ± 4.07	36.31 ± 2.95	0.94 ± 0.07
A. vittata 2g/kg body wt.	47.31 ± 4.02	49.64 ± 4.34	33.23 ± 3.26	0.96 ± 0.07
A. vittata 1 g/kg body wt.	43.80 ± 3.89	51.21 ± 4.20	34.21 ± 3.04	0.94 ± 0.09

Table 19. Antitumor activity of *Alpinia malaccensis* against DLA induced solid tumor (Tumor volume in cm^3)

Group of extracts	1st day	5th day	10th day	15th day	20th day	25th day	30th day
Control	0.78 ± 0.02	1.38 ± 0.53	2.00 ± 0.44	2.53 ± 0.63	2.71 ± 0.69	3.5 ± 0.63	4.53 ± 1.31
Cisplatin 10 mg/kg body wt.	0.91 ± 0.11	1.03 ± 0.34	1.45 ± 0.32	1.56 ± 0.35	1.49 ± 0.43	1.45 ± 0.41	1.4 ± 0.26
A. malaccensis 100 mg/kg body wt.	0.86 ± 0.18	1.03 ± 0.16	1.53 ± 0.30	1.62 ± 0.23	1.72 ± 0.23	1.83 ± 0.09	1.71 ± 0.16
A. malaccensis 500 mg/kg body wt.	0.81 ± 0.09	0.97 ± 0.12	1.21 ± 0.13	1.36 ± 0.05	1.2 ± 0.12	1.15 ± 0.10	1.00 ± 0.16

Table 20. Synergistic effect of *Alpinia malaccensis* and Cisplatin against DLA induced solid tumor (Tumor volume in cm^3)

Groups of extracts	1st day	5th day	10th day	15th day	20th day	25th day	30th day
Control	0.78 ± 0.02	1.38 ± 0.53	2.00 ± 0.44	2.53 ± 0.63	2.71 ± 0.69	3.5 ± 0.63	4.53 ± 1.31
Cisplatin 10 mg/kg body wt.	0.91 ± 0.11	1.03 ± 0.34	1.45 ± 0.32	1.56 ± 0.35	1.49 ± 0.43	1.45 ± 0.41	1.4 ± 0.26
A. malaccensis 100 mg/kg body wt.	0.86 ± 0.18	1.03 ± 0.16	1.53 ± 0.30	1.62 ± 0.23	1.72 ± 0.23	1.83 ± 0.09	1.71 ± 0.16
A. malaccensis 100 mg/kg body wt. + Cisplatin	0.81 ± 0.21	1.00 ± 0.32	1.38 ± 0.09	1.39 ± 0.20	1.21 ± 0.13	1.02 ± 0.33	0.96 ± 0.13

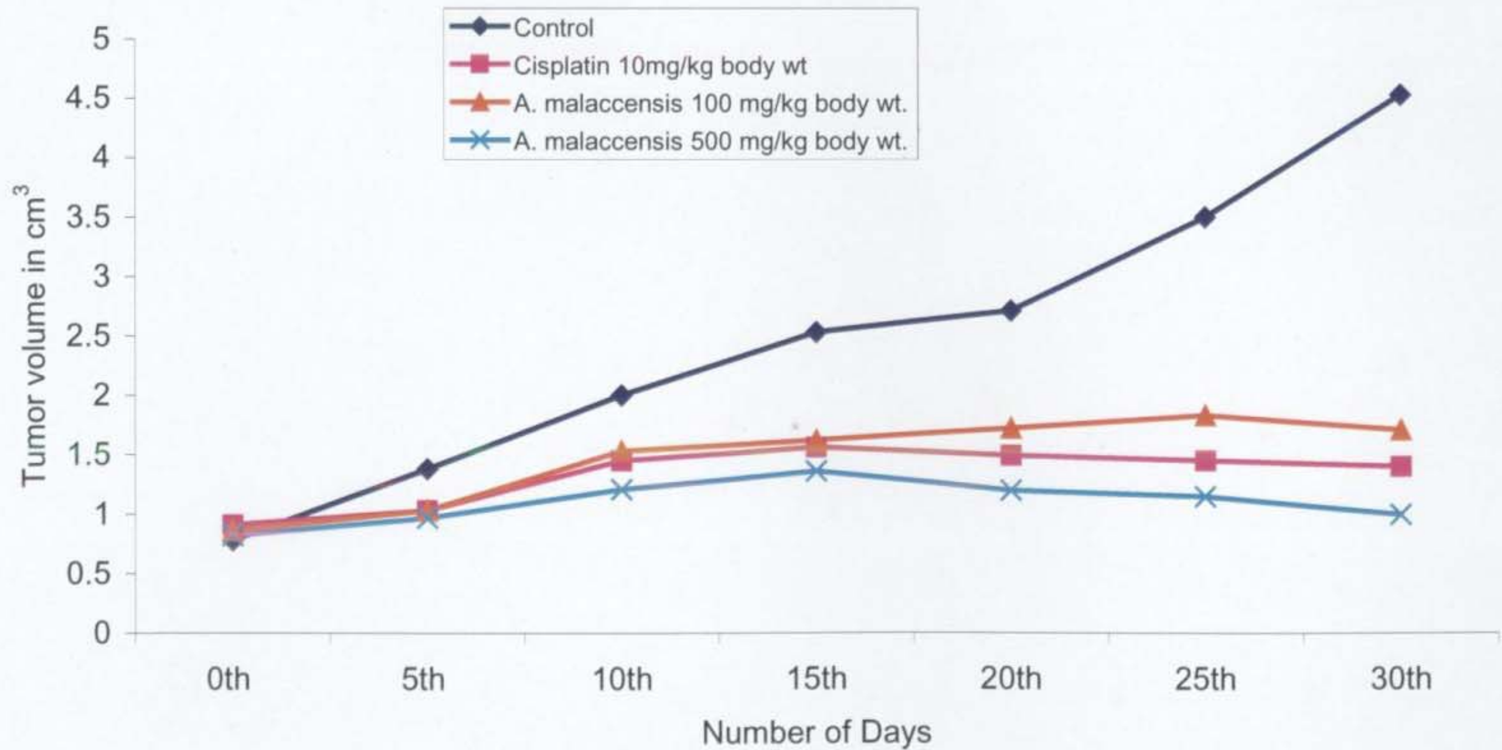


Fig. 1. Effect of methanolic extracts of *A. malaccensis* on DLA induced solid tumor (Tumor volume in cm^3)

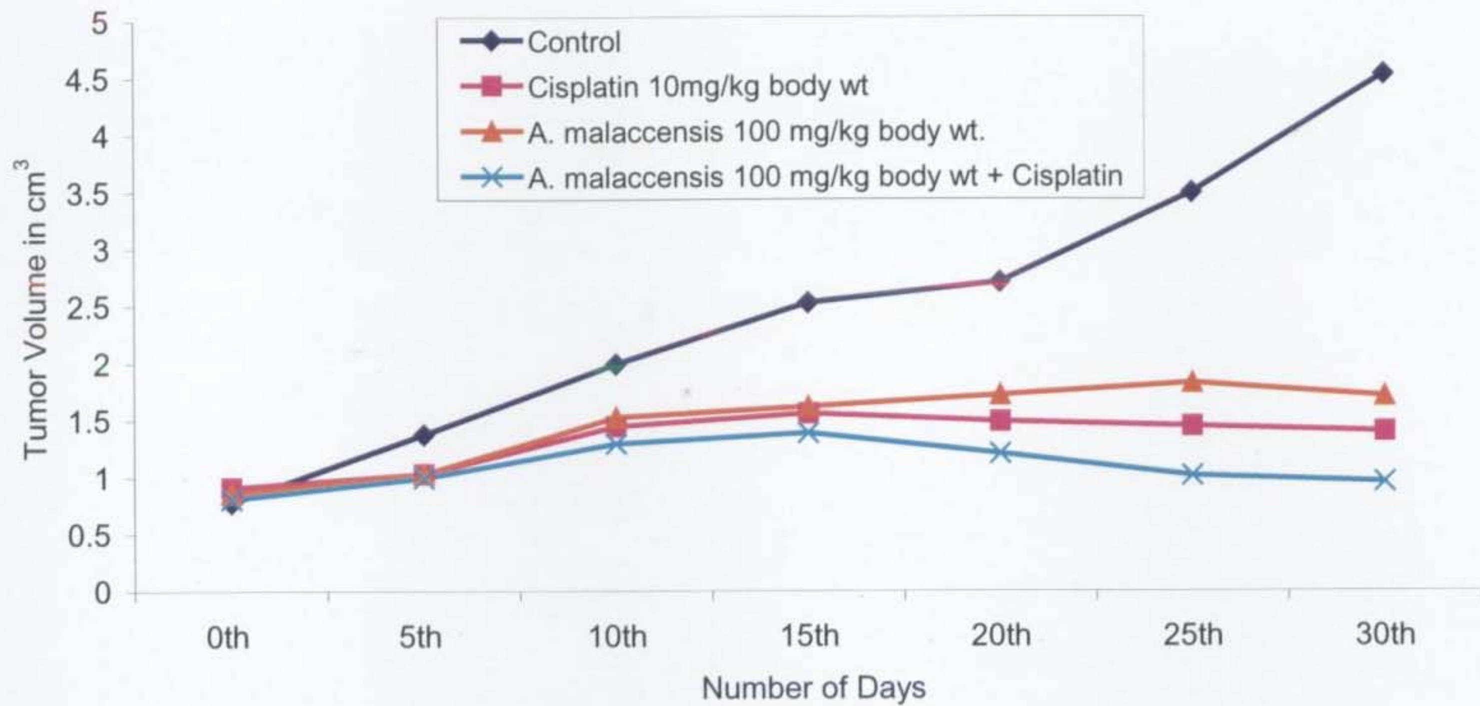


Fig. 2. Synergistic effect of methanolic extracts of *A. malaccensis* and Cisplatin on DLA induced solid tumor (Tumor volume in cm^3)

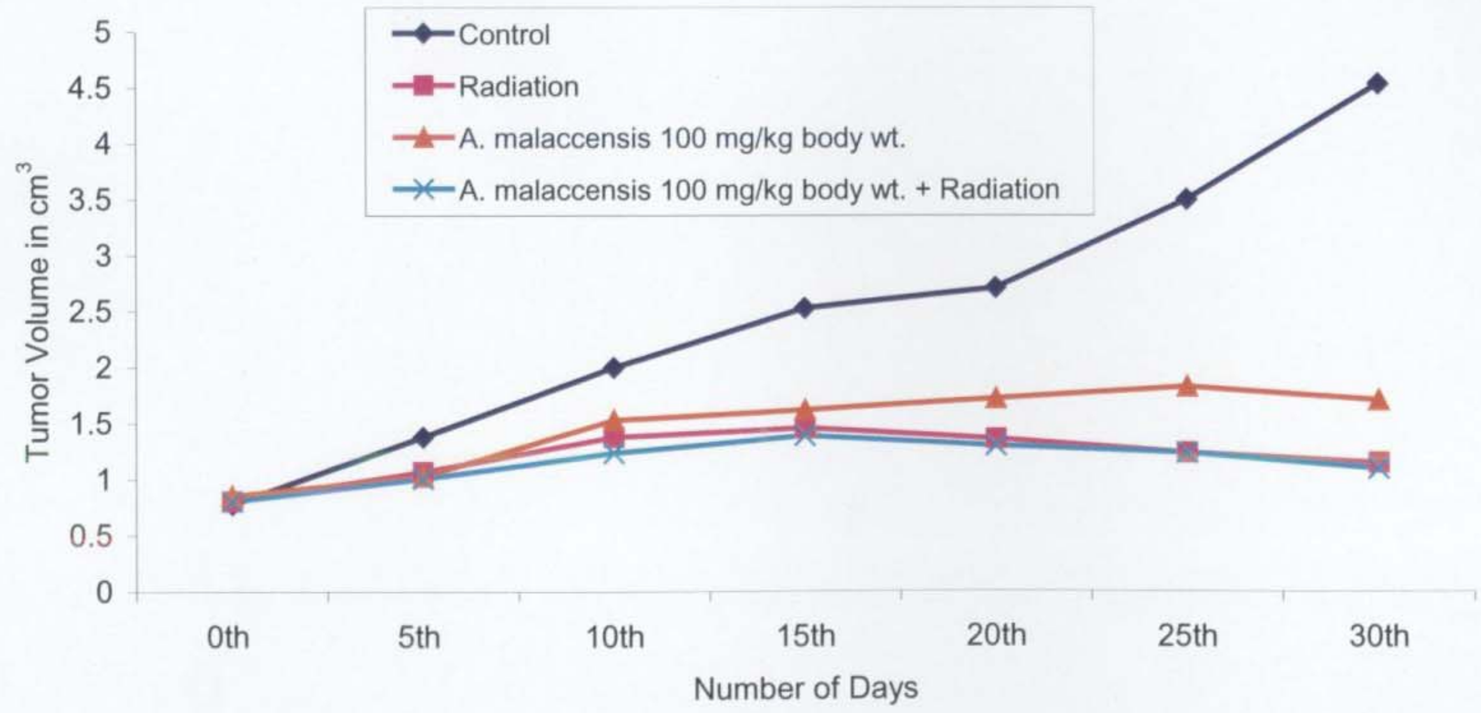


Fig. 3. Synergistic effect of *A. malaccensis* methanolic extract and Radiation on DLA induced solid tumor (Tumor volume in cm^3)

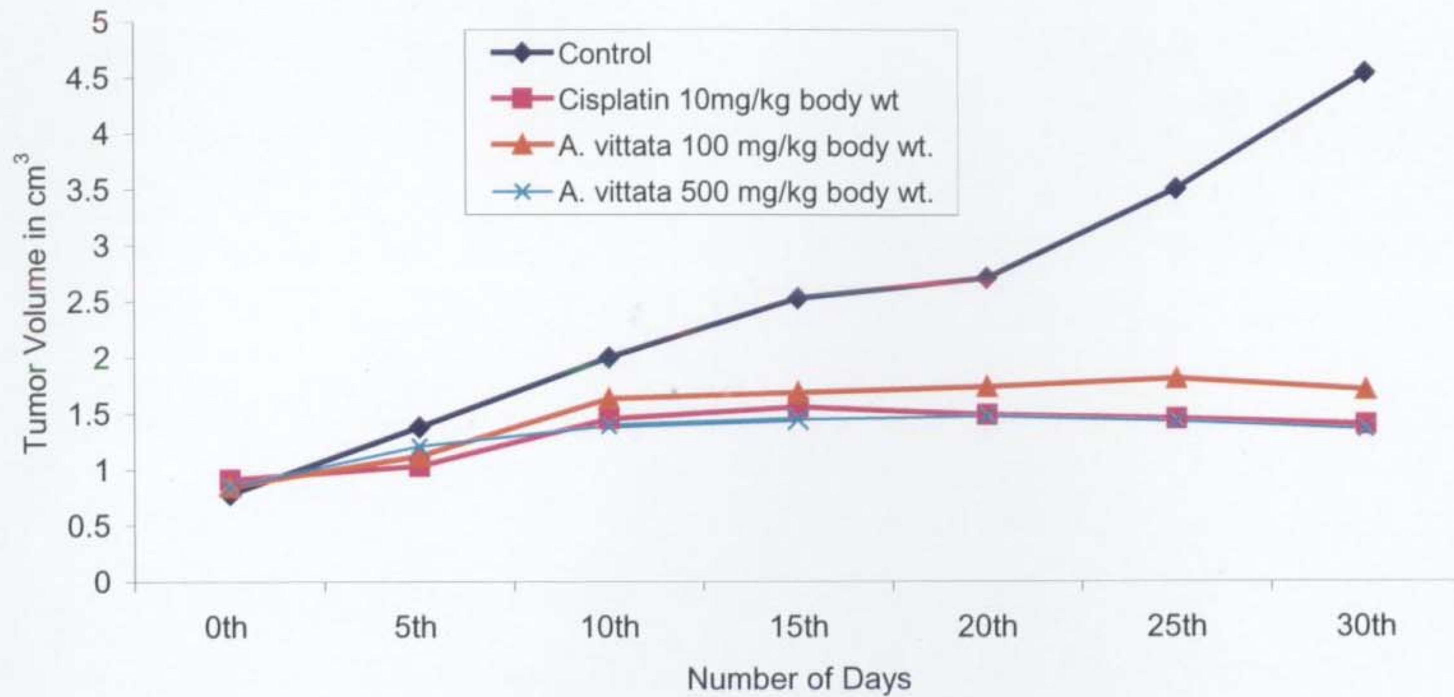


Fig. 4. Effect of *A. vittata* methanolic extract on DLA induced solid tumor (Tumor volume in cm^3)

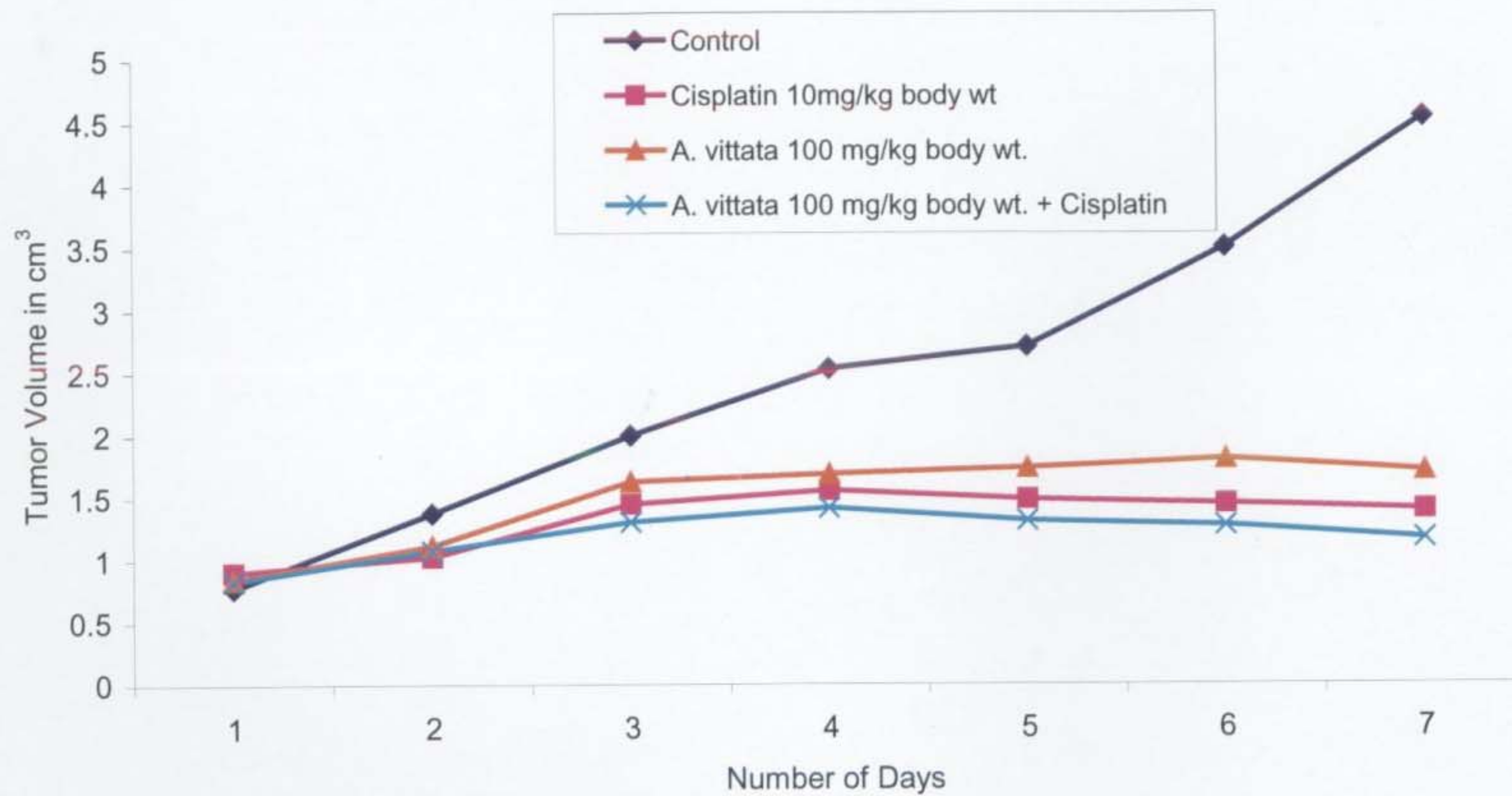


Fig. 5. Synergistic effect of *A. vittata* methanolic extract and Cisplatin on DLA induced solid tumor (Tumor volume in cm^3)

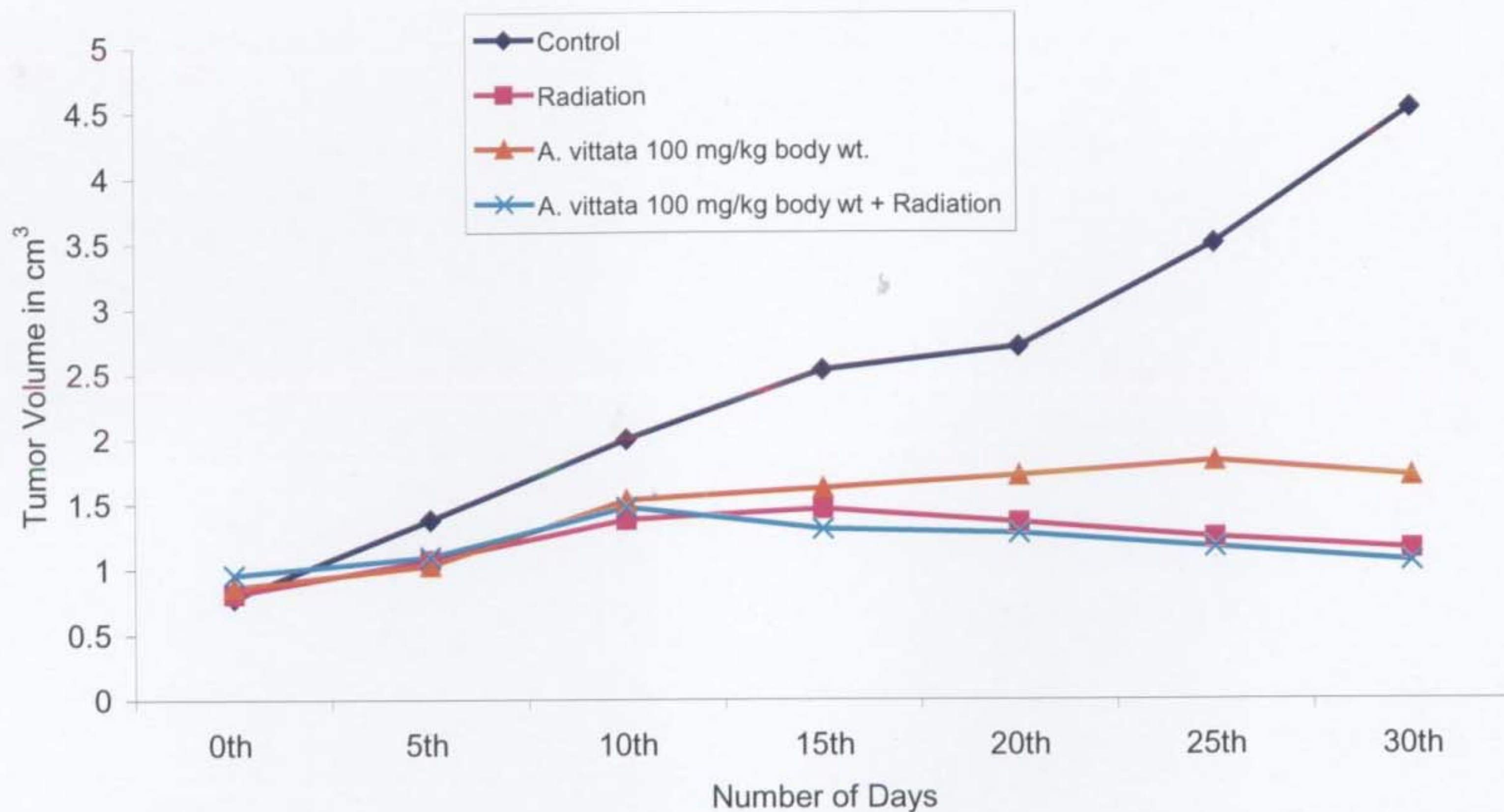


Fig. 6. Synergistic effect of *A. vittata* methanolic extract and Radiation on DLA induced solid tumor (Tumor volume in cm^3)

Table 21. Synergistic effect of *Alpinia malaccensis* and Radiation against DLA induced solid tumor (Tumor volume in cm^3)

Groups of extracts	1st day	5th day	10th day	15th day	20th day	25th day	30th day
Control	0.78 ± 0.02	1.38 ± 0.53	2.00 ± 0.44	2.53 ± 0.63	2.71 ± 0.69	3.5 ± 0.63	4.53 ± 1.31
Radiation 100 rad	0.81 ± 0.06	0.81 ± 0.13	1.38 ± 0.33	1.46 ± 0.59	1.36 ± 0.63	1.24 ± 0.44	1.18 ± 0.51
A. malaccensis 100 mg/kg body wt.	0.86 ± 0.18	1.03 ± 0.16	1.53 ± 0.30	1.62 ± 0.23	1.72 ± 0.23	1.83 ± 0.09	1.71 ± 0.16
A. malaccensis 100 mg/kg body wt. + Radiation 100 rad	0.81 ± 0.09	1.01 ± 0.17	1.24 ± 0.17	1.39 ± 0.07	1.30 ± 1.16	1.21 ± 1.13	0.96 ± 0.13

Table 22. Antitumour activity of *Alpinia vittata* against DLA induced solid tumor (Tumor volume in cm^3)

Groups of extracts	1st day	5th day	10th day	15th day	20th day	25th day	30th day
Control	0.78 ± 0.02	1.38 ± 0.53	2.00 ± 0.44	2.53 ± 0.63	2.71 ± 0.69	3.5 ± 0.63	4.53 ± 1.31
Cisplatin 10 mg/kg body wt.	0.91 ± 0.11	1.03 ± 0.34	1.45 ± 32	1.56 ± 0.35	1.49 ± 0.43	1.45 ± 0.41	1.4 ± 0.26
A. vittata 100 mg/kg body wt.	0.86 ± 0.43	1.03 ± 0.45	1.53 ± 0.26	1.62 ± 0.28	1.72 ± 0.20	1.83 ± 0.27	1.71 ± 0.16
A. vittata 500 mg/kg body wt.	0.85 ± 0.14	1.03 ± 0.14	1.39 ± 0.48	1.45 ± 0.41	1.48 ± 0.62	1.43 ± 0.52	1.36 ± 0.42

68.cc

Table 23. Synergistic effect of *Alpinia vittata* and Cisplatin against DLA induced solid tumor (Tumor volume in cm^3)

Groups of extracts	1st day	5th day	10th day	15th day	20th day	25th day	30th day
Control	0.78 ± 0.02	1.38 ± 0.53	2.00± 0.44	2.53 ± 0.63	2.71 ± 0.69	3.5 ± 0.63	4.53 ± 1.31
Cisplatin 10 mg/kg body wt.	0.91 ± 0.11	1.03 ± 0.34	1.45 ± 0.32	1.56 ± 0.35	1.49 ± 0.43	1.45 ± 0.41	1.4 ± 0.26
A. vittata 100 mg/kg body wt.	0.86 ± 0.43	1.03 ± 0.45	1.53 ± 0.26	1.62 ± 0.28	1.72 ± 0.20	1.83 ± 0.27	1.71 ± 0.16
A. vittata 100 mg/kg body wt. + Cisplatin	0.84 ± 0.16	1.09 ± 0.12	1.31 ± 0.12	1.42 ± 0.11	1.32 ± 0.18	1.28 ± 0.18	1.17 ± 0.23

68.00

Table 24. Synergistic effect of *Alpinia vittata* and Radiation against DLA induced solid tumor (Tumor volume in cm^3)

Groups of extracts	1st day	5th day	10th day	15th day	20th day	25th day	30th day
Control	0.78 ± 0.02	1.38 ± 0.53	2.00 ± 0.44	2.53 ± 0.63	2.71 ± 0.69	3.5 ± 0.63	4.53 ± 1.31
Radiation 100 rad	0.81 ± 0.06	0.81 ± 0.13	1.38 ± 0.33	1.46 ± 0.59	1.36 ± 0.63	1.24 ± 0.44	1.18 ± 0.51
A. vittata 100 mg/kg body wt.	0.86 ± 0.43	1.03 ± 0.45	1.53 ± 0.26	1.62 ± 0.28	1.72 ± 0.20	1.83 ± 0.27	1.71 ± 0.16
A. vittata 100 mg/kg body wt. + Radiation 100 rad	0.96 ± 0.162	1.10 ± 0.13	1.48 ± 0.14	1.31 ± 0.13	1.28 ± 0.21	1.17 ± 0.10	1.06 ± 0.12

63EE

Table 25. Effect of methanolic extracts of *Alpinia malaccensis* and *Alpinia vittata* on EAC cells induced ascites tumor

Groups of extracts	No. of days survived	% of increase in life span
Control	22 ± 1.48	--
Cisplatin	36.57 ± 1.27	64.27
A. malaccensis 100 mg/kg body wt.	28 ± 0.861	26.29
A. malaccensis 500 mg/kg body wt.	31.57 ± 1.42	41.31
A. vittata 100 mg/kg body wt.	27.56 ± 1.643	23.27
A. vittata 500 mg/kg body wt.	29 ± 1.429	30.80

Table 26. Statistical analysis table for antitumor experiments conducted on *Alpinia malaccensis and Alpinia vittata*

Experiment	Category	F	P Value
Acute toxicity studies of *Alpinia* species	Experiment Tests	0.057 1678.53	0.687 0.000
Antitumor activity of *Alpinia malaccensis* against DLA cells induced solid tumor	Experiment Days	8.41 2.86	0.001** 0.039*
Synergistic effect of *Alpinia malaccensis* and cisplatin against DLA cells induced solid tumor	Experiment Days	7.98 2.65	0.001** 0.051
Synergistic effect of *Alpinia malaccensis* and radiation against DLA cells induced solid tumor	Experiment Days	8.61 2.71	0.001** 0.047*
Antitumor activity of *Alpinia vittata* against DLA cells induced solid tumor	Experiment Days	6.26 2.37	0.004** 0.073
Synergistic effect of *Alpinia vittata* and cisplatin against DLA cells induced solid tumor	Experiment Days	7.95 3.06	0.001** 0.031*
Synergistic effect of *Alpinia vittata* and radiation against DLA cells induced solid tumor	Experiment Days	7.89 2.50	0.001** 0.061
Effect of *Alpinia* species on EAC cells induced solid tumor	Experiment	73.09	0.000

* indicates significance at 5% level.
** indicates significance at 1% level.
(No sign – Not significant)

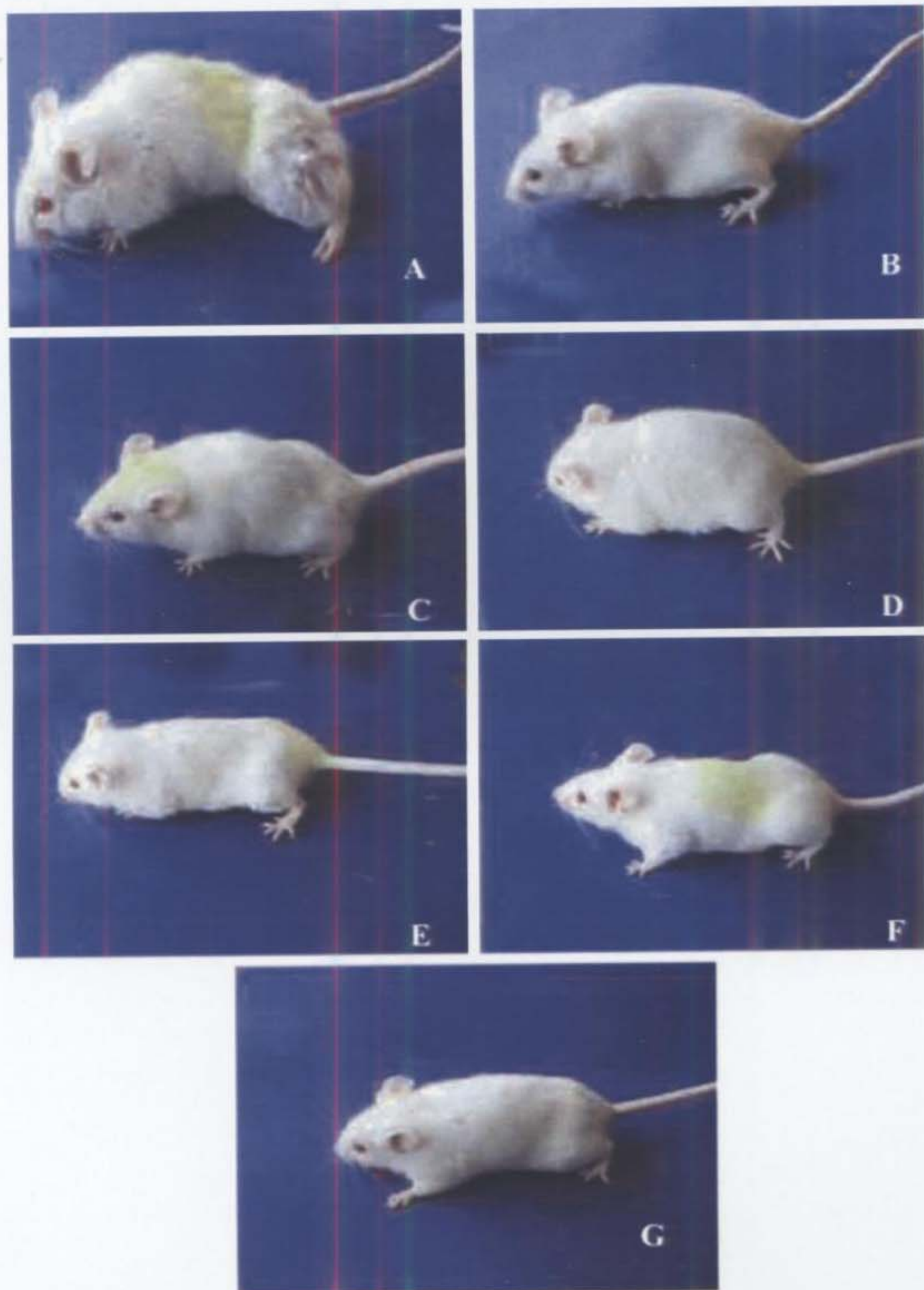


Plate X. Effect of methanolic extract of *Alpinia malaccensis* on DLA induced solid tumor

A-Control (solid tumor), B-Cisplatin 10 mg/ Kg body weight, C-100rad radiation, D-Extract 100 mg/Kg body weight, E-Extract 500 mg/Kg body weight, F-Cisplatin 10 mg/Kg body weight + extract 100 mg/ Kg body weight, G- 100rad radiation + extract 100 mg/ Kg body weight

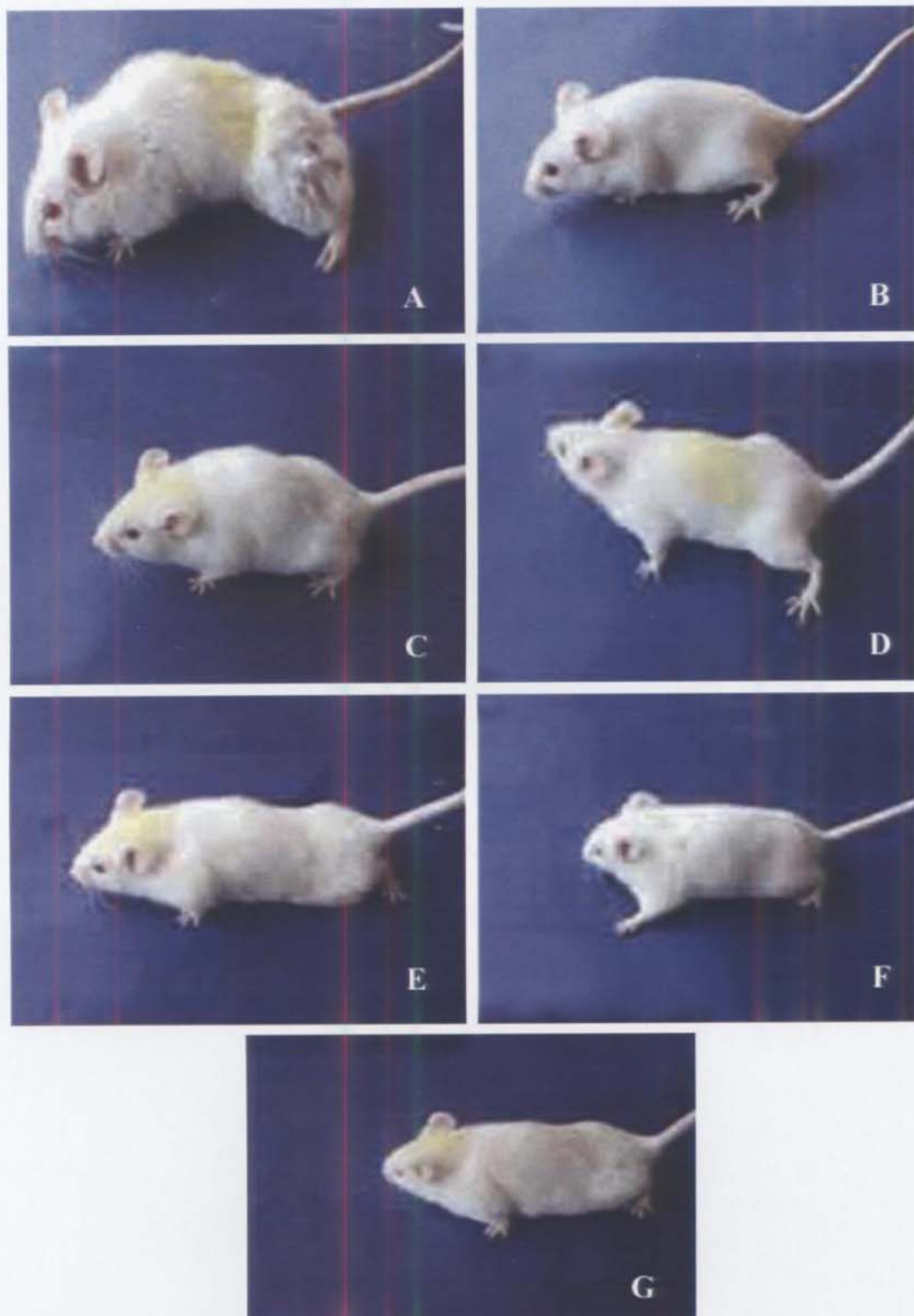


Plate X1. Effect of methanolic extract of *Alpinia vittata* on DLA induced solid tumor

A-Control (solid tumor), B-Cisplatin 10 mg/ Kg body weight, C-100rad radiation, D-Extract 100 mg/Kg body weight, E-Extract 500 mg/Kg body weight, F-Cisplatin 10 mg/Kg body weight + extract 100 mg/ Kg body weight , G- 100rad radiation + extract 100 mg/ Kg body weight

68:00

Table 27. Antioxidant activities of *Alpinia* rhizome extracts in different antioxidant systems

Antioxidant assays	*Alpinia* species						
	A. calcarata	*A. galanga*	*A. malaccensis*	*A. purpurata*	*A. smithiae*	*A. vittata*	*A. zerumbet*
1. Superoxide radical scavenging activity	+	+	+	-	+	+	-
2. Hydroxyl radical scavenging activity	-	+	+	+	+	+	-
3. Nitric oxide radical scavenging activity	-	+	-	-	+	+	-
4. Inhibition of lipid peroxidation	-	+	+	+	+	-	-

68.PP

Table 28. Concentration dependent radical scavenging activities of *Alpinia* rhizome extracts

Sample	% of Inhibition (IC_{50}) in antioxidant assays			
	Superoxide radical scavenging activity (μg/ml)	Hydroxyl radical scavenging activity (μg/ml)	Nitric oxide radical scavenging activity (μg/ml)	Inhibition of lipid peroxidation (μg/ml)
1. *A. calcarata*	24	--	--	--
2. *A. galanga*	42	73	70	83
3. *A. malaccensis*	85	136	--	114
4. *A. purpurata*	--	37	--	82
5. *A. smithiae*	48	58	57	58
6. *A. vittata*	46	124	65	--
7. *A. zerumbet*	--	--	--	--

The concentration of the samples causing 50% inhibition of radicals are referred to as their respective IC_{50} values.

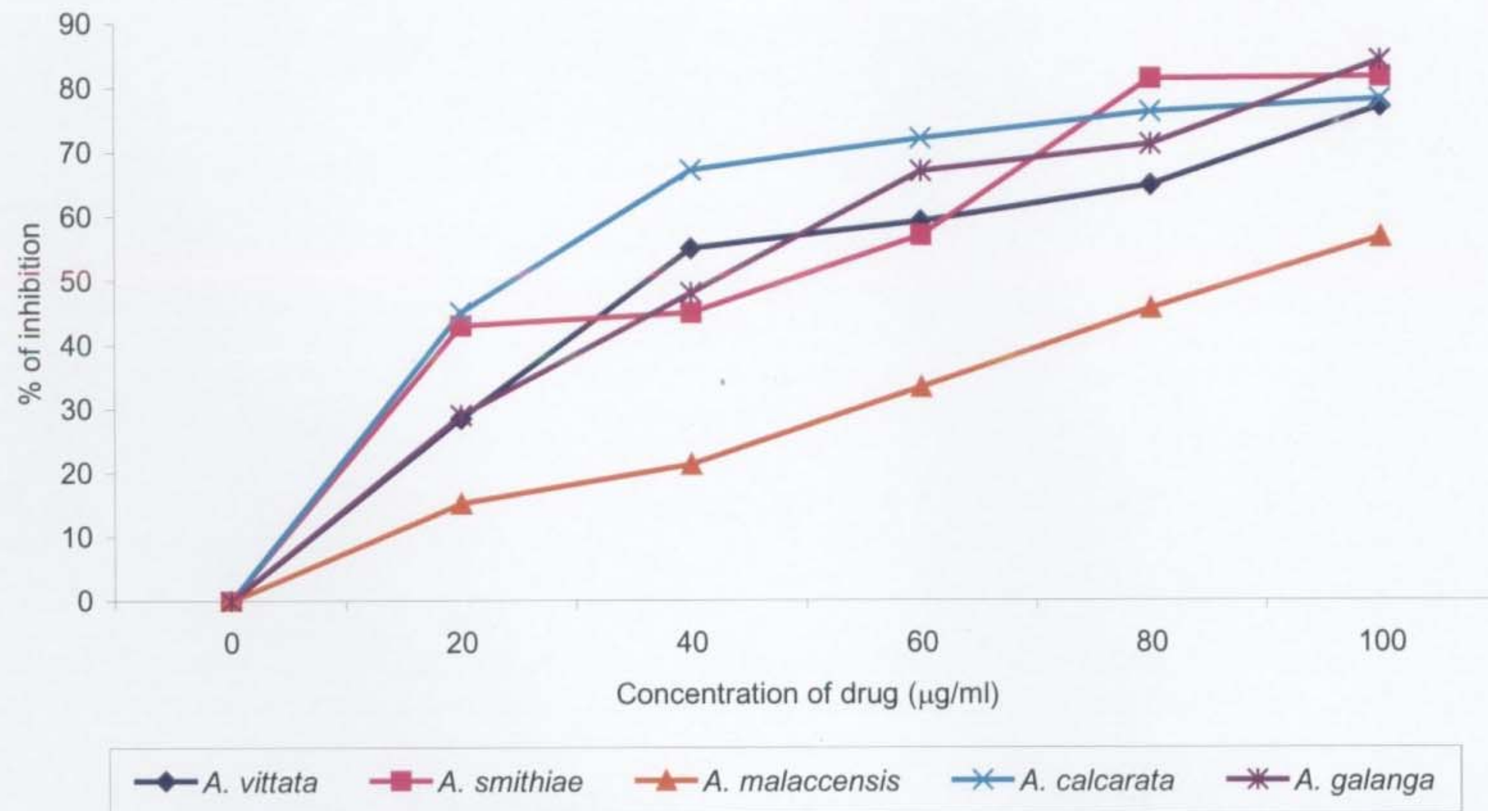


Fig. 7. Superoxide radical scavenging activity of the effective *Alpinia* species

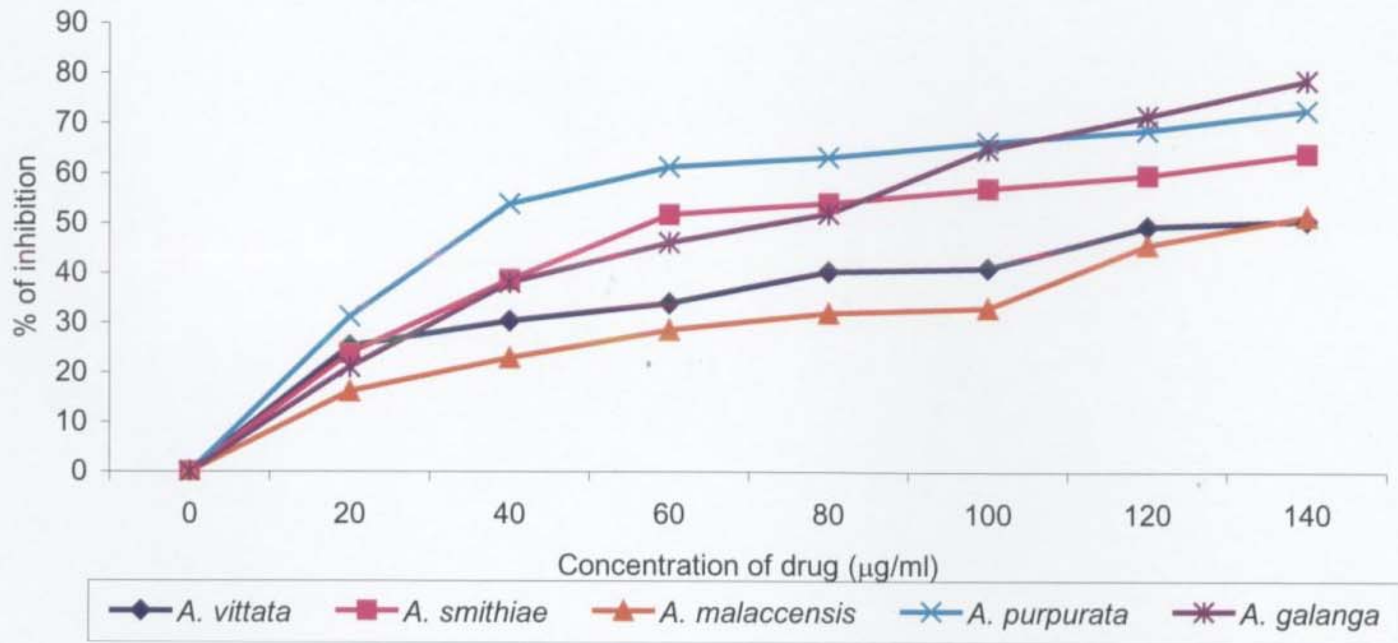


Fig. 8. Hydroxyl radical scavenging activity of the effective *Alpinia* species

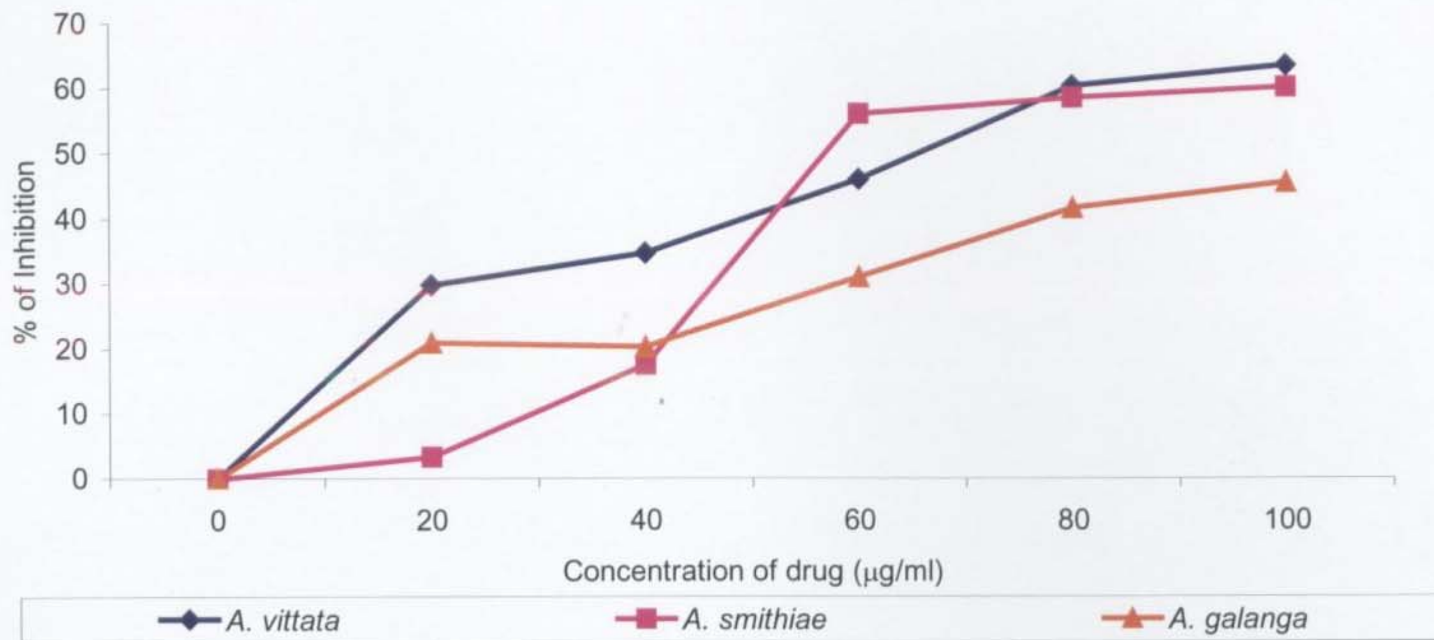


Fig. 9. Nitric oxide radical scavenging activity of the effective *Alpinia* species

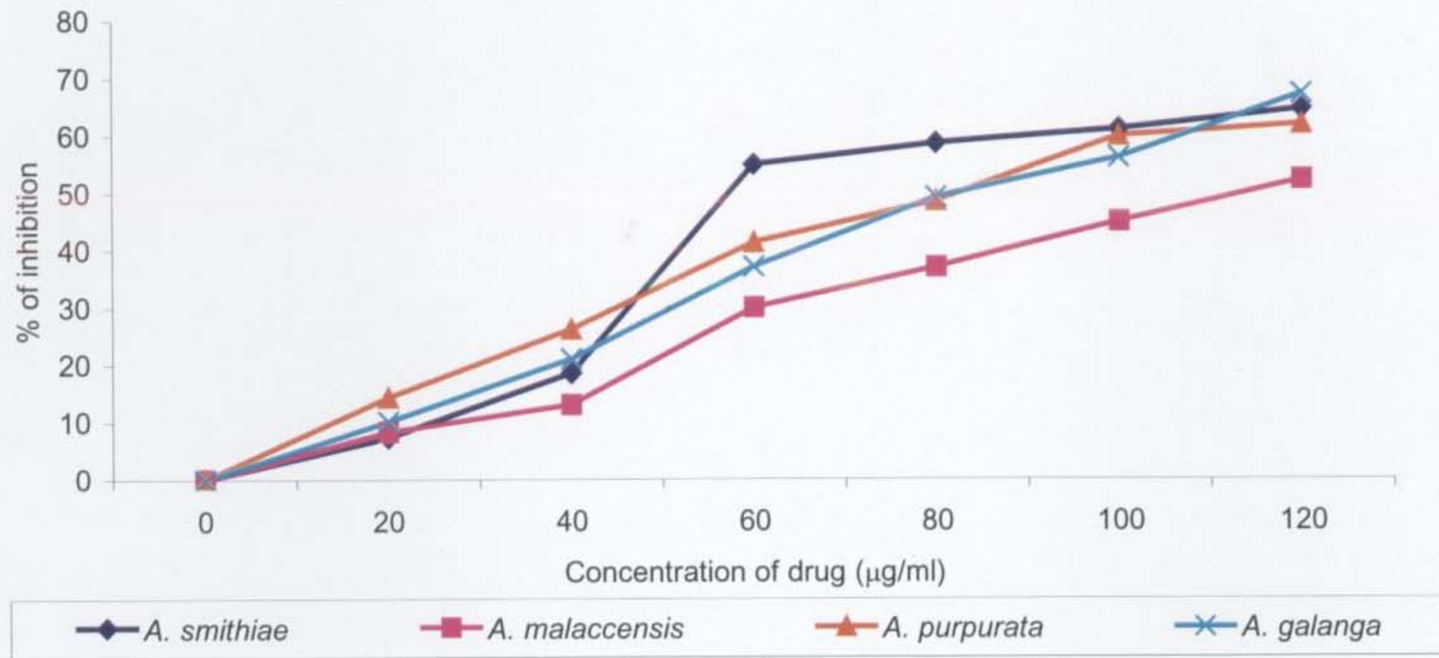


Fig. 10. Activity of the effective *Alpinia* species on the inhibition of lipid peroxidation

63·00

Table 29. Essential oil composition of seven species of *Alpinia* L.

A. calcarata

No.	Retention time	Chemical compounds	Class of compounds	Composition in %
1	3.19	Camphene	Monoterpenoid	6.62
2	3.67	β-pinene	"	7.06
3	3.94	Myrcene	"	0.64
4	4.64	p-cymene	"	0.68
5	4.72	Limonene	"	4.14
6	4.81	1,8-cineole	"	36.94
7	5.46	γ-terpinene	"	0.58
8	6.23	α-thujone	"	0.96
9	7.89	L-camphor	"	3.75
10	9.03	Terpinene-4-ol	"	0.94
11	9.53	β-fenchyl alcohol	"	4.02
12	10.59	β-fenchyl acetate	"	17.56
13	15.57	α-pinene	"	0.64
14	16.36	Epizonaren	Sesquiterpenoid	0.85
15	16.60	Valencene	"	2.68
16	16.89	Methyl cinnamate	Monoterpenoid	0.88
17	24.83	Azulenol	Sesquiterpenoid	8.03
18	27.37	α-selinene	"	3.03

A. galanga

No.	Retention time	Chemical compounds	Class of compounds	Composition in %
1	3.61	Sabinene	Monoterpenoid	0.39
2	3.66	β-pinene	"	1.12

No.	Retention time	Chemical compounds	Class of compounds	Composition in %
3	3.93	Myrcene	Monoterpenoid	0.87
4	4.45	α-terpinene	"	0.43
5	4.72	Limonene	"	2.37
6	4.82	1,8-cineole	"	63.31
7	5.45	γ-terpinene	"	0.9
8	9.02	Terpinene-4-ol	"	2.14
9	9.52	Camphene	"	0.96
10	13.05	β-fenchyl acetate	"	0.7
11	17.12	Geranyl acetate	"	2.23
12	17.93	Methyl eugenol	Phenol	0.5
13	18.05	β-caryophyllene	Sesquiterpenoid	0.62
14	19.37	α-humulene	"	1.27
15	19.91	β-elemene	"	11.49
16	20.48	Germacrene-D	"	0.69
17	21.77	β-bisabolene	"	0.5
18	21.89	α-farnesene	"	0.7
19	22.32	β-sesquiphellandrene	"	0.59
20	22.69	Acetyl eugenol	Phenol	5.68
21	33.93	Nerolidol	Sesquiterpenoid	2.54

A. malaccensis

No.	Retention time	Chemical compounds	Class of compounds	Composition in %
1	3.19	Camphene	Monoterpenoid	0.96
2	3.66	β-pinene	"	3.34
3	3.93	Myrcene	"	1.2

No.	Retention time	Chemical compounds	Class of compounds	Composition in %
4	4.20	α-phellandrene	Monoterpenoid	12.9
5	4.63	p-cymene	"	1.33
6	4.71	Limonene	"	4.21
7	6.23	α-thujone	"	0.86
8	6.99	Fenchol	"	12.19
9	9.52	α-terpineol	"	0.6
10	10.57	β-fenchyl acetate	"	6.17
11	18.05	Trans-caryophyllene	Sesquiterpenoid	1.47
12	29.91	Zerumbone	"	54.77

A. purpurata

No.	Retention time	Chemical compounds	Class of compounds	Composition in %
1	3.67	β-pinene	Monoterpenoid	41.7
2	21.85	α-selinene	Sesquiterpenoid	58.3

A. smithiae

No.	Retention time	Chemical compounds	Class of compounds	Composition in %
1	4.34	α-pinene	Monoterpenoid	2.61
2	4.65	Camphene	"	2.15
3	5.28	β-pinene	"	6.79
4	5.60	Myrcene	"	0.75
5	6.71	1,8-cineole	"	30.94
6	7.52	γ-terpinene	"	0.57
7	8.89	α-terpinolene	"	2.83

No.	Retention time	Chemical compounds	Class of compounds	Composition in %
8	9.33	α-fenchyl alcohol	Monoterpenoid	1.06
9	11.23	L-Borneol	"	2.56
10	11.67	1,4-terpineol	"	1.7
11	12.20	α-terpineol	"	9.84
12	13.35	β-Fenchyl acetate	"	17.6
13	15.42	Citral	"	2.29
14	15.98	Geranyl acetate	"	1.23
15	18.57	α-terpinene	"	0.99
16	21.24	Trans-caryophyllene	Sesquiterpenoid	4.28
17	22.57	α-humulene	"	0.93
18	24.98	γ-cadinene	"	2.56
19	27.03	Nerolidol	"	3.94
20	27.53	Caryophyllene oxide	"	4.38

A. vittata

No.	Retention time	Chemical compounds	Class of compounds	Composition in %
1	4.32	α-pinene	Monoterpenoid	1.23
2	4.63	Camphene	"	2.51
3	5.25	β-pinene	"	3.98
4	6.58	Limonene	"	0.85
5	11.19	L-Borneol	"	1.42
6	20.21	β-elemene	Sesquiterpenoid	0.79
7	21.22	β-caryophyllene	"	1.29
8	22.17	α-selinene	"	1.15
9	23.81	Alloaromadendrene	"	5.69
10	24.14	Valencene	"	7.03
11	24.58	Germacrene A	"	1.39

No.	Retention time	Chemical compounds	Class of compounds	Composition in %
12	24.86	β-bisabolene	Sesquiterpenoid	0.52
13	25.05	Germacrene D	"	1.35
14	27.86	Fenchane	Monoterpenoid	55.19
15	30.01	Eudesmol	Sesquiterpenoid	3.37
16	30.17	Neo Intermediol	"	9.21
17	32.59	Azulene	"	0.93
18	32.95	Zerumbone	"	0.67
19	43.97	Alliodorin	"	1.43

A. zerumbet

No.	Retention time	Chemical compounds	Class of compounds	Composition in %
1	3.66	β-pinene	Monoterpenoid	0.97
2	3.93	Myrcene	"	0.17
3	4.19	α-phellandrene	"	0.18
4	4.45	α-terpinene	"	1.7
5	4.63	p-cymene	"	0.84
6	4.72	Limonene	"	0.51
7	4.78	1,8-cineole	"	3.65
8	5.46	γ-terpinene	"	3.69
9	6.24	α-terpinolene	"	0.72
10	9.05	Terpinene-4-ol	"	4.87
11	10.57	β-fenchyl acetate	"	0.38
12	18.05	Trans-caryophyllene	Sesquiterpenoid	0.52
13	19.39	α-humulene	"	4.89
14	24.29	Caryophyllene oxide	"	0.97
15	24.94	Camphene	Monoterpenoid	2.57
16	26.57	β-caryophyllene	Sesquiterpenoid	0.61
17	30.01	Zerumbone	"	72.76

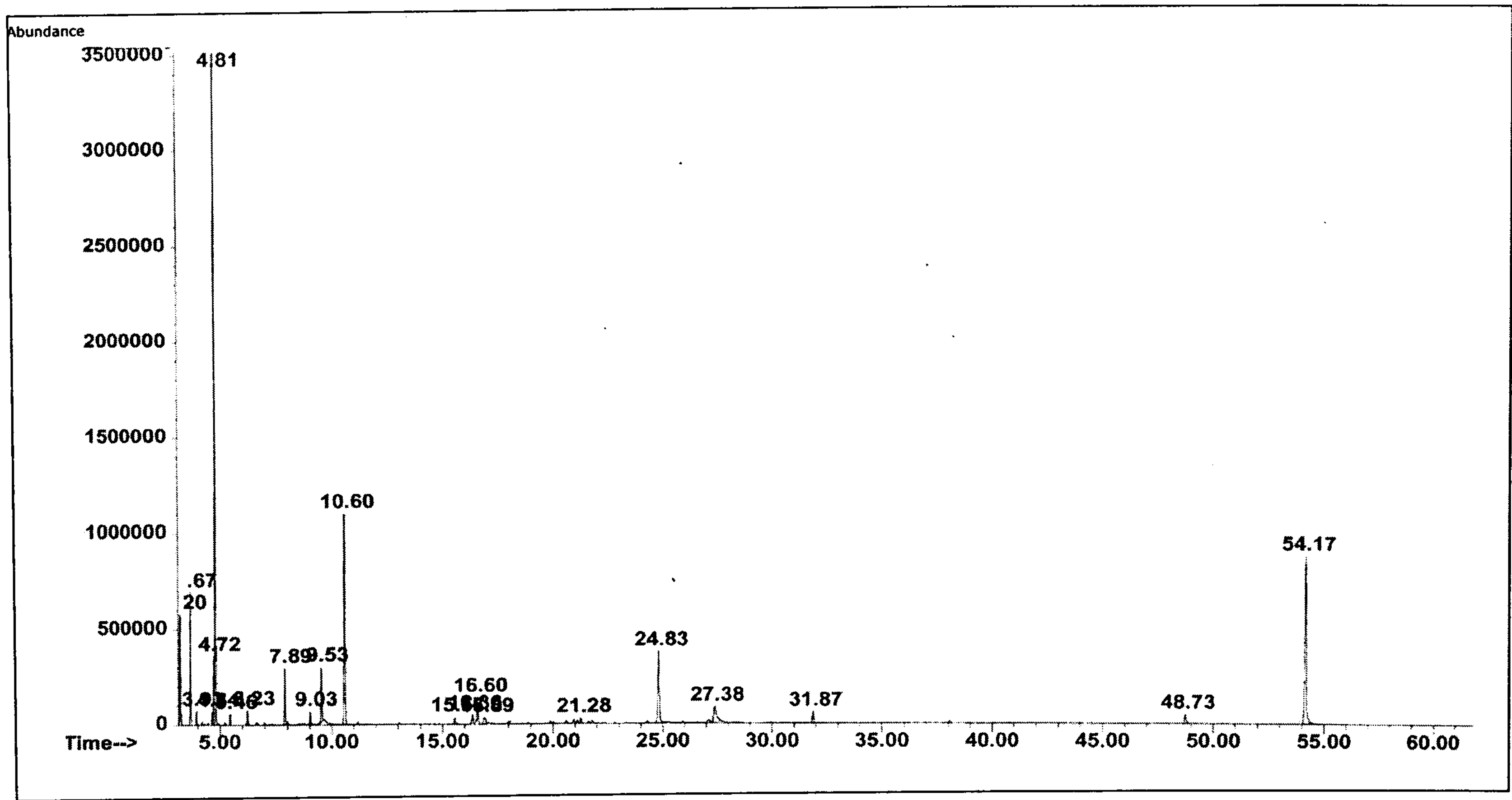


Fig.11 Gas chromatogram of *Alpinia calcarata*

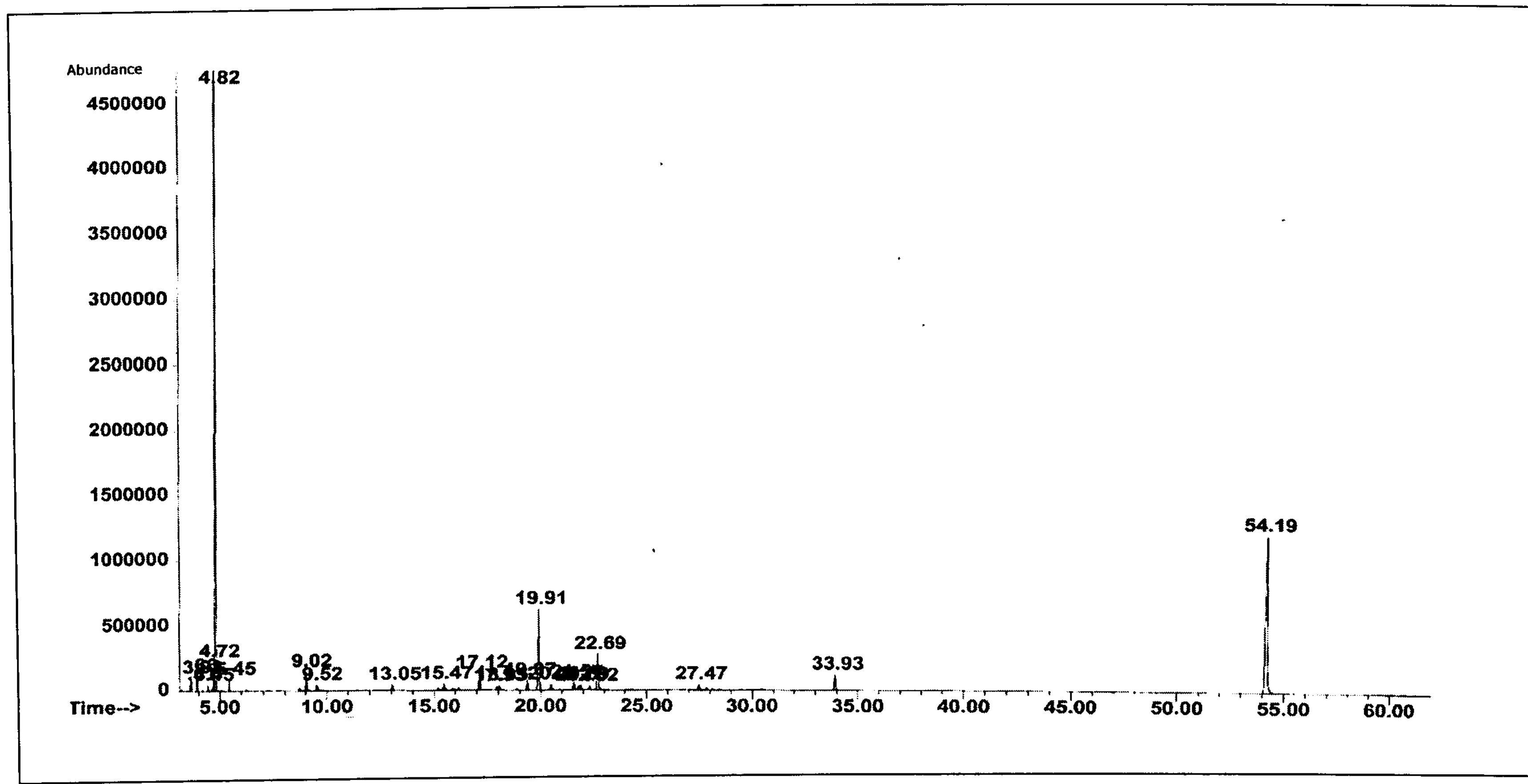


Fig. 12 Gas chromatogram of *Alpinia galanga*

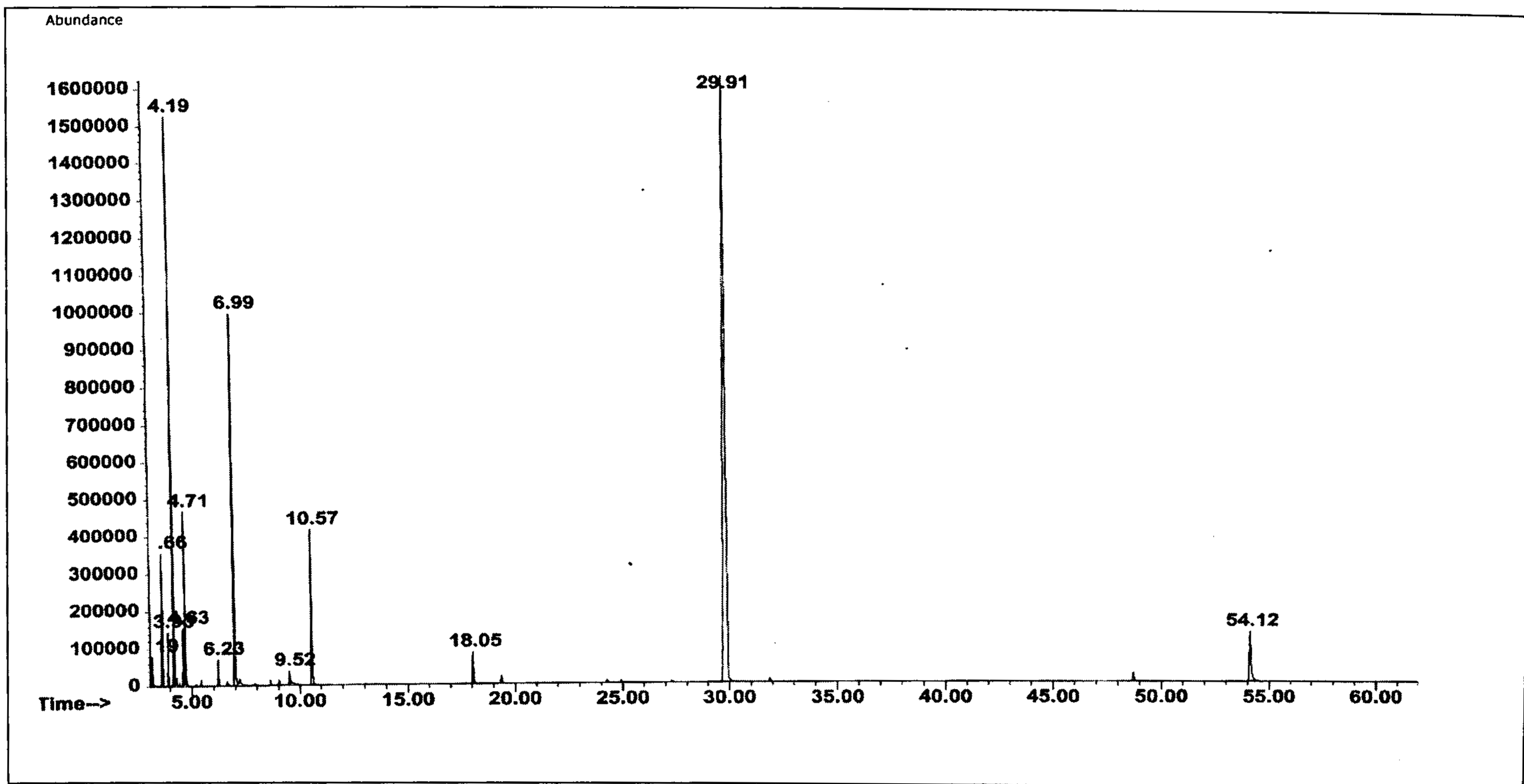


Fig. 13 Gas chromatogram of *Alpinia malaccensis*

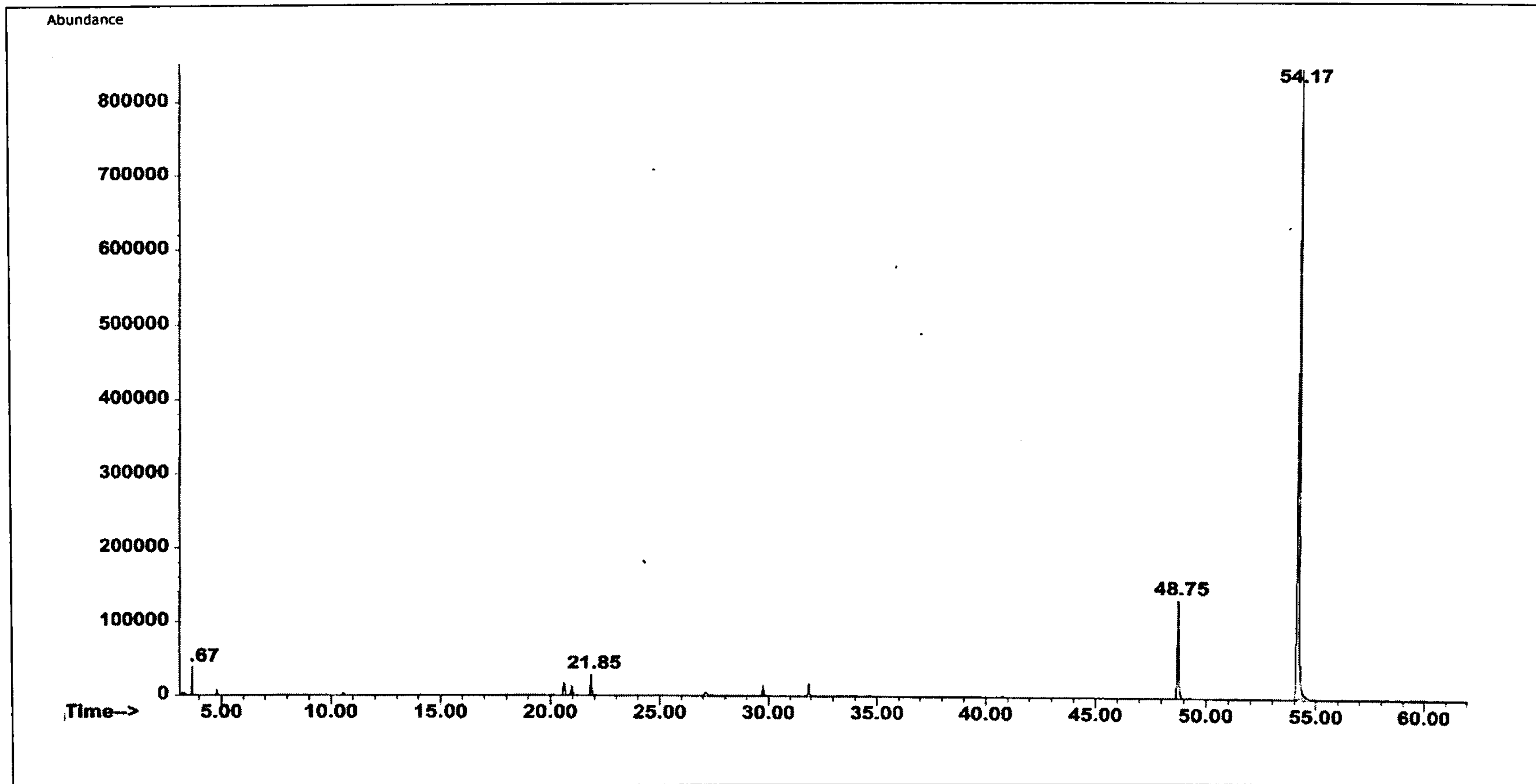


Fig. 14 Gas chromatogram of *Alpinia purpurata*

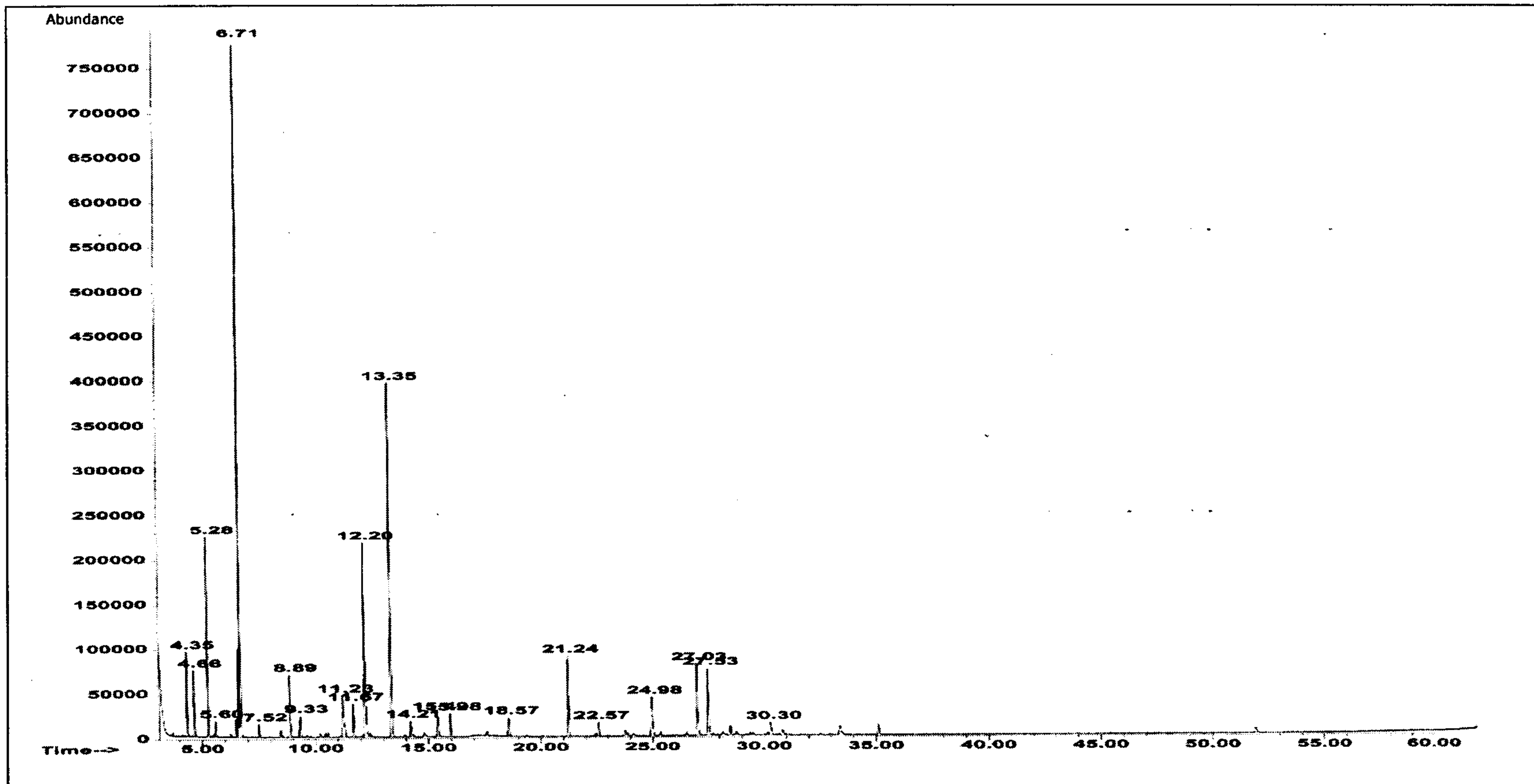


Fig. 15 Gas chromatogram of *Alpinia smithiae*

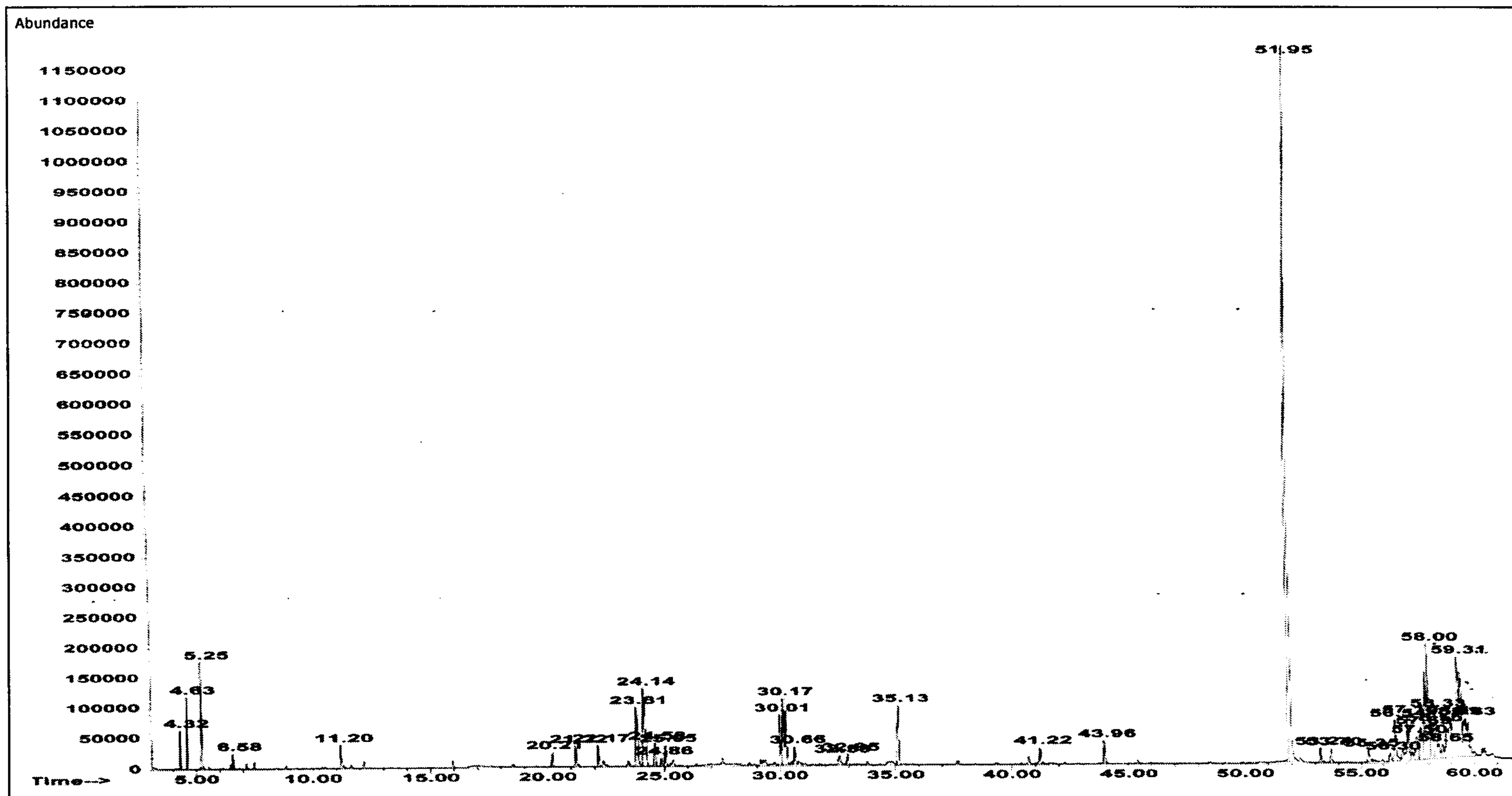


Fig.16 Gas chromatogram of *Alpinia vittata*

6 8 F F F

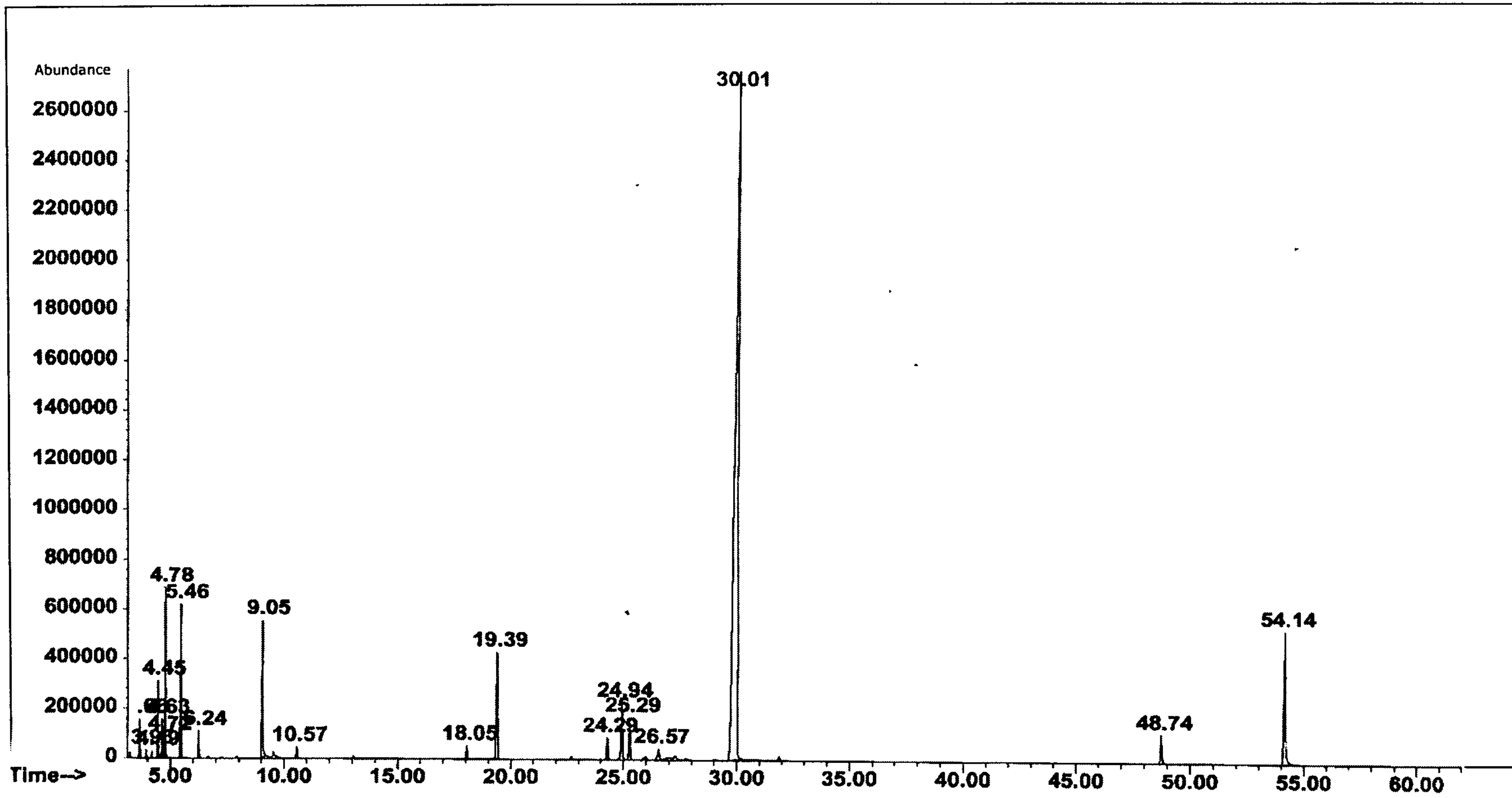


Fig. 17 Gas chromatogram of *Alpinia Zerumbet*

Camphene

Sabinene

β – Pinene

Myrcene

α - Phellandrene

Fig. 18 (i). Mass spectra of different essential oil components detected in the seven species of *Alpinia*

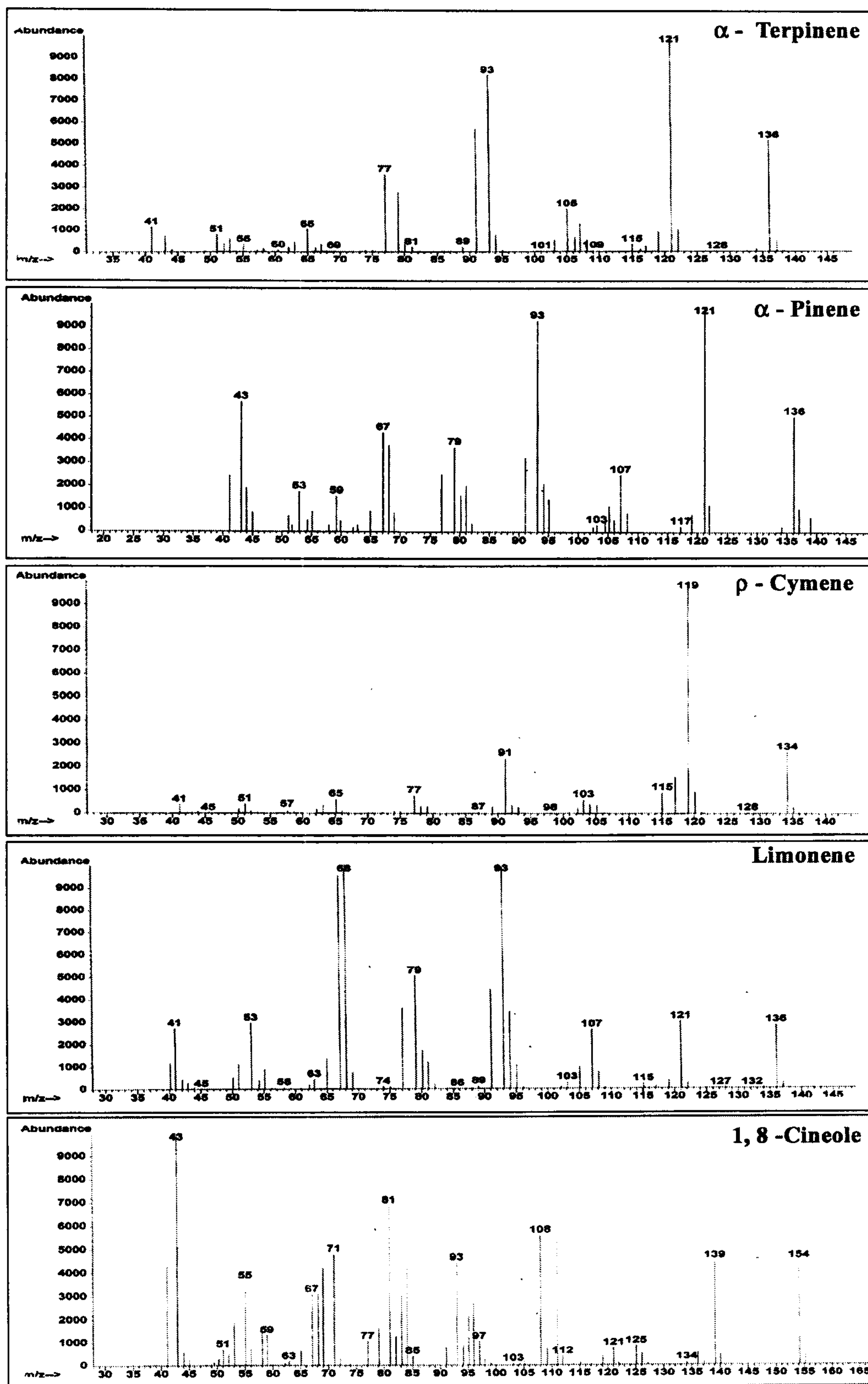


Fig. 18 (ii). Mass spectra of different essential oil components detected in the seven species of *Alpinia*

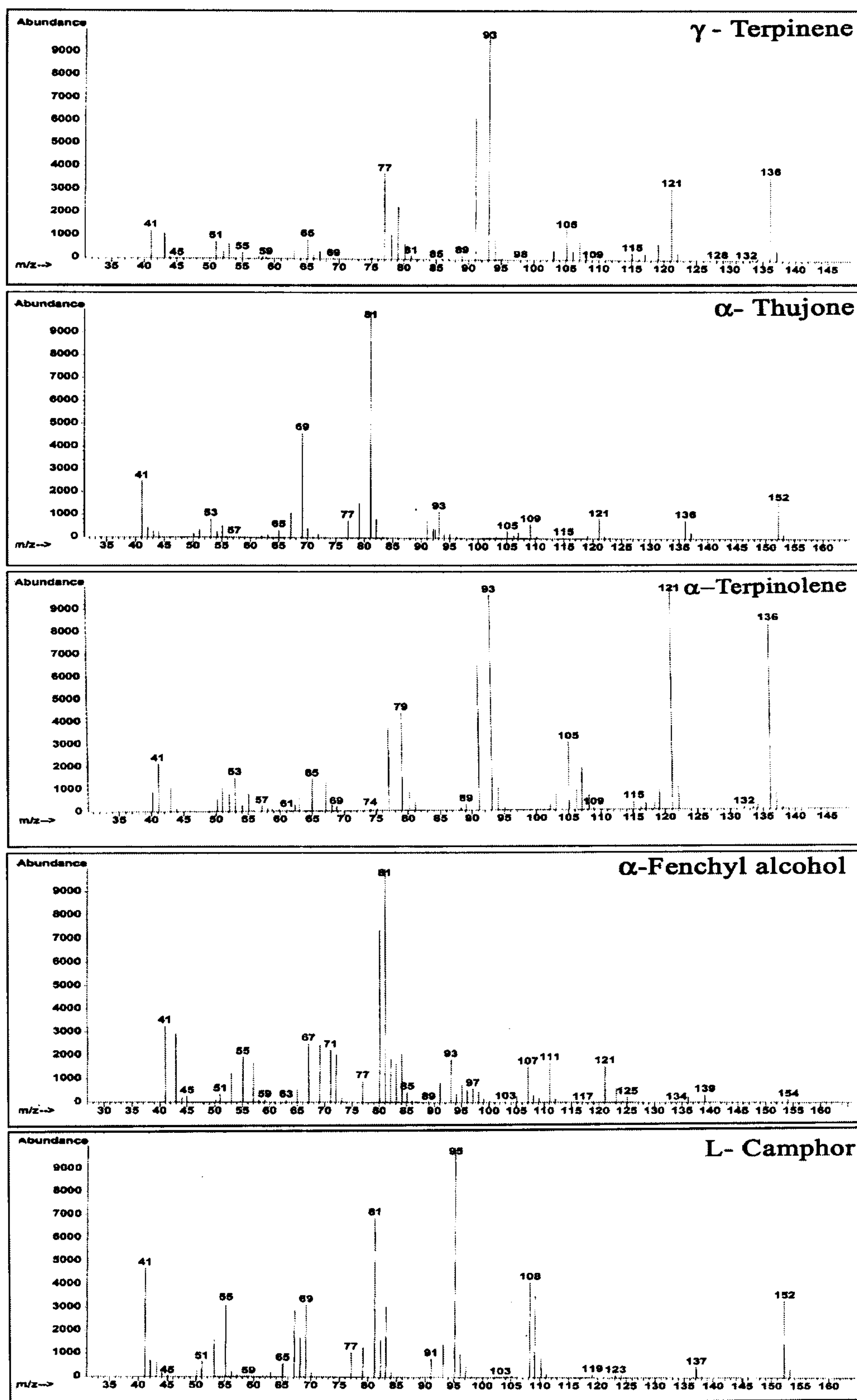
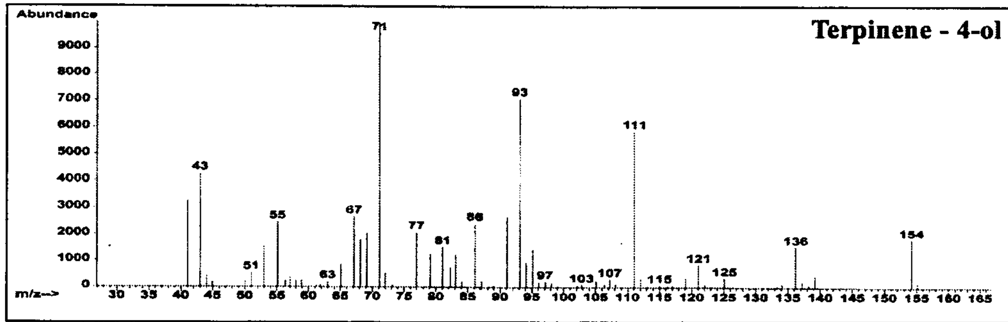
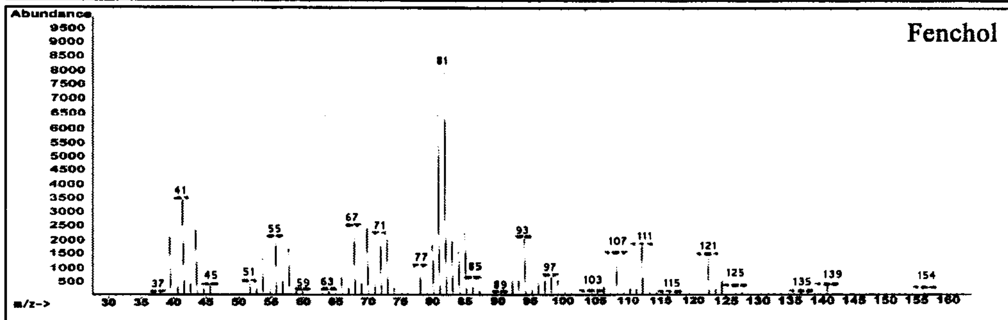
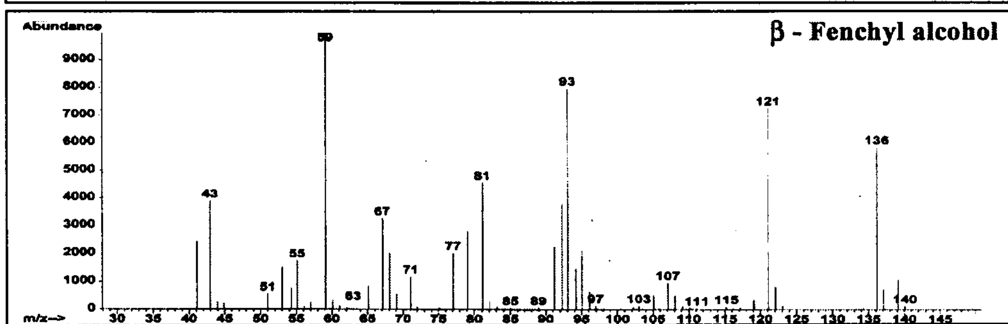
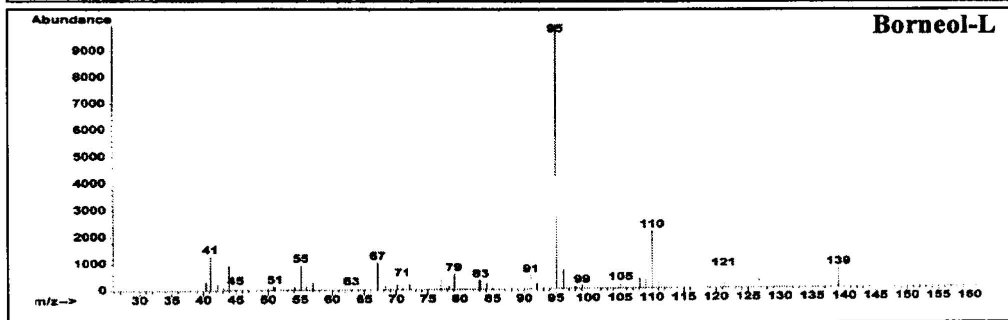


Fig. 18 (iii). Mass spectra of different essential oil components detected in the seven species of *Alpinia*









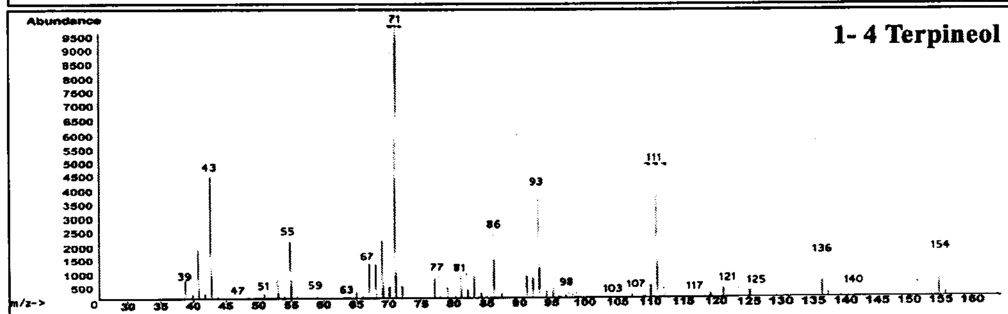


Fig. 18 (iv). Mass spectra of different essential oil components detected in the seven species of *Alpinia*

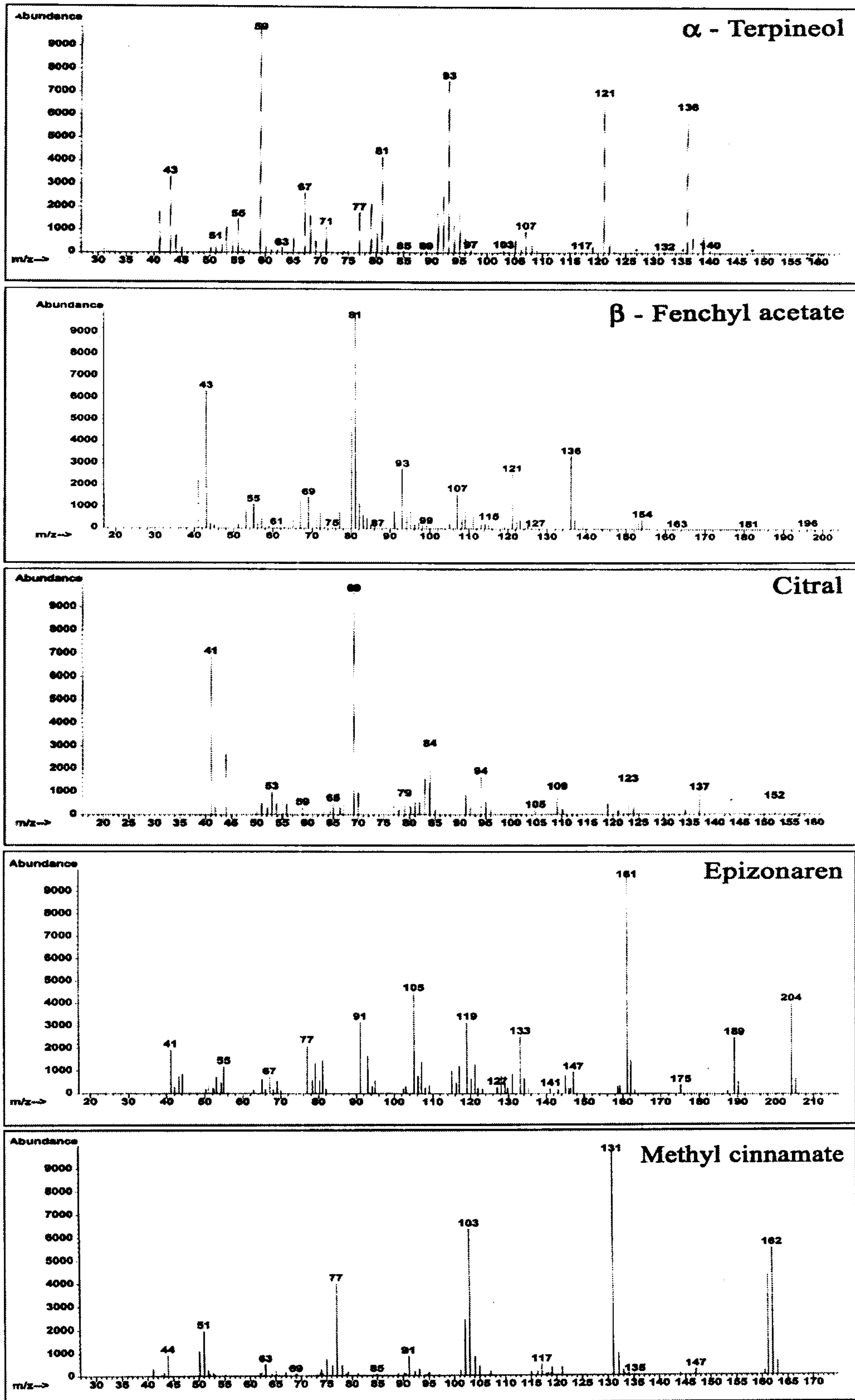


Fig. 18 (v). Mass spectra of different essential oil components detected in the seven species of *Alpinia*

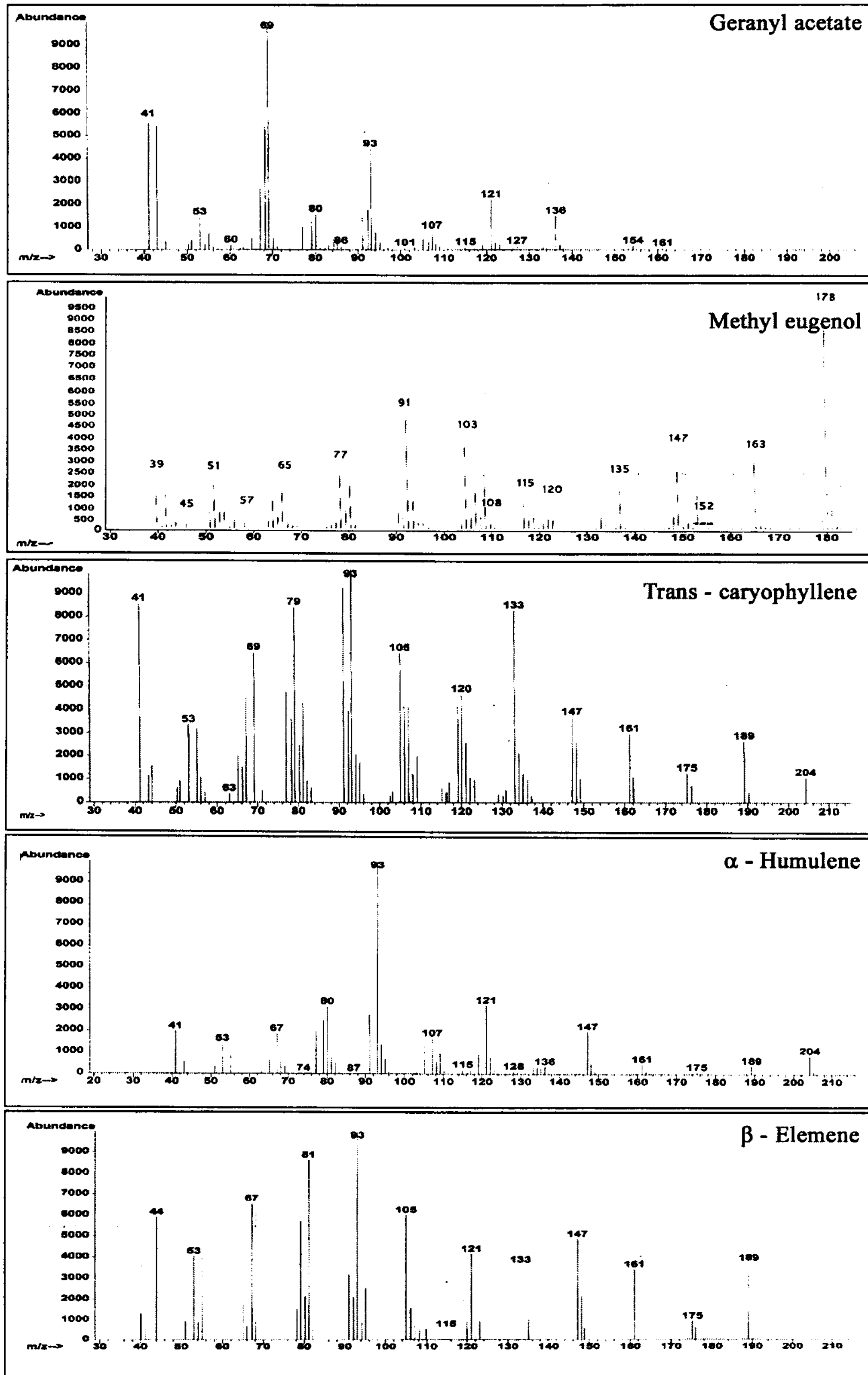


Fig. 18 (vi). Mass spectra of different essential oil components detected in the seven species of *Alpinia*

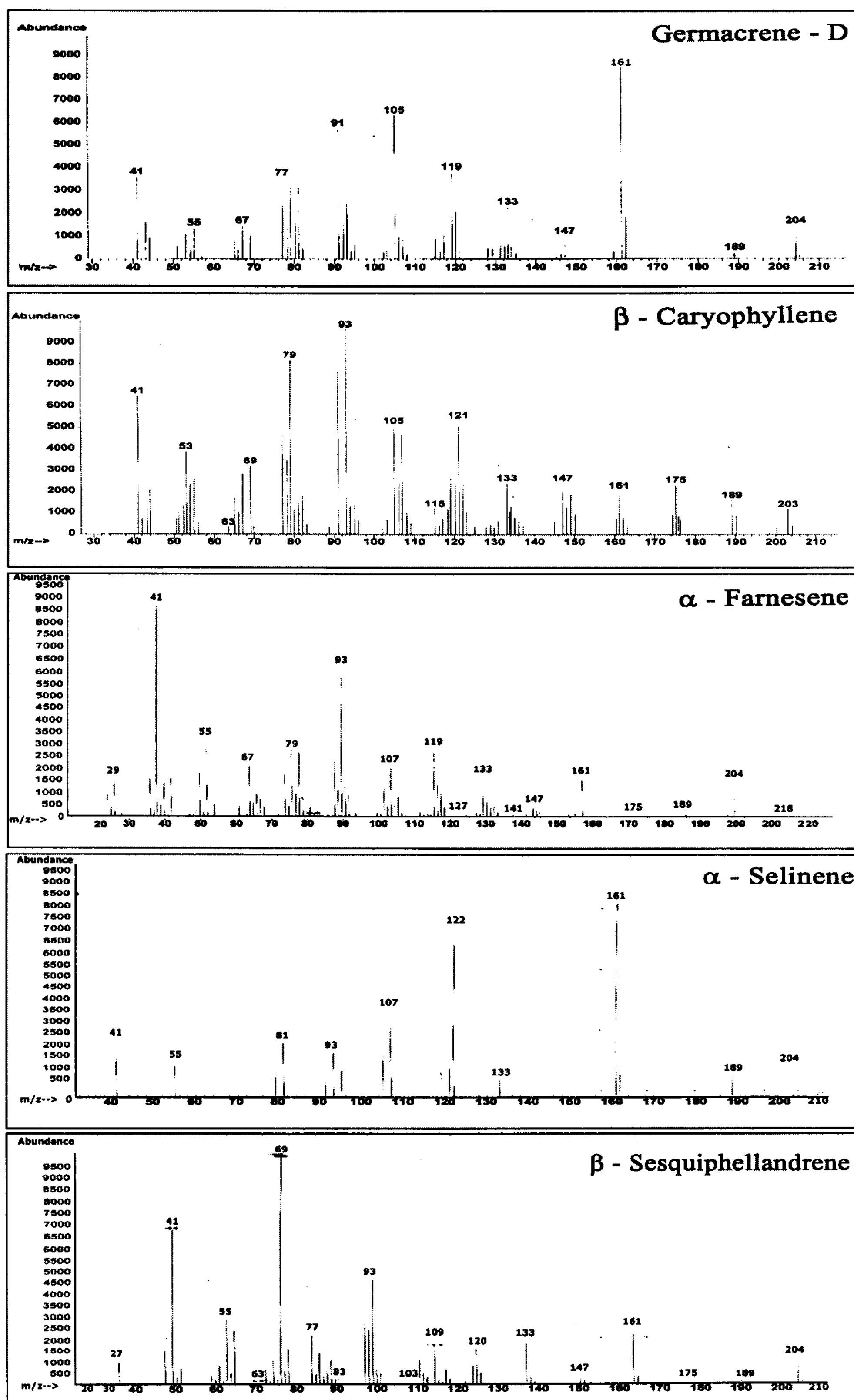


Fig. 18 (vii). Mass spectra of different essential oil components detected in the seven species of *Alpinia*

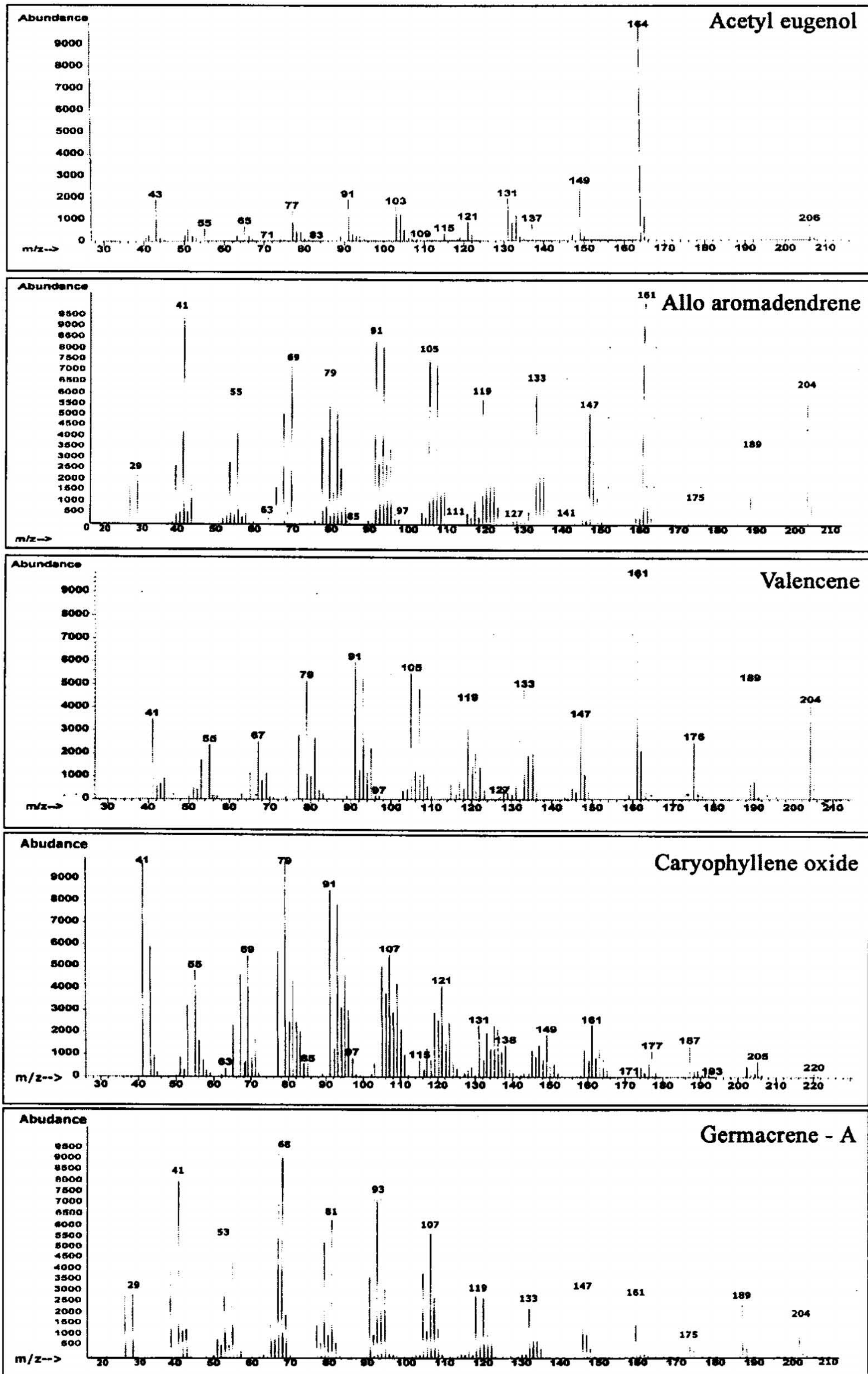


Fig. 18 (viii). Mass spectra of different essential oil components detected in the seven species of *Alpinia*

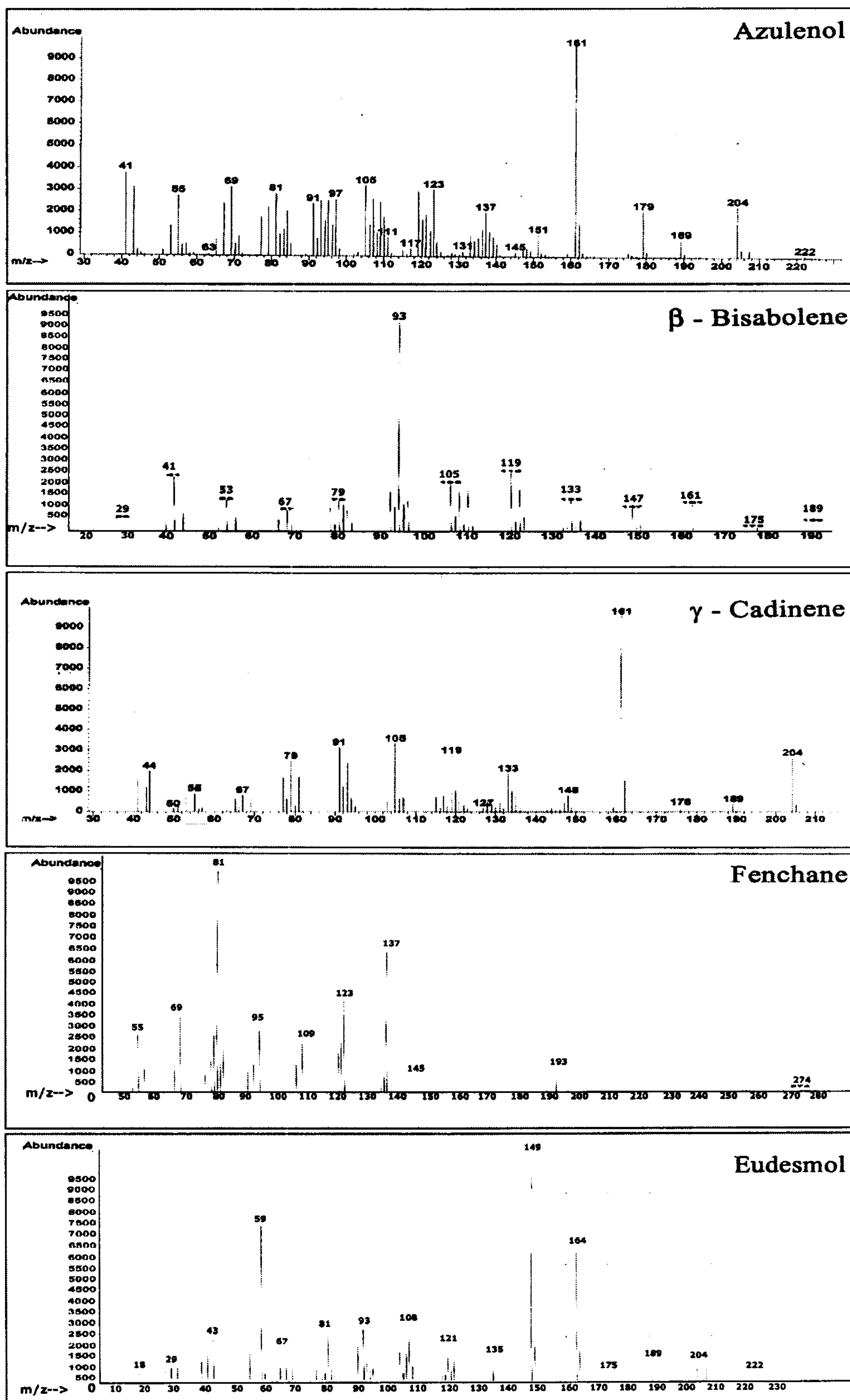


Fig. 18 (ix). Mass spectra of different essential oil components detected in the seven species of *Alpinia*

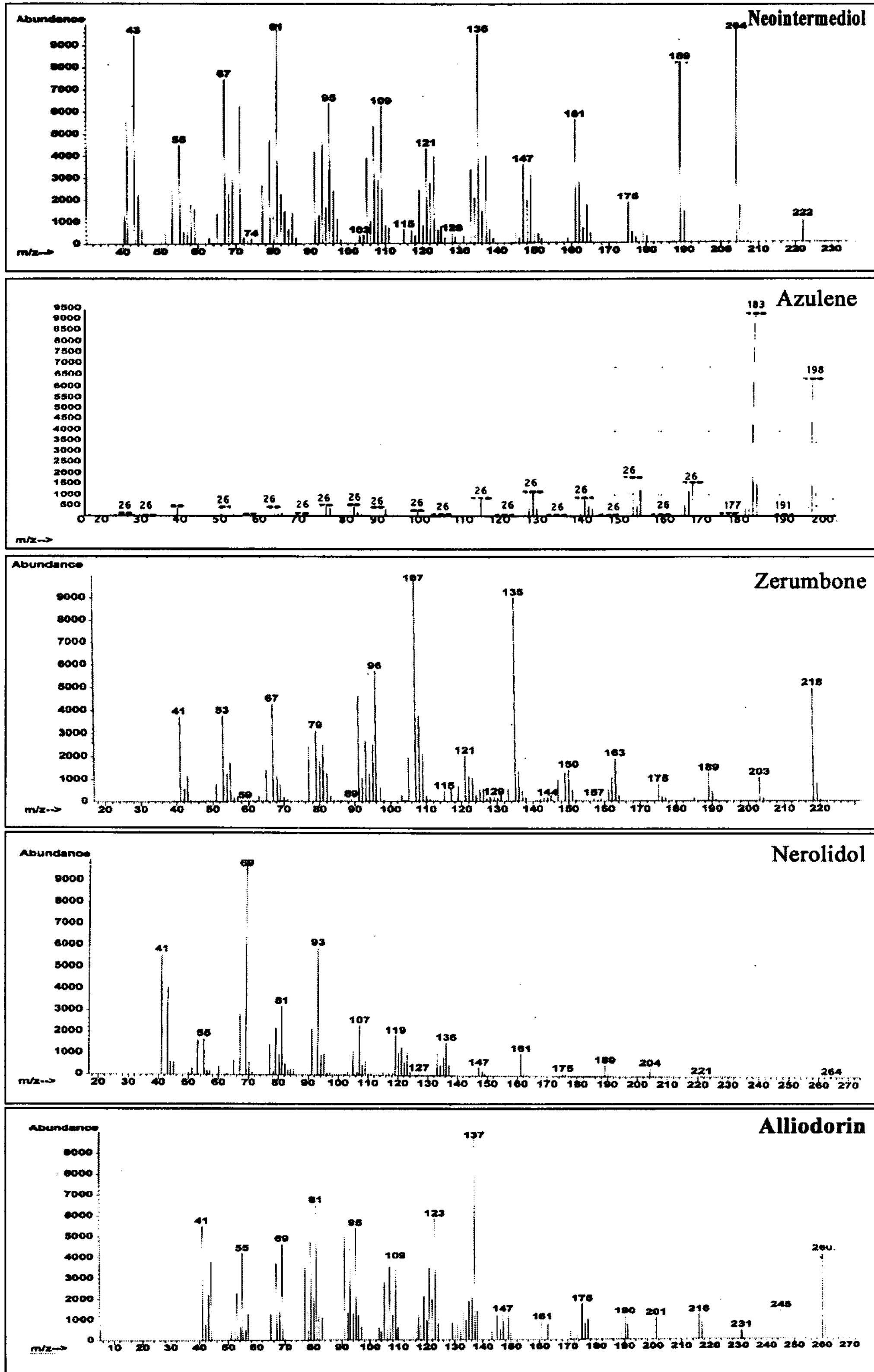


Fig. 18 (x). Mass spectra of different essential oil components detected in the seven species of *Alpinia*

Cytotoxic, antitumor, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.

Thesis submitted to the University of Calicut for the award of the degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By
JOSE MARY DAS

GENETICS AND PLANT BREEDING DIVISION
DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA, INDIA

JUNE 2007

DISCUSSION

CYTOTOXIC ASSAYS

Cytotoxicity of *Alpinia* extracts in *Allium cepa* root tip meristem

Plant derived compounds are used in folklore remedies and traditional systems of medicine for the treatment of various kinds of human ailments (Kirtikar and Basu, 1935). *Allium* test has been widely used in screening the plant extracts to reveal its cytotoxic potential (Kihlman, 1966). Chromosomal aberrations have been considered as reliable indicators of mutagenic activity (Mohandas and Grant, 1972) and there have been evidences for a correlation between chromosomal damage and toxic effects of extracts of different plants. Therefore, plant system is generally accepted as a first tier assay system for detection of the possible chromosomal damage resulting from the treatment of plant extracts.

In the present investigation mitosis was found to be normal when the *Allium cepa* roots were treated with distilled water (control) which clearly showed 2n = 16 chromosomes at the metaphase stage (Plate III). A wide spectrum of abnormalities were recorded in the *Allium cepa* roots after treating with extracts of seven species of *Alpinia* having polar and non-polar compounds and plant extracts having polar fractions alone (Plates IV-IX). Plant extracts induced severe effects on cell division. The cell division was arrested and the percentage of dividing cells reduced with increase in concentration and duration of time (Tables 2-15). The percentage of cytotoxicity and mitotic inhibition was found to be different in plant extracts comprising both polar and non-polar components and the plant extracts having polar compounds alone. The former showed severe

cytotoxicity and mitotic inhibition, whereas these were negligible on the latter.

The present study revealed that treatment of *Allium cepa* root meristems with extracts containing both polar and non-polar fractions of seven *Alpinia* species had a detrimental effect on the test material *Allium cepa*. Treatment not only brought down the frequency of dividing cells but also produced a good number of anomalies in the mitotic cells. There was a marked decrease in the mitotic index and a gradual increase in the percentage of chromosomal abnormalities as concentration of plant extracts and the time of treatment increased. Such an inverse relationship between the mitotic index, dosage and time of treatment and a direct relation between the concentration and time of treatment to percentage of abnormalities had been reported earlier by various workers working with different plant extracts (Veronica *et al.*, 2001; Williams, 1996).

Antimitotic effects induced by the plant extracts in the present investigation leading to severe mitotic inhibition and mitotic arrest at different stages varied with plant extracts, their concentrations and treatment durations (Plates IV-IX). The mitotic depression may be due to the inhibitory effects of chemical components present in the extracts leading to decrease in the mitotic index (Tables 2-15) which can result from obstruction of the onset of prophase or from an arrest of the subsequent mitotic phases. The chemical components of the extracts due to their action on the spindle apparatus, caused an arrest in metaphase, producing high percentage of metaphase abnormalities. The reduction in the mitotic index may be mainly due to the inhibitory action of the chemical components of the extracts on the onset of mitosis inspite of metaphase arrest. This lead to the conclusion that extracts caused a combined effect on the spindle formation and also showed a

mitodepressive effect. The pattern of mitotic inhibition induced by the seven plant extracts are more or less the same. But high percentage of abnormalities and low mitotic indices were noticed in the treatments using *Alpinia malaccensis* and *Alpinia vittata* extracts containing polar and non-polar fractions. Similar effects were shown by *A. smithiae* and *A. zerumbet*. Effects of other three plant extracts seems to be moderate.

The most significant effect of the extracts evidenced from the experiments were the arrest of the mitotic cycle either at interphase or at different stages in the M phase in treated cells. Reduction in the number of dividing cells resulted either from mitotic inhibition or from mitotic arrest at interphase. In all the treatments, the decrease in the mitotic index value in the root meristem of *Allium cepa* with increasing concentration is attributed to mitotic inhibition. A decrease in the mitotic activity was clearly noticed when the roots were treated with high concentrations. Such a decrease in the mitotic index indicates that the components of the extract interferes in the normal sequence of mitosis, thereby leading to the prevention of a large number of cells from entering into the prophase stage. Such reduction in the mitotic activity could be due to the inhibition of DNA synthesis (Schneiderman *et al.*, 1971). Mitotic inhibition by chemical components of the plant extracts has been attributed to blocking of mitotic cycle during interphase, which may result from prolonged G_2 period or to the inhibition of DNA synthesis (Chand and Roy, 1981). In the present investigation, the mitotic inhibitory effect observed during the treatments might be due to the presence of mito-inhibitory principles in the *Alpinia* extracts.

The effect of antimitotic substances is said to be mitodepressive if mitotic index is decreased in the cell population studied or mitostatic if it results in the disappearance of all mitosis (Deysson, 1968). Thus in the

present study the extracts of all the seven species of *Alpinia* seem to be mitodepressive. The mitodepressive activity of all the plant extracts are dose and duration dependent.

Usually the cytotoxic effects observed in animals include spindle disturbances as well as clastogenic effects. However in plants, the effect is mainly on the spindle (Talukdar *et al.*, 1985). The cytotoxic plant extracts act on the cells in three different ways. (1) Prophase inhibitor (2) Inhibitor of mitotic spindle formation and orientation, compounds being termed as mitoclastic agents and (3) Inhibitor of cell plate and cell wall formation between daughter nuclei resulting in binucleate and multinucleate cells (Ray and Barman, 1987). Cytological aberrations were classified as clastogenic aberrations attributable to the direct action in chromosomes and non-clastogenic (physiological) abberations attributable to spindle abnormalities.

The cytotoxic studies with the aqueous extracts of seven species of *Alpinia* on *Allium cepa* root meristem showed many clastogenic and non-clastogenic abnormalities. The major clastogenic abnormalities observed include nuclear lesions, chromosome bridges, chromosome stickiness, agglutination of chromosomes, chromosome fragments and ring chromosome. Whereas, non-clastogenic abnormalities noticed were chromosome clumping, chromosome scattering, micronucleus, multipolarity, diagonal metaphase, diagonal anaphase, ball metaphase, binucleate cells, polyploidy, early movement of chromosomes, *etc.* The most frequent abnormalities observed in all treatments were nuclear lesions and binucleate cells (Plates IV-IX; Tables 2-15).

The observations of the present study is a clear indication of the clastogenic and non-clastogenic property of the *Alpinia* extracts, which is evident from the direct actions on chromosomes and spindle

abnormalities. The lowering of mitotic index might have been achieved by the inhibition of DNA synthesis at the S phase (Sudhakar *et al.*, 2001). It may be also due to the slowing of the rate of cell progression through mitosis (Sharma and Sahu, 1977) or due to the obstruction of the onset of prophase or due to the arrest of one or more mitotic phases (Kabarity and Mallalah, 1980). The present investigation with the aqueous rhizome extracts of *Alpinia* finally upset nucleic acid metabolism leading to disturbances in DNA and protein synthesis and thus resulted in various abnormalities both at nuclear and chromosome levels on *Allium cepa* root meristem.

Frequently observed clastogenic abnormalities

Nuclear lesions

Nuclear lesions (Plate IV A) was the major abnormality noticed when root tip cells were treated with both types of plant extracts in all the seven species of *Alpinia* studied (Tables 2-15). Previous reports confirm the occurrence of nuclear lesions, induced by plant derived chemicals in *Allium cepa* root meristem (Mercykutty and Stephen, 1980). It may be due to the disintegration of portion of nuclear material by the action of plant extracts.

Chromosome bridges

Chromosome bridge formation (Plate VIII H-P; Plate IX M & N) is observed in all the treatments of the present study. Mitotic bridges may arise due to stickiness or due to formation of dicentric chromosomes by breakage and reunion (Raj and Rao, 1972). Double and multiple bridges occur as a result of fusion between broken chromosomes (Wong Young and Woo Young, 1993). Single, double or triple bridges are formed due to the breaking up of chromosomes followed by proximal chromatid

reunion (Grant, 1978) and also due to general stickiness of chromosomes (Abraham and Koshy, 1979). The anaphase bridges may arise due to effect of chemicals in breaking the protein moiety of nucleoprotein backbone (Patnaik *et al.*, 1984).

Chromosomal stickiness

Stickiness of chromosomes (Plates IV P; VII C & D; VIII M & N; IX F & L; Tables 2-15) was another major abnormality observed in almost all the treatments of the present study. According to Darlington (1942), stickiness is due to the disturbances in the nucleic acid metabolism of the cell. Stickiness has been interpreted as the result of depolymerization of DNA (Darlington, 1942), partial dissolution of nucleoproteins (Kaufman, 1956) and stripping of protein covering of DNA in chromosomes (Stephen, 1979). Induction of stickiness is also manifested as the cytotoxic effect of chemical substances (Panda and Sahu, 1985). There could be some substances present in the plant extracts which affect the DNA structure, perhaps resulting in physical depolymerization of DNA. This together with or without partial dissolution of nucleoproteins (Mercykutty and Stephen, 1980) could account for the stickiness of chromosomes.

Chromosome agglutination

Agglutination of chromosomes (Plate VII E; Tables 4 & 10) is an extreme type of chromosomal stickiness observed frequently in the treatments with the extracts of *Alpinia galanga* and *A. smithiae* containing polar and non-polar fractions. The extreme stickiness resulting to agglutination of chromosomes is probably due to the heterochromatinization of chormosomes resulting in denaturation of

nucleic acid and thus making the chromosome contour adhesive (Grundmann, 1966).

Ring chromosome

A small ring chromosome was noticed in different treatments of *Allium cepa* with *Alpinia malaccensis* and *A. smithiae* extracts (Table 6 & 10; Figs. VII A & IX E). Induction of ring chromosomes suggests the possibility of two breaks that occur in the same chromosome. Sax (1940) opined that the two breaks that occurs in the same chromosome after the process of rejoining may form a ring chromosome.

Rarely observed clastogenic abnormalities

A wide spectrum of other clastogenic abnormalities were found to occur in some treatments, which appeared to be rare in the present investigation. The different infrequent clastogenic aberrations scored in *Allium cepa* root tip meristem after treatment with *Alpinia* extracts are as follows:

(a) Hyperchromasia (Plate IV B; Table 6), where the interphase nucleus takes up intense stain than normal probably due to abnormal heterochromatinization.

(b) Bizzare nucleus (Plate IV C; Table 4), where nucleus shows distortion in shape due to the effect of cytotoxicants present in the extract.

(c) Nuclear leakage (Plate IV D; Tables 11, 14) due to the probable breakage of nuclear membrane at one region of the interphase nucleus.

(d) Nuclear disintegration (Plate IV E; Table 12) as a result of disruption of nuclear membrane and chromatin.

(e) Nuclear diminution (Plate IV F; Table 12), where the interphase nucleus condenses abnormally.

(f) Nuclear fragmentation (Plate IV G; Table 8) and fragmentation leading to the formation of chromatin globules (Plate IV M; Table 8) arises as a result of the disruption of nuclear membrane and chromatin, followed by compartmentalization and realignment of the broken parts into separate subnuclear structures.

(g) Nuclear budding (Plate IV H & I; Tables 5, 14) results probably from the excessive production of chromatin materials, especially in the 'S' phase leading to the formation of an accessory appendage.

(h) Giant cell with a giant nucleus (Plate V A; Table 10), nuclear dissolution in a giant cell (Plate V B; Tables 10, 12), giant polyploid cell with chromosome fragments (Plate V C; Tables 7, 12) and giant cell with isochromatid gaps (Plate V D; Tables 10, 12) are extremely clastogenic aberrations that were scored rarely in the present investigation. In all these instances the giant cell formation initiates probably in the preceding abnormal G_1, S and G_2 phases, followed by the respective clastogenic conditions.

(i) Isochromatid gaps were observed in the metaphase chromosomes of ordinary sized aberrant cells (Plate VII A; Table 6) along with a ring chromosome (mentioned earlier) and in giant cells (Plate V D; Tables 10, 12 – mentioned earlier). These chromatid gaps may not be true breaks, but they might have been formed due to localized uncoiling of the chromosomes.

(j) Pulverization of chromosomes (Tables 13, 14) during metaphase (Plate VII F), anaphase (Plate IX I) and telophase (Plate IX K) may be due to the induced premature condensation of the chromosomes, followed by the disintegration of all the chromosomes.

(k) Coalesced ends of chromosomes (Plate VII H; Table 15) are probably due to the induction of stickiness at the telomeric regions of the chromosomes.

(l) Chromosome fragments (Plate VII J; VIII A; Tables 5, 8) is probably the result of breaking at the fragile sites of chromosomes. Fragments can arise due to the changes brought about in the viscosity of the cytoplasm by the cytotoxic extracts.

(m) Contorted chromosomes (Plate VII K; Tables 3, 6) arise probably as a result of dissolution of the protein associated with the chromosomes.

(n) Hypercondensed chromosomes (Plate VII L; IX D; Tables 4, 14) may be the result of elimination or disintegration of heterochromatin or abnormal condensation and tighter coiling induced by the cytotoxic extracts.

(o) Abnormal chromosomal associations (Plate VII M; Tables 11, 12) are probably due to the end to end adhesion of chromosomes.

Frequently observed non-clastogenic abnormalities

Ball metaphase

Ball metaphase (Plate VII B & C; Tables 2, 3, 6, 8-10, 11, 12 & 14) was noticed in most of the treatments (0.125%, 0.25%, 0.5% and 1%) of

all plant extracts. It is a form of C-mitosis with characteristically clumped chromosomes. The ball metaphase is followed by either a complete degeneration of the cell or a state similar to interphase (Barber and Callan, 1943). It may be due to the localized activity of spindle apparatus at the centre so that chromosomes were arranged in such a way that their centromeres remain at the equator and arms radiating in different directions and orienting in the form of a ball.

Polyploidy

Polyploid cells were noticed in some treatments (Plates V C; VII O & P; Tables 2, 6, 7, 10, 12 & 14) with extracts of *A. calcarata, A. malaccensis, A. smithiae, A. vittata* and *A. zerumbet*. Minija *et al.* (1999) observed that polyploidy may be due to the inhibition of spindle mechanism. Herichova (1973) noticed polyploid cells on application of spindle destructing chemicals. Nagpal (1994) observed that occurrence of hyperploid cells may be attributed to the spindle inhibition, lack of anaphase movement or failure of cell plate formation.

Binucleate cells

The formation of binucleate cells during interphase is a major abnormality observed in all concentrations of plant extracts (Plate IV K & L; Tables 2-15). Ene-Obong and Amadi (1987) noted that delay or failure of cytokinesis could account for the occurrence of binucleate and multinucleate cells. Binucleate cells also may arise due to the suppression of cell plate formation in the early telophase. Hence neither the cell plate nor the cell wall appeared at the equatorial plane in the treated cells (Sato and Tanaka, 1972).

Stellate anaphase

In the present investigation, stellate anaphase is observed in various treatments (Plate VI A; VIII H; IX G; Tables 2, 6, 9, 10, 12, 14) of *A. calcarata, A. malaccensis, A. purpurata, A. smithiae, A. vittata* and *A. zerumbet* extracts. Amar (1965) considered this phase as a fore step of the complete disturbance of the spindle. Star shaped arrangement may be due to the clumping of daughter chromosomes into star like configurations near the polar region of the cell.

Micronucleus formation

Presence of micronucleus (Plate IV J; Table 6) was observed only during treatment with *A. malaccensis* extracts (polar and non-polar fractions). Micronucleus may originate from a lagging chromosome of anaphase or from a chromosome fragment (Badr and Ibrahim, 1987). The formation of micronuclei may be due to the action of chemicals present in the extract on the spindle apparatus leading to unequal separation of chromosomes at anaphase. The larger group of daughter chromosomes form a larger nucleus and the smaller group form a micronucleus.

Multipolarity

Multipolarity of chromosomes (Plates VI D; VIII K; IX H; Tables 2, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15) was noticed in most of the treatments of *Alpinia* extracts in the present investigation. A possible explanation might be the pronounced inactivation of spindle apparatus, that the chromosomes could not regain the normal position inside the cell. Position and number of poles determine multipolar condition. The number of poles in a cell depends on the position of the assemblage of RNA and polysaccharides which remains distributed either in the form of

sol or gel (Prasad, 1974). Occurrence of tripolar cells indicate the inhibition of cytokinesis (Somasekhar and Gowda, 1984).

Diagonal metaphase, anaphase and telophase

Diagonal orientation of chromosome groups at metaphase and anaphase were observed in most of the treatments (Plates VII I; VIII F; IX B; IX C; IX N; Tables 2, 3, 4, 5, 6, 9, 10, 11, 13, 14, 15). This may be due to the slight tilt in the spindle apparatus induced by the failure and improper functioning of the spindle apparatus (Deena and Thoppil, 2000).

Early movement

Early movement of chromosomes were noticed in most of the treatments (Plate VIII E; Tables 2, 3, 6, 7, 9, 11, 12, 13). This abnormality may be due to the disturbance in the spindle mechanism (Tajo and Thoppil, 1998).

Clumping

Clumping of chormosomes was another most frequent abnormality observed in the treatments (Plate VII G; Tables 2, 3, 4, 5, 6, 9, 10, 12, 14). Clumping of chromosomes is attributed to the increased concentration of the cytotoxicant (Pritchard and Court, 1968).

Misorientation

Misorientation of chromosomes were noticed in the treatments of extracts of *Alpinia calcarata, A. purpurata* and *A. smithiae* and *A. zerumbet* (Plates VI C, VII N, VIII O, IX O; Tables 2, 3, 9, 11, 15). Disturbed function of the spindle apparatus may be the reason for the occurrence of misorientation of chromosome groups (Das *et al.*, 1968).

Non-synchronization

Non-synchronization movement of chromosomes was a frequently observed abnormality in different treatments (Plate VI B, VIII F, IX A, IX P; Tables 6, 9, 10, 12, 14). The non-synchronous movement of chromosomes may be due to severe imbalances in the spindle mechanism. It may be due to the multipolar nature of the mitotic spindle apparatus (Minija *et al.*, 1999).

Scattering of chromosomes

Scattering of chromosomes was observed in the treatments with polar fraction of *Alpinia galanga* and polar and non-polar fractions of *Alpinia purpurata* (Plates VIII B; Tables 5, 8). Disturbances in the mitotic spindle may result in the scattering of chromosomes (Darlington, 1942).

Rarely observed non-clastogenic abnormalities

(a) Tetranucleate cells (Plate IV N; Table 7) may be the result of preceeding multipolar mitosis or failure of cell plate formation.

(b) Nuclear vacuolization (Plate IV O; Tables 2, 6, 14) results due to the formation of a clear zone of cytoplasm, devoid of chromosomes during prophase, probably due to the change in viscosity of the cytoplasm, brought about by the extract.

(c) Stellate metaphase observed in diploid and polyploid cells (Plate VII O; Table 14) in a few treatments may be due to the clumping of metaphase chromosomes in a star like pattern. This can arise due to the disturbance of the spindle apparatus.

(d) Equatorial separation of chromosomes (Plate VIII C; IX J; Tables 4, 7) can be due to a distortion of the spindle apparatus leading to a

tilt in the equatorial organization of metaphase chromosomes or a change in the direction of movement of daughter chromosomes during anaphase.

(e) Disturbed anaphase (Plate VIII D; Tables 3, 15) may be due to loss of activity of microtubules in the spindle fibres. Inhibition of spindle formation can also lead to disturbed anaphase.

(f) Stathmo-anaphase (Plate VIII G; Table 11) may be the case, where the daughter chromosomes do not separate fully, but they remain connected together by partial overlapping of chromosome arms. This may also be due to abnormal functioning of the spindle apparatus.

In the present investigation, a careful screening of data reveals that the percentage of cytotoxicity and mitotic inhibition was found to be severe in the aqueous extracts of *Alpinia* species containing both polar and non-polar fractions. However, these effects were negligible in *Alpinia* water extracts containing polar compounds alone. This clearly indicates that, it is the non-polar compounds present in *Alpinia* aqueous extracts, which is mainly responsible for the cytological aberrations and mitotic inhibition.

Cytotoxicity of *Alpinia* extracts on *in vitro* cell lines

Cancer is perhaps the most progressive and devastating disease posing a threat of mortality to the entire world despite significant advances in medical technology for its diagnosis and treatment. This area therefore is a most promising site for the discovery of novel biologically active substances from plants (Burkhill, 1966; Murakami, 1999). Hence various screening studies were carried out to discover the scientific basis for the efficacy of traditional plants in treating cancer and to elucidate

chemical basis for any activity. It is also commonly recognized that bioactive compounds are more likely to emerge from screening programmes guided by traditional medicine than from random screening.

The results of the present study using water extracts of seven species of *Alpinia* did not show cytotoxity against Daltons lymphoma ascites (DLA) and Ehrlich ascites carcinoma (EAC) is in concordance with the earlier reports. Saetung *et al.* (2005) reported that the water extracts of twelve Thai medicinal plants did not exhibit cytotoxic activities against human cell lines. However, methanolic extracts of *A. malaccensis* and *A. vittata* were found to be cytotoxic against DLA and EAC cell lines where as the other five species of *Alpinia* showed weak cytotoxicity (Table 16). Lantz *et al.* (2005) showed that water soluble extracts of turmeric were not cytotoxic and did not exhibit any biological activity where as the organic extracts of turmeric were cytotoxic. Zaeoung *et al.* (2005) reported the cytotoxic activity of the methanolic extract of five species of Zingiberaceae against colon adenocarcinoma and breast adenocarcinoma cell lines. They have further observed that the monoterpenes, sesquiterpenes and phenyl propanoids could be responsible for the cytotoxic activity. The difference in cytotoxic activity of methanolic extracts of the seven species of *Alpinia* against DLA and EAC cell lines seem to be attributed to the chemical composition of these plants, which may even vary depending upon the environmental conditions. Murakami *et al.* (1998) showed variations in the cytotoxic activity of *Alpinia* species, which may be dependent on variations in ecology or collection time, although the possibility of chemovars cannot be ruled out.

The methanolic extracts of the cytotoxic species, *viz., A. malaccensis* and *A. vittata* in short duration studies were further screened for their efficacy in long term cytotoxic assays using L_{929} and A_{549} cell lines. The

results indicated that both of them were considerably cytotoxic against both cell lines and reduced cell viability (Table 17). Chung *et al.* (2001) reported that the crude ethanolic extract of *Glycyrrhiza uralensis* accelerate apoptosis of A_{549} cells possibly due to its chemical components in the crude extract. It also showed the role played by the sample in limiting the initiation of carcinogenesis and result in effectively inhibiting the growth of cancer cells. Missiry *et al.* (2000) indicated the influence of melatonin on specific receptors in tumor cells and can induce, in part, apoptosis of EAC cells and phase delay in cell cycle. The cytotoxic potential of methanolic extracts of *A. malaccensis* and *A. vittata* revealed by long term assays using L_{929} and A_{549} cell lines, may be due to the specific effect of the major chemical constituents or due to the combination effect of all chemical compounds in totality.

Sylvestre *et al.* (2005) suggested that myrcene, limonene, α-phellandrene containing extracts, is cytotoxic against human lung carcinoma cell lines-A_{549}. Long duration of treatment of these compounds containing extracts showed higher cell growth inhibition due to sesquiterpene enrichment. Moteki *et al.* (2002) showed that suppression of growth by 1, 8-cineole in leukemia cell lines, results from the induction of apoptosis by this compound. Zhangli *et al.* (2006) suggested that the antitumor effect of D-glucosamine hydrochloride derivatives may be due to its cytocidal and immunomodulating properties. Sylvia *et al.* (2003) indicated that diterpene and sesquiterpene fractions of *Copaifera multijuga* have reduced cell viability when incubated with melanoma cell-lines and these fractions have tumouricidal activity in the melanoma in both models *in vivo* and *in vitro*.

The results of the present study is in concurrence with earlier reports of Thippeswamy *et al.* (2006), Vimala *et al.* (1999), Chung *et al.* (2001) and Zaeoung *et al.* (2005) and Zhou Bingnan *et al.* (1997).

The variations among cytotoxic ability of *Alpinia* species was well documented by Murakami *et al.* (1998). Sylvia *et al.* (2003) observed a positive correlation between *in vitro* cytotoxic properties and *in vivo* antitumour activities. Zhangli (2006) observed the antitumour activity of extract may be due to cytocidal properties. Shylesh *et al.* (2000) revealed that the methanolic extract of *Emilia sonchifolia* was found to be cytotoxic to Daltons lymphoma, Ehrlich ascites carcinoma and mouse lung fibroblast cells (L_{929}) but not toxic to normal human lymphocytes under *in vitro* conditions. Oral administration of the extract reduced the development of solid tumors and ascites tumors and increased the life span of these tumor-bearing mice. Kirana *et al.* (2003) indicated that zerumbone, a sesquiterpene can induce apoptosis. Tanaka *et al.* (2001) suggests that dietary zerumbone has a beneficial effect on chemically induced colonic pre-neoplastic progression in rats and provides an effective dietary chemo-preventive approach to cancer chemoprevention. Hence it seems probable that the cytotoxic ability of *A. malaccensis* and *A. vittata* may be due to the chemical components present in these extract which may induce apoptosis and may be responsible for the antitumour effect.

ANTITUMOUR ASSAYS

After a quarter century of rapid advances, cancer research has generated a rich and complex amount of knowledge revealing cancer to be a disease involving dynamic changes in the genome. Hahn (1999) observed that tumorigenesis is a multistep process formally analogous to

Darwinian evolution, in which a succession of genetic changes, each conferring one another, a type of growth advantage, which leads to the progressive conversion of normal cells into cancer cells.

Mechanism that suppress tumorigenesis often involve modulation of signal transduction pathways, leading to alterations in gene expression, arrest of cell cycle progression or apoptosis. Apoptosis is a mode of cell death used by multicellular organisms to eradicate cells in adverse physiological and pathological settings. Several studies have demonstrated that apoptosis may be involved in cell death induced by chemotherapeutic agents including cisplatin and camptothecin. There is ample evidence that the efficiency of antitumour agents is related to the intrinsic ability of the target tumour cells to respond to these agents by apoptosis (Cohen, 1993).

In the present investigation, acute toxicity studies were conducted on the methanolic extracts of *A. malaccensis* and *A. vittata* to check the probable toxic effects of these extracts at higher and lower doses in Swiss albino mice. The results indicate that the extracts of *A. malaccensis* and *A. vittata* are not toxic to the test organism (Table 18). The minor differences found among the values both after administration of higher and lower doses, when compared to the control are insignificant (Table 26) and hence can be neglected. The antitumor activity of methanolic extracts of *A. malaccensis* against DLA induced solid tumor (Fig. 1; Plate X; Table 19) was found to be more pronounced when compared with that of *A. vittata* (Fig. 4; Plate XI; Table 22). The phytochemicals, especially terpenoids and dietary antioxidants are known to decrease the risk of cancer. In nature, terpenoids are biosynthesized by tandem reaction of the phosphorylated isoprene unit bearing five carbons. According to the number of combined isoprene units they are classified into mono (C_{10}),

sesqui (C_{15}) and bi (C_{20}) and triterpenoids (C_{30}) and so on. Crowel (1999) suggested that dietary terpenoids have anticarcinogenic activities after conducting a variety of rodent experiments. The results of the antitumor assay is in accordance with the earlier reports that zerumbone and an array of terpenoid components are capable of checking cancer.

In *A. malaccensis*, the major chemical component zerumbone, either singly or in combination with other monoterpenoids and sesquiterpenoids (Table 29) seems to be responsible for the potential antitumor effect. Murakami *et al.* (1999) noticed that α and β unsaturated carbonyl group is a notable moiety of zerumbone, a sesquiterpenoid and intriguingly, α-humulene, a zerumbone analogue lacking this functional group has been found to be virtually inactive to disrupt the induced biochemical pathway for Epstein – Bar virus activation. Narisawa *et al.* (1992) have shown the cancer preventive and therapeutic potential of sesquiterpenoids. Murakami *et al.* (2002) indicated that zerumbone, a sesquiterpene can induce apoptosis and has distinct potentials in anti inflammation, chemoprevention and chemotherapy strategies. It also suppresses tumor promoter TPA (12-0-tetradecanoyl phorbol-13 acetate). Murakami *et al.* (2003) reported that oral administration of zerumbone markedly suppressed dextran sulfate sodium induced colitis in mice. Murakami *et al.* (2004) suggested that the simultaneous application of the drug will affect the promotive and initiation stages of carcinogenesis. Kirana *et al.* (2003) showed the antitumour activity of extract of *Zingiber aromaticum* and its bioactive sesquiterpenoid, zerumbone. They indicated that zerumbone can be used as an anticancer agent, possibly by its apoptosis inducing and antiproliferative influences. Tanaka (2001) demonstrated that zerumbone can inhibit azoxymethane induced rat abberant crypt foci and phorbol ester induced papilloma formation in

mouse skin as a further indication for its efficacy to prevent colon and skin cancers. Therefore, zerumbone is a promising dietary agent that has distinguishable potentials for use in antiinflamation, chemoprevention and chemotherapy strategies. Hoffman (2002) suggests that zerumbone, a sesquiterpenoid can suppress the pro-inflammation of caner cells and at the same time it will not inhibit the proliferation of normal cells. Murakami (2002) reported that zerumbone inhibits the proliferation of colon cancer cells and induces apoptosis in them, while having less effect on normal cell proliferation. Takada *et al.* (2005) showed the antiproliferative and anti inflammatory activities of zerumbone found in *Zingiber zerumbet* and also pointed out that zerumbone inhibits the gene expression induced by carcinogens and this inhibition may provide a molecular basis for the prevention and treatment of cancer by zerumbone.

In the case of *A. vittata*, the major chemical constituent fenchone – a monoterpenoid – either alone or together with other co-existing monoterpenoids and sesquiterpenoids (Table 29) might have been the cause for its antitumor effect. The cancer preventive ability of monoterpenoids was reported by Kawamori *et al.* (1996). The results of Liebermanns-Burchardt test on the methanolic extract of *A. malaccensis* and *A. vittata* also indicates the presence of terpenoids in both the plants. Hence these terpenoids present in the extracts may be responsible for the antitumor activity of the extracts of *A. malaccensis* and *A. vittata*.

Baatout *et al.* (2004) showed the chemopreventive nature of *Curcuma* on human cancer cells. However, when *Curcuma* extracts and radiation were applied together, the results indicates that human cancer cells showed a radiation sensitising effects. This may be of immense help to selectively induce apoptosis in cancer cells.

The results of the combination therapy in the present study using terpenoid containing methanolic extracts of *A. malaccensis* and *A. vittata* and radiation (Fig. 3, 6; Plate X, XI; Tables 21, 24) and also the plant extracts with the standard drug cisplatin (Fig. 2, 5; Plate X, XI; Tables 20, 23) showed a synergistic effect in the antitumor activity.

The effect of methanolic extracts of cytotoxic species of *Alpinia* on EAC cells induced ascites tumor harbouring mice reveals that both plants possess moderate antitumor effect, when compared with the control. When both these plants were compared *A. malaccensis* shows a higher percentage of increase in life span than *A. vittata*, in both higher and lower doses (Table 25). Earlier study conducted by Thippeswamy *et al.* (2006) reports that the growth of EAC cells and formation of ascites tumor were inhibited by the extract of a zingiberaceous plant, *Curcuma aromatica*.

Apart from the antitumor assays conducted with the methanolic extracts of *A. malaccensis* and *A. vittata* on EAC cells induced ascites tumor, all the other experiments conducted on DLA induced solid tumor were found to be significant (Table 26). Based on the earlier reports and the results of the present study it can be assumed that the cytotoxic nature of the *Alpinia* extracts together with apoptosis induction resulted in the antitumor potential of the rhizome extracts of *A. malaccensis* and *A. vittata*.

ANTIOXIDANT ASSAYS

Antioxidant activities

Carcinogenic agents are powerful generators of free radicals that damage the cells and its components. Free radicals (Reactive oxygen species) are low molecular weight metabolites reactive enough to damage essential biological molecules including nucleic acid (Groot, 1994). The

free radicals are known to interact directly with genomic DNA, damage specific genes that control cell growth and differentiation during initiation and promotion phases and stimulate faster growth of malignant cells (Troll *et al.*, 1985). Anti oxidants are substances that can provide free radicals, an electron which becomes a companion to their unpaired electron thus eliminating the threat of gene alterations leading to cancer (Thomas, 2000). Antioxidants are compounds that help to inhibit free radicals such as super oxides, hydroxy radicals, nitrogen free radical species, *etc.* and are capable of decreasing the localized O_2 concentration, thereby reducing molecular oxygen's oxidation potential, metabolising lipid peroxides to non-radical products. Thus antioxidants regulates free radical damage (Lakenbrink, 2000).

The results of the present study is in accordance with the previous reports that methanolic extracts containing terpenoids posses anti oxidant activity (Table 27). The extracts of *Alpinia calcarata, A. galanga, A. malaccensis, A. smithiae* and *A. vittata* showed superoxide radical scavenging activity. The hydroxyl radical scavenging activity was observed in the methanolic extract of *A. galanga, A. malaccensis, A. purpurata, A. smithiae* and *A. vittata.* The nitric oxide radical scavenging activity was noticed in *A. galanga, A. smithiae* and *A. vittata.* Lipid peroxidation activity was noticed in the extract of *A. galanga, A. malaccensis, A. purpurata* and *A. smithiae.* Concentration dependent radical scavenging activities of the seven species of *Alpinia* (Table 28) reveal that the maximum percentage of inhibition (IC_{50}) for superoxide radical scavenging activity was shown by *A. malaccensis* (Fig. 7). In the case of hydroxyl radical scavenging activity, *A. malaccensis* shows the maximum IC_{50} value followed by *A. vittata* (Fig. 8). Nitric oxide radical scavenging activity was found to be the maximum in *A. galangal,* followed by *A. vittata*

(Fig. 9). Maximum inhibition of lipid peroxidation was shown by *A. malaccensis* (Fig. 10). Zaeoung *et al.* (2005) studied the free radical scavenging activities of Zingiberaceae plants and reported that methanolic extracts showed strong free radical scavenging activity than water extracts and volatile oils. Matsuda *et al.* (2006) reported that 80% acetone extract from the rhizomes of *Alpinia officinarum* were found to inhibit nitric oxide production.

The antioxidant activities of alcoholic extracts of *A. malaccensis, A rafflessiana, A. nutans, A. mutica* were studied by Habash *et al.* (2000). Padma *et al.* (2006) reported that methanolic extract of *Alpinia galanga* and *Alpinia allughas* showed strong free radical scavenging activity *in vitro.*

In the light of the antioxidant assays conducted with the help of four different assays it seems probable that the prominent antioxidant potential exhibited by *A. malaccensis* in most of the assays and the substantial antioxidant effects shown by other species of *Alpinia* may be due to the terpenoids and phenols present in their methanolic extracts. Nakamura (2004) provide biological evidence that zerumbone, a sesquiterpenoid has significant ability to suppress oxidative stress possibly through induction of endogenous antioxidants such as xenobiotic metabolising enzymes. Considering the oxidative damage in carcinogenesis, the antioxidant effect of zerumbone can be exploited as a cancer chemo-preventive agent targeted towards inflammation related carcinogenesis. Murakami *et al.* (2004) demonstrated that zerumbone effectively suppressed superoxide anion generations and is a promising agent for the prevention of both tumor initiating and promoting processes through induction of anti oxidative and phase II metabolising enzymes as well as attenuation of pro inflammatory signalling path ways.

The implications of free radicals in different steps of carcinogenesis was well documented by Player (1982). The antitumour activity of antioxidants may be due to induction of apoptosis (Putul *et al.*, 2000). Sreejayan *et al.* (1997) indicated the free radical scavenging activities of curcumin and observed that the anticarcinogenic activity of this compound could be partly explained by its free radical scavenging properties. The role of oxygen derived free radicals in carcinogenesis was demonstrated by Fantone (1982) and McCord (1974). The antiinflammatory activity of antioxidants was also demonstrated by Srimal (1973). Surh (1999) suggested that a variety of terpenoids and phenolic substances present in dietary and medicinal plants posses striking antioxidant properties which contribute to their cancer chemo-preventive potential. Hence it can be stated that the free radical scavenging activity of methanolic extracts of *Alpinia* species at lower concentrations may be one of the basis for the antitumour activity of these extracts at higher concentrations. At higher concentrations these extracts act as a prooxidant rather than an antioxidant. A very similar observation made by Preethi *et al.* (2005) confirms this potential.

PHYTOCHEMICAL ASSAYS

GC-MS analysis of essential oils of *Alpinia* species

Essential oils are products, generally, of rather complex composition comprising the volatile principles contained in the plants, and are more or less modified during the biosynthetic process (Bruneton, 1995). In the present investigation the essential oil of the seven species of *Alpinia* were obtained by hydrodistillation and the chemical analysis was carried out using GC - MS analysis [Figs. 11 - 17, 18 (i) – 18 (x)]. Identification of the main components was carried out by the comparison

of both GC retention times and MS data against those of reference standards. In the present investigation a wide spectrum of essential oil components were detected by GC-MS in the seven species of *Alpinia* (Table 29). Many of these chemical compounds were detected earlier in the essential oil of other species of *Alpinia* (Table 1). Generally essential oils are poorly soluble in water, but soluble in many organic solvents like acetone, methanol, ethylene glycol, ethanol, *etc.* Biological activity of an essential oil is related to its chemical composition. The biological activity of the essential oils may be attributable both to their major and minor non-polar components (alcoholic, phenolic, terpenic, or ketonic compounds) present in these oils. It is possible that these non-polar components may act together synergistically to contribute to the toxicity of the totality of the tested oil.

Alpinia calcarata

The essential oil of *Alpinia calcarata* is dominated by monoterpenes, 1-8 cineole (36.94%), β-fenchyl acetate (17.56%), limonene (4.14%), camphene (6.62%), β-pinene (7.06%), myrcene (0.64%), p-cymene (0.68%), γ-terpinene (0.58%), α-thujone (0.96%), L-camphor (3.75%), terpinene 4-ol (0.94%), β-fenchyl alcohol (4.02%), α-pinene (0.64%) and methyl cinnamate (0.88%). The sesquiterpenoids present were epizonaren (0.85%), valencene (2.68%), azulenol (8.03%), and α-selinene (3.03%).

The rhizome oil of *Alpinia calcarata* is dominated by monoterpenoids (85.41%) and hence it falls under the monoterpenoid class of essential oils. A considerable amount of sesquiterpenoids are also present (14.59%). The major monoterpenoid is 1-8 cineole, followed by fenchyl acetate, so it fall under cineole > fenchyl acetate chemotype (Fig. 11; Table 29). Earlier workers, Arambewala *et al.* (2005), Kaul Pran *et al.*

(2005) and Choudhary *et al.* (2003) have confirmed the occurrence of many of these essential oil components in *Alpinia calcarata*.

Alpinia galanga

The essential oil obtained from *Alpinia galanga* is rich in a monoterpenoid, 1-8 cineole (63.31%). The other monoterpenoids identified were sabinene (0.39%), β-pinene (1.12%), myrcene (0.87%), α-terpinene (0.43%), limonene (2.37%), γ-terpinene (0.90%), geranyl acetate (2.23%), terpinene-4-ol (2.14%), camphene (0.96%) and β-fenchyl acetate (0.70%).

The sesquiterpenes were β-caryophyllene (0.62%), α-humulene (1.27%), β-elemene (11.49%), germacrene D (0.69%), β-bisabolene (0.50%), α-farnesene (0.70%), β-sesquiphellandrene (0.59%) and nerolidol (2.54%). The phenolic compounds present are methyl eugenol (0.5%) and acetyl eugenol (5.68%).

The major class of compounds identified in this taxa is monoterpenoids (75.42%), sesquiterpenoids (18.4%) and phenolic compounds (6.18%). Since 63.31% of 1-8 cineole is present in the rhizome oil, it fall under cineole chemotype. The result of the present study is in concurrence with the earlier reports of Leopold *et al.* (2003), Raina (2002) and Mallavarapu *et al.* (2002).

Alpinia malaccensis

The essential oil of *A. malaccensis* is dominated by a sesquiterpenoid, zerumbone (54.77%). The other sesquiterpenoid present in the rhizome oil is trans-caryophyllene (1.47%). Monoterpenoids present are fenchol (12.19%), α-phellandrene (12.9%), limonene (4.21%),

camphene (0.96%), β-pinene (3.34%), myrcene (1.2%), p-cymene (1.33%), α-thujone (0.86%), α-terpineol (0.6%) and β-fenchyl acetate (6.17%).

Sesquiterpenoids are the major class of compounds obtained in this study (56.24%). Substantial amount of monoterpenoids are also present (43.76%). Since the dominant compound is zerumbone, the rhizome essential oil of *A. malaccensis* falls under zerumbone chemotype (Fig. 13; Table 29). Previous report by Roy (1998) confirms the presence of many of these components in the essential oil of *A. malaccensis*.

Alpinia purpurata

The major components of *Alpinia purpurata* are α-selinene, a sesquiterpenoid (58.3%) and β-pinene, a monoterpenoid (41.7%).

The essential oil of *Alpinia purpurata* is dominated by α-selinene together with β-pinene. So the rhizome essentiall oil of this plant falls under α-selinene > β-pinene chemotype (Fig. 14; Table 29). Sadaquat Ali *et al.* (2002) reported the occurrence of β-pinene in the essential oil of *Alpinia purpurata*.

Alpinia smithiae

The chemical components identified in the GC-MS analysis of *A. smithiae* are monoterpenoids and sesquiterpenoids (Fig. 15; Table 29). Monoterpenoids are 1,8-cineole (30.94%), β-fenchyl acetate (17.60%), α-terpineol (9.84%), α-pinene (2.61%), camphene (2.15%), β-pinene (6.79%), myrcene (0.75%), γ-terpinene (0.57%), α-terpinolene (2.83%), α-fenchyl alcohol (1.06%), L-borneol (2.56%), 1,4-terpineol (1.70%), citral (2.29%), geranyl acetate (1.23%) and α-terpinene (0.99%).

The sesquiterpenoids identified were trans-caryophyllene (4.28%), α-humulene (0.93%), γ-cadinene (2.56%), nerolidol (3.94%) and caryophyllene oxide (4.38%).

Monoterpenoids (83.91%) are the dominant class of compounds present in this species. Sesquiterpenoids (16.09%) are also present in this plant. Since major components identified in this plant are 1-8 cineole, the plant may fall under cineole chemotype. Many of the essential oil components are earlier reported by Roy. (1998).

Alpinia vittata

The major component isolated from the rhizome essential oil of *Alpinia vittata* is a monoterpenoid, fenchane (55.19%). Other monoterpenoids identified are α-pinene (1.23%), camphene (2.51%), β-pinene (3.98%), limonene (0.85%) and L-borneol (1.42%). The sesquiterpenoids identified are β-caryophyllene (1.29%), β-elemene (0.79%), α-selinene (1.15%), alloaromadendrene (5.69%), valencene (7.03%), germacrene-A (1.39%), β-bisabolene (0.52%), germacrene-D (1.35%), eudesmol (3.37%), neo intermediol (9.21%), zerumbone (0.67%), alliodorin (1.43%) and azulene (0.93%).

The rhizome oil of *A. vittata* was found to be composed of 65.18% monoterpenoids and 34.82% sesquiterpenoids (Fig. 16; Table 29).

Previous works on the essential oil is not available. The present study reveals the dominance of a monoterpenoid compound, fenchane. Hence this oil belongs to the fenchane chemotype.

Alpinia zerumbet

The major constituents of the rhizome essential oil of *Alpinia zerumbet* are monoterpenoids and sesquiterpenoids. The monoterpenoids

observed were β-pinene (0.97%), myrcene (0.17%), α-phellandrene (0.18%), α-terpinene (1.70%), p-cymene (0.84%), limonene (0.51%), 1,8-cineole (3.65%), γ-terpinene (3.69%), camphene (2.57%), α-terpinolene (0.72%), terpinene-4-ol (4.87%) and β-fenchyl acetate (0.38%). The sesquiterpenoids detected were trans-caryophyllene (0.52), α-humulene (4.89%), caryophyllene oxide (0.97%), zerumbone (72.76%) and β-caryophyllene (0.61%).

The essential oil of *A. zerumbet* rhizome is dominated by sesquiterpenoids (79.75%) and monoterpenoids (20.25%). Sadaquat Ali *et al.* (2002) reported the presence of many terpenoids in this plant. Since the essential oil is dominated by a sesquiterpenoid zerumbone, the rhizome oil of the plant falls under zerumbone chemotype (Fig. 17; Table 29).

The GC-MS analyses revealed that all the seven species of *Alpinia* are rich in the non-polar compounds, viz. terpenoids. Monoterpenoids were found to be the major class of compounds in the rhizome essential oils of *A. calcarata, A. galanga, A. smithiae* and *A. vittata*. Whereas in *A. malaccensis. A. purpurata* and *A. zerumbet,* the rhizome oils seem to be dominated by sesquiterpenoids. So it can be stated that the non-polar compounds, terpenoids present in these species of *Alpinia* are responsible for their potential cytotoxic, antitumor and antioxidant activities.

Supplementary test for terpenoids in the cytotoxic species of *Alpinia.*

Liebermann – Burchardt test

GC-MS studies can give a thorough information on the volatile non-polar fractions of the seven species of *Alpinia* contained in their

essential oils. However, in the present investigation cytotoxic and antitumor assays were conducted on the methanolic extracts of the potential cytotoxic species *A. malaccensis* and *A. vittata.* So the supplementary phytochemical test, *viz.* Liebermann – Burchardt test was conducted to find out whether the methanolic extracts of the cytotoxic species is dominated by either the volatile non-polar compounds or by other non-volatile non-polar/ polar compounds. The results of the Liebermann – Burchardt test confirms the dominance of volatile non-polar compounds, *viz.* terpenoids in the methanolic extracts of *A. malaccensis* and *A. vittata*. Previous reports confirm that zerumbone and other terpenoids present in *Alpinia* species are the chemical principles responsible for cytotoxic, antitumor and antioxidant potentialities.

The highlights of the present investigation are as follows:

- The seven species of *Alpinia, viz., A. calcarata, A. galanga, A. malaccensis, A. purpurata, A. smithiae, A. vittata* and *A. zerumbet* were found to be cytotoxic by the *Allium cepa* assay.
- These plants exhibited prominent mitotic inhibition in *Allium cepa* root tip meristem, which is an indication of their probable antitumour activity.
- *In vitro* cytotoxic short duration assays conducted on DLA and EAC cell lines revealed the prominent cytotoxic activities of methanolic extracts of *A. malaccensis* and *A. vittata.*
- Long duration *in vitro* assays conducted on L_{929} and A_{549} cell lines confirmed cytotoxic potential of methanolic extracts of *A. malaccensis* and *A. vittata.*

- Acute toxicity studies conducted on Swiss albino mice with the methanolic extracts of *A. malaccensis* and *A. vittata* proved that they can be used as antitumor drug, since they proved to be nontoxic by the SGOT, SGPT, ALP and creatinine assays.
- The methanolic extracts of the cytotoxic species, *A. malaccensis* and *A. vittata* show remarkable antitumour activity against DLA induced solid tumor. The activity was more in the former than the latter.
- The synergistic action of both the cytotoxic species increases when the methanolic extract was administered along with the standard drug cisplatin and with a standard dose of radiation.
- The life span of ascites (EAC) tumor harbouring mice when treated with methanolic extracts of the cytotoxic species were found to increase moderately in a dose dependent manner.
- Assays conducted with 4 different tests reveal the antioxidant potential of most of the species of *Alpinia* at lower doses, with the most prominent being *A. malaccensis*, followed by *A. vittata* and *A. galanga*.
- GC-MS analyses of the essential oils reveal that all the seven species of *Alpinia* were dominated by terpenoids (monoterpenoids and sesquiterpenoids) together with a few phenols.
- *A. malaccensis* was found to belong to a zerumbone chemotype and *A. vittata* falls under the fenchane chemotype.
- Liebermann-Burchardt test conducted as a supplementary test reveal the dominance of terpenoids in the methanolic extracts of *A. malaccensis* and *A. vittata*.

The above mentioned outcome of the present investigation can be concluded in such a manner that, all the species of *Alpinia* investigated reveals remarkable cytotoxic activity. However, the potential cytotoxic activity exhibited by *A. malaccensis* and *A. vittata* may be exploited for cancer chemoprevention, since both these plants also exhibits remarkable antitumour activity. The terpenoids present in these cytotoxic species, especially zerumbone and fenchane may act as potential anticancer agents. The cytotoxic ability together with the antioxidant efficacy of *A. malaccensis* and *A. vittata* may be responsible for the observed antitumour effect.

Future research should concentrate to isolate and characterize the active chemical principles in these cytotoxic species of *Alpinia*. Efforts can then be made to find out the probable mechanism of antitumour and antioxidant activities followed by clinical drug experiments.

Cytotoxic, antitumor, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.

Thesis submitted to the University of Calicut
for the award of the degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By

JOSE MARY DAS

GENETICS AND PLANT BREEDING DIVISION
DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA, INDIA

JUNE 2007

SUMMARY

Cancer is a disease which results from the abnormal growth and uncontrolled division of cells. Carcinogenesis is a multistage process that consists of at least three separate but closely linked processes – initiation, promotion and progression. In multistage carcinogenesis, promotion stage is closely linked to oxidative and inflammatory tissue damage. A substance with pronounced antioxidant and antiinflammatory effect is anticipated to act as antitumour promotor. A wide variety of terpenoids like zerumbone derived from different taxa of Zingiberaceae have been reported to have marked antioxidant and antiinflammatory effects which contribute to their cancer chemopreventive potential.

Most of the members of Zingiberaceae are used in folklore medicine around the world. Hence the present study was initiated with *Alpinia* spp. that are used in traditional medicine. The genus *Alpinia* Roxb. is the largest genus of the family with over 250 species. They are mainly distributed in the tropical and subtropical Asia. Seven species of *Alpinia*, i.e., *A. calcarata, A. galanga, A. malaccensis, A. purpurata, A. smithiae, A. vittata* and *A. zerumbet* were used in the present study. The plants are aromatic with rhizomatous stem.

The rhizomes of *Alpinia* species were collected and crude extracts having both polar and non-polar compounds and the extracts having polar compounds alone was prepared. The meristematic root tips of *Allium cepa* were used for testing the cytotoxic property of both types of extracts at different concentrations (0.125%, 0.25%, 0.50% and 1%) and at different time durations (½ hr, 1 hr and 2 hrs).

NB35625

TH
584.39 JOS/C

The abnormalities noticed were both clastogenic and non-clastogenic types. The major clastogenic abnormalities observed were nuclear lesions, chromosome bridges and chromosome stickiness. The non-clastogenic abnormalities detected were ball metaphase, diagonal anaphase, binucleate cells, polyploidy, micronucleus, multipolarity of chromosomes, star anaphase and non synchronous movement of chromosomes. The frequent abnormalities observed were binucleate cells and nuclear lesions. The mitotic index value and frequency of abnormalities showed gradual decrease and increase respectively when the concentration of the extract and duration of treatment was increased. But percentage of cytotoxicity was found to be more in plant extracts comprising both polar and non-polar compounds. Whereas, the cytotoxicity was negligible in extracts containing polar compounds alone.

The water extracts and methanolic extracts of the powdered rhizome of *Alpinia* species were further studied to reveal its cytotoxic potential *in vitro* in DLA and EAC cell lines using short duration assays. The water extracts of all *Alpinia* species studied showed weak cytotoxic activity. The methanolic extracts of *A. vittata* and *A. malaccensis* were found to be cytotoxic against both cell lines. The other five species showed weak activity. The two cytotoxic species, which were found to be effective in short duration assays were further observed for its toxicity against long duration cell lines A_{549} and L_{929}. The results indicated that both extracts are considerably cytotoxic against both cell lines, with *A. malaccensis* being more effective than *A. vittata.*

The methanolic extracts of the cytotoxic species, *A. malaccensis* and *A. vittata* were screened for their antitumour ability induced by DLA and EAC cell lines in Swiss albino mice. The oral administration of the methanolic extracts (100 mg/kg and 500 mg/kg body weight) of *A.*

malaccensis and *A. vittata* significantly inhibited the formation of solid tumour induced by DLA. These *Alpinia* species extracts also showed a synergistic action when administered along with standard drug cisplatin and with a standard dose of radiation.

The ascites tumor (EAC) harbouring mice when treated with methanolic extracts (100 mg/kg and 500 mg/kg body weight) of *A. malaccensis* and *A. vittata* were found to increase life span in a dose dependant manner.

The free radicals are implicated as the main cause of diseases like cancer, inflammation, *etc.* Hence the free radical scavenging activity of methanolic extracts of *Alpinia* species were carried out against superoxide radical, hydroxyl radical, nitric oxide radical and lipid peroxidation. Superoxide radical scavenging activity was found to be maximum in *A. malaccensis.* Hydroxy radical scavenging activity was found to be maximum in *A. malaccensis* followed by *A. vittata.* Nitric oxide radical scavenging activity was found to be prominent in *A. galanga* followed by *A. vittata.* Maximum inhibition of lipid peroxidation was shown by *A. malaccensis.*

Aromatic essential oil was obtained from the shade dried and powdered rhizome of the seven species of *Alpinia* after hydrodistillation in a clevenger apparatus at 100°C for 4 hrs. The extracted oil was dried over anhydrous sodium sulfate. The volatile non-polar chemical components were detected by gas chromatographic analysis. The components *viz.*, monoterpenoids, sesquiterpenoids and few phenols were identified by comparison of both GC retention time and MS data against those of reference standards. The major and minor components of the oil may act synergistically to contribute to the toxicity of the oil.

The methanolic extracts were further analysed by a supplementary test, namely Liebermann-Burchardt test to confirm the presence of volatile non polar compounds, *viz.* terpenoids in the cytotoxic species of *Alpinia*. The pink colouration obtained in the reaction mixture indicated the presence of terpenoids.

Hence it can be concluded that the cytotoxic nature of the extracts of *A. malaccensis* and *A. vittata* may be due to the chemical components (terpenoids). Terpenoids present may be the potential anticancer agents that induced apoptosis. The cytotoxic ability together with its antioxidant efficacy may be responsible for the observed antitumour effect. However further investigations are necessary to isolate and characterize the active principles and to find out their mechanism of action.

Cytotoxic, antitumor, antioxidant and phytochemical assays in some species of *Alpinia* Roxb.

Thesis submitted to the University of Calicut
for the award of the degree of
DOCTOR OF PHILOSOPHY IN BOTANY

By

JOSE MARY DAS

GENETICS AND PLANT BREEDING DIVISION
DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
KERALA, INDIA

JUNE 2007

REFERENCES

Abraham, S. and Koshy, M. P. 1979. Mutagenic potential of green chillies. Cytologia **44**: 221-225.

*Ahn, B. Z. and Lee, J. H. 1989. Cytotoxic and cytotoxicity – potentiating effects of the *Curcuma* root on L 1210 cell. Saengyak **20**: 223-226.

*Alan, H. 1999. Medicines from nature: Are natural products still relevant to drug discovery. Tips **20**: 196-198.

Ali, Y., Oktay, M. M. and Vahit, B. 2001. Antioxidant activity of the leaves of *Cydonia vulgaris* Turk. J. Med. Sci. **31:** 23-27.

Alkofahi, A., Batshoun, R., Owais, W. and Najib, N. 1997. Biological activity of some Jordanian medicinal plant extracts. Part II. Fitoterapia **68(2)**: 163-168.

Amer, S. M. 1965. Cytological effects of pesticides. 1. Mitotic effects of N-methyl-1-naphthyl carbamate "Sevin". Cytologia **30**: 175-181.

Anis, K. V. and Kuttan, R. 1999. Role of berberine as an adjuvant response modifier during tumour therapy in mice. Pharm. Pharmacol. Commun. **5**: 697- 701.

Arambewela, Lakshmi, S. R., Kumaratunge, A. A., Menka, O., Noel, L. and Du, L. 2005. Volatile oils of *Alpinia calcarata* grown in Srilanka. J. of Essent. Oil Res. **17**: 358-360.

Ayuso, M. J. and Garcia, M. D. 1988. Preliminary study and antimitotic activity of leaves from *Quercus rotundifolia*. Ann. De La Real Acad. Farm. **54(2)**: 315-319.

Baatout, S., Deeradji, H., Jacquet, P., Ooms, D., Michaux, A. and Mergeay, M. 2004. Effect of *Curcuma* on radiation induced apoptosis in human cancer cells. Int. J. Oncology **24**: 321-329.

Babu, B. H., Shylesh, B. S. and Padikala, J. 2002. Tumor reducing and anticarcinogenic activity of *Acanthus ilicifolius* in mice. J. Ethanopharmacol. **79**: 27-33.

Badr, A. and Ibrahim, A. C. 1987. Effect of herbicide Glean on mitosis, chromosomes and nucleic acids, in *Allium cepa* and *Vicia faba* root meristems. Cytologia **52**: 293-302.

Barber, H. N. and Callan, H. G. 1943. The effects of cold and colchicine on mitosis in the newt. Proc. Roy. Soc. *B.* **131**: 250.

Bishayee, S. and Balasubramanian, A. S. 1979. Assay of lipid peroxide formation. J. Neurochem. **18**: 909-913.

Borah, S. P. and Talukdar, J. 2002. Studies on the cytotoxic effects of castor seeds. Cytologia **67**: 235-243.

Brod, J. and Sirota, J. H. 1980. Non-protein nitrogen, urea, urate, creatine and creatinine. In: Varley, H., Gouen Lock, A. H., Beel, M. (eds). Practical Clinical Biochemistry Vol. I, 5th Edn. William Hainmann Medical Books Ltd, London. pp.478-480.

Brunetone, J. 1995. Pharmacognosy and phytochemistry. Phytotherapy Res. **18**: 435-448.

Bruzell, E. M., Morisbak, E. and Hanne, H. T. 2005 Studies on curcumin and curcuminoids. XXIX Photoinduced cytotoxicity of curcumin in selected aqueous preparations. Photochem. Photobiol. Sci. **4**: 523-530.

Buchbauer, G. 2000. The detailed analysis of essential oils leads to the understanding of their properties. Perfum. and Flavor. **25**: 64-67.

Burkhill, I. H. 1966. A Dictionary of the Economic Products of the Malay Peninsula, Vol. 11. Crown Agents for the Colonies, London, U.K. pp. 1306-1312, 1720-1721, 2117.

Calixio, J. B. 2000. Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). Braz. J. Med. Biol. Res. **33(2)**: 179-189.

Carmia, B. 2004. Dietary antioxidants and human cancer. Integrative Cancer Ther. **3(4)**: 333-341.

Carnesecchi, S., Schneider, Y., Ceraline, J., Duranton, B., Gosse, F., Seiler, N. and Raul, F. 2001. Geraniol a component of plant essential oils inhibits growth and polyamine biosynthesis in human colon cancer cells. J. Pharmacol. Exp. Ther. **298**: 197-200.

Chand, S. and Roy, S. C. 1981. Effects of herbicide 2,4-dinitrophenol on mitosis, DNA, RNA and protein synthesis in *Nigella sativa* L. Biol. Plant. **23**: 198-202.

Chauhan L. K. S., Saxena, P. N. and Gupta. S. K. 1999. Cytogenetic effects of cypermethrin and fenvalerate on the root meristem cells of *Allium cepa*. Environ. and Exp. Bot. **42**: 181-189.

Chowdhury, J. U., Yusuf, M. and Husain, M. 2003. Composition of the rhizome oil of *Alpinia calcarata.* Indian Perfum. **47**: 355-357.

Chung, W. T., Seo, H. L., Jong, D. K., Nak, S. S., Back, H., Shin, Y. L., Chang, Y. Y. and Hycon, Y. L. 2001. Effect of the extracts from *Glycyrrhiza uralensis* Fisch. on the growth characteristics of human cell lines: Anti tumor and immune activation activities. Cytotechnology **37**: 55-64.

Claudio, J. B., Anibal, G. A., Malta, Y., Dardo, A. M., Ana, G. R. and Aurelio, S. 2004. Evaluation of the genotoxicity of aqueous extracts of *Ilex paraguariensis* St. Hil. (Aquafoliaceae) using the *Allium* test. Cytologia. **69(2)**: 109-117.

Clevanger, J. F. 1928. Apparatus for determination of volatile oil. J. Amer. Pharm. Assoc. **13**: 346.

Cohen, J. J. 1993. Apoptosis. Immunology Today **14**: 126-130.

Cox, P. A. 1994. The ethnobotanical approach to drug discovery: strengths and limitations. In: Chadwick, D. J. and March J., (eds.). CIBA foundation symposium 185 – Ethnobotany and the search for new drugs. John Wiley and Sons, Chichester. pp. 25-41.

Cragg, G. M. 1997. Natural product in drug discovery and development. J. Nat Prod. **60**: 52-60.

Crowell, P. L. 1999. Prevention and therapy of cancer by dietary monoterpenes. J. Nutr. **129**: 7755-7785.

Cross, M. and Dexter, T. M. 1987. Growth factors in development transformation and tumorigenesis. Cell **64**: 271-280.

Darlington, C. D. 1942. Chromosome chemistry and gene action. Nature **149**: 66-69.

Das, N. T., Raj, S. A. and Rao, R. B. V. 1968. Cytological studies in *Vicia faba* treated with asafoetida. Cytologia **33(1)**: 100-111.

Deena, M. J. and Thoppil, J. E. 2000. Cytotoxic properties of *Lantana camara* . J. Cytol. Genet. **1**: 111-116.

Devita, V. T., Hellman, S., and Rosenberg, S. A. 1993. Cancer: Principles and Practice of Oncology, 4th ed. Philadelphia: JB Lippincott.

Deysson, G. 1968. Antibiotic substances. In: G. H. Bounes and J. F. Danielli (eds.). International Review of Cytology. Acad. Press, New York, pp. 99-148.

Doll, S. R. 1992. The lesson of life: Keynote address to the nutrition and cancer conference. Cancer Res. **44**: 2024-2029.

Dubey, N. K., Takeya, K. and Itokawa, H. 1997. Citral: A cytotoxic principle isolated from the essential oil of *Cymbopogon citratus* against p 388 leukemia cells. Curr. Sci. **73**: 22-24.

Elizabeth, K. and Rao, M. N. A. 1990. Oxygen radical scavenging activity of curcumin. Int. J. Pharm. **58**: 237-240.

Ene Obong, E. E. and Amadi, O. C. 1987. Mito depressive effects of water extracts of *Boerhaavia diffusa* and *Vernonia amygdalina* on *Allium cepa* root mitosis. Cytologia **52**: 469-474.

Fantone, J. C. and Ward, P. A. 1982. Role of oxygen derived free radicals and metabolites in leukocyte dependent inflammatory reactions. Am. J. Pathol. **107**: 349

Fiskejo, Y. 1985. The *Allium* test as a standard in environmental monitoring. Hereditas **102**: 99-112.

Forsberg, L. and Morgensten, R. 2001. Oxidative stress, human genetic variation and disease. Arch. Biochem. and Biophys. **389(1)**: 84-93.

Fransworth, N. R. 1984. The role of medicinal plant in drug development. In: Krogsgaard, L. P., Christenzen S. B. and Kofod H. (eds.). Natural Products Development. Balliare, Tindal and Cox, London. pp. 8-98.

Gowooni, P., Lee, E., Min, H. Y., Choi, H. Y., Han, A., Lee, S. and Seo, E. Y. 2002. Evaluation of cytotoxic potential of Indonesian medicinal plants in cultured human cancer cells. Nat. Prod. Sci. **8(4)**: 165-169.

Grant, W. F. 1978. Chromosomal aberration in plants as monitoring system. Environ. Health Perspect. **27**: 347-351.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. C., Wishnok, J. S. and Tannenbaum, S. R. 1982. Analysis of nitrate, nitrite and ^{15}N in biological fluids. Ann. Biochem. 126: 131-136

Groot, H. 1994. Reactive oxygen species in tissue injury. Hepato Gastro Entrology **41**: 328-332.

Grundmann, E. 1966. General Cytology. Edward Arnold Ltd., London. p.428.

Habash, M., Amran, M., Mackeen, M. M., Lajis, N. H., Kikuzaki, H., Nakatani, N., Rahman, A. A. and Ghafar, A. M. A. 2000.

Screening of Zingiberaceae extracts for antimicrobial and antioxidant activities. J. Ethanopharmacol. **72**: 403-410.

Hahn, W. C., Counter, C. M., Lundberg, A. S., Berijersbgern, R. L. and Weinberg, R. A. 1999. Creation of human tumor cells with defined genetic elements. Nature **400**: 464-468.

Han, A. R., Lee, E., Min, H. Y., Kim, H. R., Lee, S. K. and Seo, E. Y. 2003. A potential cytotoxic principle of *Zingiber casumunar*. Nat. Prod. Sci. **9(2)**: 109-111.

Heilmann, J., Brun, R., Mayr, S., Rali, T. and Sticher, O. 2001. Minor cytotoxic and antibacterial compounds from the rhizomes of *Amomum aculeatum*. Phytochemistry. **57(8)**: 1281-1285.

Herichova, A. 1973. Cytological study of the effect of isopropyl N-(3-chlorophenyl) carbamate on the division and ultra structure of meristematic cells of barely (*Hordeum sativum* L.) *Physiol. Plant.* **6**: 85-107.

Hoffman, A., Spetner, L. M. and Bruke, M. 2002. Redox regulated mechanism may account for zerumbone's ability to suppress cancer cell proliferation. Carcinogenesis **23**: 1961-1962.

Houdret, J. 2000. A Visual Dictionary of Herbs. South water, U. K. p.1.

Ikuo, N., Kuttan, R., Oku, H., Faramarz, A., Hiroko, A., and Kunio, Y. J. 1992. Supressive effects of curcumin on lipid peroxidation in rats by carbon tetrachloride or ^{60}Co irradiation. J. Clin. Biochem. Nutr. **13**: 23-29.

Itokawa, H., Morita, H., Sumitomo, T., Totsuka, N. and Takeya, 1987. Antitumor principles from *Alpinia galanga*. Planta Med. **53**: 32-33.

John Ruby, A., Kuttan, G., Dinesh, B. K., Rajasekharan, N. and Kuttan, R. 1996. Antitumor and free radical scavenging activity of synthetic curcuminoids. Int. J. Pharm. **131**: 1-7.

Johnson, I. S., Armstrong, J. G. and Gorman, M. 1963. The *Vinca* alkaloids: a new class of oncolytic agents. Cancer Res. **23**: 1390-1397.

Kabarity, A. and Mallalah, G. 1980. Mito-depressive effect of Khat extract in the meristematic region of *Allium cepa* root tip. Cytologia **45**: 733-738.

Kalpana, C. and Venugopal Padmanabhan, M. 2004. Curcumin ameliorates oxidative stress drug nicotine induced lung toxicity in Wilstater rats. The Ital. J. of Biochem. **52 (2)**: 82 – 86.

Kanjana Pothi, D., Panthog, A., Lertprasertssuki, N., Taesotikul, T., Rujjanawate, C., Kaecopint, D., Sudthayakorn, R., Choochote, W., Chaithong, U., Jitpakdi, A. and Pitasawat. B. 2004. Toxicity of crude rhizome extract of *Kaempferia galanga* L. J. Ethnopharmacol. **90**: 359-365.

Kantarjian, H. M., O'Brien, S. and Anderlini, P. 1996. Treatment of myeloginous leukemia: Current status and investigational options: Blood **87**: 3069-3081.

Katiyar, S. K., Agarwal, R. and Mukhtar, H. 1996. Inhibition of tumor promotion in SENCAR mouse skin by ethanol extract of *Zingiber officinale* rhizome. Cancer Res. **56(5)**: 1023-1030.

Kaufmann, B. P. 1956. Cytochemical studies of changes induced in cellular materials by ionising radiations. Ann. N. Y. Acad. Sci. **59**: 553.

Kawamori, T., Tanaka, T., Hirose, Y. and Mori, H. 1996. Inhibitory effects of d-limonene on the development of colonic aberrant crypt foci induced by an azoxymethane in F_{344} rats. Carcino genesis **17**: 369-372.

Khattak, S., Saeed, U. R., Hamid, U. S., Waquar, A. and Mansoor, A. 2006. Biological effects of indigenous medicinal plant *Curcuma longa* and *Alpinia galanga*. Fitoterapia **76**: 254-257.

Kihlman, B. A. 1966. The Action of Chemicals on Dividing Cells. Prentice Hall Inc., Engle Wood Cliffs, New Jersey.

Kim, W. A., Akira M., Masakao, A. B. E., Yoshio, O., Yasu, J. M., Marshall, V. W. and Hajime, O. 2005. Suppressive effects of Mioga ginger and ginger constituents on reactive oxygen and nitrogen species generation, and the expression of inducible pro inflammatory genes in macrophages. Anti oxidants and Redox signalling. **7(11,12)**: 1621 – 1629.

Kim, M., Kang, H. J., and Moon, A. 2001. Inhibition of invasion and induction of apoptosis by curcumin in H – raj – transformed MCF 10A human breast epithelial cells. Arch. of Pharmacol. Res. **24 (4)**: 354-394.

Kind, P. R. N. and King, E. J. 1954. Estimation of plasma phosphatase by determination of hydrolysed phenol with antipyrene. J. Clin. Pathol. **7**: 321-326.

Kinzler, K. W., Nilbert, M. C., Su, L. K., Vogel Stein, B., Bryan, T. M., Levy, D. B., Smith, K. J., Presising, A. C., Hedge, P., Mekechine, D., Finniear, R., Markham, A., Groffen, J., Boguski, M. S., Altschul, S. F., Horil, A., Ando, H., Miyishi, Y., Miki, Y., Nishisho, I. and Nakamura, Y. 1991. Identification of FAP locus genes from chromosome 5q21. Science **253**: 661- 670.

Kirana, C., Mc Intosh, G. H., Record, I. R. and Jones, G. P. 2003. Antitumor activity of extract of *Zingiber aromaticum* and its bioactive sesquiterpenoid zerumbone. Nutr. Cancer. **45(2)**: 218-225.

Kirtikar, K. R. and Basu, B. D. 1935. Indian Medicinal Plants. Bishen Singh Mahendra Pal Singh, Dehra Dun Vol. II, pp. 1914-1915.

Koo-Byung, S., Lee, W. C., Chang, Y. C. and Kim, C. H. 2004. Protective effects of Alpinae Oxyphallae Fructus (*Alpinia oxyphylla* Miq.) water extracts on neurons from ischemic damage and neuronal cell toxicity. Phytotherapy Res. **18(2)**: 142-148.

Kozhimizu, K., Ohigashi, H., Tokuoa, H., Kondu, A. and Yamagucha, K. 1988. Screening of edible plants against possible anti tumor promoting activity. Cancer Lett. **39**: 247-257.

Kaul Pran, N., Rao, R., Bhaskaruni, R., Kamla, S., Arun, K. B., Mallavarapu, G. R., Ramesh, S. 2005. Volatile constituents of essential oils isolated from different parts of *Alpinia calcarata*. J. Essent. Oil Res. **17**: 7-9.

Kuttan, R., Bhanumathi, P., Nirmal, K. and George, M. C. 1985. Potential anti cancer activity of turmeric. Cancer Lett. **29**: 197-202.

Lahlou, M. and Berrada, R. 2003. Composition and insecticidal activity of essential oil of three chemotypes of *Rosmarinum officinalis* L. acclimatized in Morocco. Flavour and Fragrance J. **18**: 124-127.

Lakenbrink, C., Lapczynski, S., Maiwald, B. and Engelhardt, U. H. 2000. Flavanoids and other polyphenols in consumer brews of tea and other caffeinated beverages. J. Agric. Food Chem. **48**: 2848-2852.

Lantz, R. C., Chen, G. J., Solyom, A. M., Jolad, S. D. and Timmermann, B. N. 2005. The effect of turmeric extracts on inflammatory mediator production. Phytomedicine **12**: 445-452.

Lee, C. C. and Peter, H. 2005. Cytotoxicity of plants from Malaysia and Thailand used traditionally to treat cancer. J. Ethanopharmacol. **100**: 237-243.

Lee, S. E., Shin, H. T., Hwang, H. J. and Kim, J. H. 2003. Antioxidant activity of extracts from *Alpinia kastumudai* seed. Phytother. Res. **17(9)**: 1041-1047.

Leeja, L. and Thoppil, J. E. 2004. Cytotoxic potential of extracts of *Artemisia nilgirica.* Cell and Chromosome Res. **24**: 21-26.

Leemol, D. and Girija K. 1998. Suppressive effects of cyclophosphamide-induced toxicity by *Withania somnifera* extract in mice. J. Ethanopharmacol. **82**: 209-214.

Leopold, J., Gerhard, B., Shafi, M. P. and Leela, N. K. 2003. Analysis of the essential oils of the leaves, stems, rhizomes and roots of the medicinal plant *Alpinia galanga* from Southern India. Acta Pharm. **53**: 73-81.

Levan, A. 1949. Chemically induced chromosome reactions in *Allium cepa* and *Vicia faba.* Cold Spring Harbor Symposium on Quantitative Biology **16**: 238.

Liang, V., Go, W., Debra, A. W. and Ritva, B.. 2001. Diet, nutrition and cancer prevention: Where are we going from here? J. Nutr. **131**: 3121s-3126s.

Mackeen, M. M., Ali, A. M., EI-Sharkawy, S. H., Manap, M. Y., Salleh, K. M. and Lajis, K. K. 1999. Antimicrobial and cytotoxic properties of some Malaysian traditional vegetables (ulam). Int. J. Pharmacognosy **35(3)**: 174-178.

Mallavarapu, G. R., Laxmi, R., Sreenivasayer, R., Dimri, B. P., Rao, B. R. R., Kaul, N. N. and Bhattacharya, A. K. 2002. Composition of volatile oils of *Alpinia galanga* rhizomes and leaves from India. J. Essential Oil Res. **14(6)**: 397-399.

Mates, J. M., Franscisca, M. and Sanchez-Jimenez, F. M. 2000. Role of reactive oxygen species in apoptosis: Implications for cancer therapy. Int. J. of Biochem. Cell Biol. **32**: 157-170.

Matsuda, H., Pongpiri Yadacha, Y., Morikawa, T., Ochi, M. and Yoshikawa, M. 2003. Gastro protective effect of phenyl propanoids from the rhizomes of *Alpinia galanga* in rats: Structural requirements and mode of action. Eur. J. Pharmacol. **471**: 59-67.

Matsuda, H., Shin, A., Tomoko, K., Toshio, M. and Masayuki Y. 2006. Inhibitors from the rhizomes of *Alpinia officinarum* on production of nitric oxide in lipopolysaccharide activated macrophages and the structural requirements of diarylheptanoids for the activity. Bioorg. Med. Chem. **14**: 138-142.

Matthes, H. W. D., Luu, B. and Ourisson, G. 1980. Cytotoxic components of *Zingiber zerumbet, Curcuma zeodaria* and *C. domestica*. Phytochemistry **19(12)**: 2643-2650.

Mc Cord, J. M. 1974. Free radicals and inflammation: Protection of synovial fluid by superoxide dismutase. Science **185**: 529-531.

Mc Cord, J. M. and Fridovich, I. 1969. Super oxide dismutase an enzymatic function for erythrocuprein. J. Biol. Chem. **244**: 6049-6055.

McGuire, W. P., Hoskins, W. J. and Brady, M. F. 1996. Cyclophosphamide and cisplatin compared with paclitaxel and cisplatin in patients with stage III and IV ovarian cancer. N. Engl. J. Med. **334**: 1-6.

Mehta, K., Pantazis, P., Mc Queen, T. and Aggarwal, B. B. 1997. Anti proliferative effect of curcumin (diferloyl methane) against human breast tumor cell lines. Anticancer Drugs **8(5)** 470-481.

Menon G. L., Ramadasan K. and Girija, K. 1999. Anti metastatic activity of *Curcuma catechu*. Cancer Lett. **141**: 159-165.

Mercykutty, V. C. and Stephen, J. 1980. Adriamycin induced genetic toxicity as demonstrated by *Allium* test. Cytologia **45**: 769-777.

Minija, J., Tajo, A. and Thoppil, J. E. 1999. Mitoclastic properties of *Mentha rotundifolia*. J. Cytol. Genet. **34(2)**: 169-171.

Missiry-El, M. A. and Abd El-Aziz, A. F. 2000. Influence of melatonin on proliferation and anti oxidant system in Ehrlich ascites carcinoma cells. Cancer Lett. **151**: 119-125.

Mohandas, T. and Grant, W. F. 1972. Cytogenetic effect of 2,4-D and amitrol in relation to nuclear volume and DNA content in some higher plants. Can. J. Genet. Cytol. **14**: 773- 778.

Moon, Y., Heo, S., Sohn, J. and William, W. A. 2001. Antigenotoxicity of galangin as a cancer chemo preventive agent candidate. Mutat. Res. **488**: 135-150.

Morse, M. A. and Stoner, G. D. 1993. Cancer prevention principles and prospects. Carcinogenesis **14**: 1737-1746.

Moteki, H., Hiroshige, H., Yayoi, Y., Hirotaka, K., Kunio, M. and Takashi, K. 2002. Specific induction of apoptosis by 1,8-cineole in two human leukaemia cells lines, but not in a human stomach cancer cell line. Oncology Reports **9**: 757-760.

Murakami, A., Ryohei, H., Takuji, T., Kuan, K., Hajime, O. and Ratu, S. 2003. Suppression of dextran sodium sulfate induced colitis in mice by zerumbone, a sub tropical ginger sesquiterpene and nimesulide, separately and in combination. Biochem. Pharmacol. **66**: 1253-1261.

Murakami, A., Takuji, T., Ji-Yoon, H., Young – Joou, S., Ha Won, K., Kyuichi, K. and Yoshimasa, N. 2004. Zerumbone, a sesquiterpene in sub tropical ginger suppresses skin tumor initiation and promotion stages on ICR mice. Int. J. Cancer **110**: 481-490.

Murakami, A., Kozhimizu, K. and Ohigashi, H. 1998. Chemo prevention with food phytochemicals, screening, rodent studies and action mechanisms. J. Med. Food **1**: 29-37.

Murakami, A., Ohigashi, H. and Kozhimizu, K. 1994. Possible antitumor promoting properties of traditional Thai food items and source of their active constituents. Asian Pac. J. Clin. Nutr. **3**: 185-191.

Murakami, A., Ohigashi, H. and Kozhimizu, K. 1999. Chemo prevention, insights into biological mechanism and promising food factors. Food Rev. Int. **15**: 335-395.

Murakami, A., Takahashi, D., Kinoshita, T., Kozhimizu, K., Kim, H. W., Yoshihiro, A., Nakamura, Y., Jiwajinda, S., Terao, J. and Ohigashi, H. 2002. Zerumbone, a south east Asian ginger sesquiterpene, markedly suppresses free radical generation, pro inflammatory protein production and cancer cell proliferation accompanied by apoptosis: The alpha, beta - unsaturated carbonyl group is a pre-requisite. Carcinogenesis **23(5)**: 795-802.

Muraoka, O., Manabu, F., Genzoh, T., Michinori, K., Toshie, M., Iwao, T. and Masayuki, Y. 2001. Absolute stereo structures of novel norcadinane and trinoreudesmane type sesquiterpenes with nitric oxide production inhibitory activity from *Alpinia oxyphylla*. Bioorg. Med. Chem. Lett. **11**: 2217-2220.

Nagpal, A. and Grover, I. S. 1994. Genotoxic evalution of systemic pesticides in *Allium cepa*. Nucleus **37(3)**: 99-105.

Nakamura, Y., Chiho, Y., Akiramurakami, H. O., Toshihiko, O. and Koji, U. 2004. Zerumbone, a tropical ginger sesquiterpene, activates phase II drug metabolizing enzymes. FEBS Letters **572**: 245-250.

Nan-Peng, H. Y., Zhao-Jia, Y. and Feng-Ying, Z. Y. 2004. Chemical composition of the essential oils of two *Alpinia* species from Hainan Island, China. Biosci. **59(3/4)**: 157-160.

Narisawa, T., Fukaura, Y. and Asakawa, Y. 1992. Inhibitory effect of cryptoporic acid E on colon carcinogenesis in rats and mice Jap. J. Cancer Res. **83**: 830-834.

Nguyen, X. D., Trinh, D. C., Do, D. R. and Leclercq, P. A.1994a. Chemical composition of the leaf oil of *Alpinia chinensis* from Vietnam. J. Essential Oil Res. **6(1)**: 91-92.

Nguyen, X. D., Trinh, D. C., Do, D. R. and Leclercq, P. A. 1994b. Chemical composition of the leaf oil of *Alpinia breviligulata* from Vietnam. J. Essential Oil Res. **6(2)**: 181-182.

Nguyen, X. D., Trinh, D. C., Do, D. R. and Leclercq, P. A. 1994c. Constituents of the seed and fruit peel oils of *Alpinia breviligulata*. J. Essential Oil Res. **6(3)**: 295-297.

Nguyen, X. D., Trinh, D. C., Do, D. R. and Leclercq, P. A. 1994d. Constituents of the flower oil of *Alpinia speciosa* from Vietnam. J. Essential Oil Res. **6(4)**: 433-434.

Nguyen, X. D., Trinh, D. C., Do, D. R. and Leclercq, P. A. 1994e. Chemical composition of the flower oil of *Alpinia chinensis* from Vietnam. J. Essential Oil Res. **6(6)**: 637-638.

Odukoya, A., Olukemi, O., Sofidiya, M., Olywatoyin, A., Austin, O., Lawal, B. M. and Tade, I. O. 2005. Antioxidant activity of Nigerian dietary species. EJEAF Chem. **4(6)**: 1086-1093.

Ohkawa, H., Ohishi, N., Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem. **95**: 351.

Ojima, I., Chakravarty, S., Inou, T., Lin, S., He, L., Horwitz, S. B. S., Kuduk, S. D. and Danish Efksy, J. 1999. A common pharmacophore for cytotoxic natural products that stabilize microtubules. *Proc.* Natl. Acad. Sci. USA. 4256-4261.

Padma, S. V., Vandana, T. L., Warjeet, S. and Swapana, N. 2006. Antioxidant properties of some exclusive species of Zingiberaceae family of Manipur. EJEAF. Chem. **5(2)**: 1318-1322.

Pal, S., Choudhuri, T., Chattopadhyay, S., Bhattacharya, A., Datta, G. K., Das, T. and Sa, G. 2001. Mechanism of curcumin induced apoptosis of Ehrlich's Ascites Carcinoma cells. Biochem. Biophys. Res. Commun. **288**: 658-665.

Panda, B. B. and Sahu, U. K. 1985. Induction of abnormal spindle function and cytokinesis inhibition in mitotic cells of *Allium cepa* by the organophosphate insecticide 'Fensulphothion'. Cytobios **42**: 147-155.

Panizzi, L., Flamini, G., Cioni, P. L. and Morelli, I. 1993. Composition and antimicrobial properties of essential oils of four mediteranian Lamiaceae. J. Ethanopharmacol. **39**: 167-170.

Pascual, M., Garcia, M. D., Toiro, M. U. and Suen, T. 1987. Antimitoitic activity of aqueous extracts and phenol fraction of *Erica andevalensis*. Farmaco-Ed-Prat. **42**: 111-115.

Patnaik, S., Saran, B. L. and Patnaik, S. N. 1984. Effect of Zarda (processed tobacco leaf) extract on the chromosomes of *Allium cepa* L. Cytologia **49**: 807-814.

Player, T. 1982. Free Radicals and Cancer. In: Mc Brein, D. C. H. and Slater, T. F. (eds.). Academic Press, London, pp.173-195.

Pooter, H. L., Aboutabl, E. A. and El Shabrawy, A. O. 1995. Chemical composition and antimicrobial activity of essential oil of leaf, stem, and rhizome of *Alpinia speciosa* grown in Egypt. Flavour Fragrance J. **10(2)**: 63-67.

Powel , R. G., Weisleder, D. and Smith, C. R. 1970. Structures of harringtonine, isoharringtonine and homoharringtonine. Tetrahedron Lett. **11**: 815-818.

Prasad, A. B. 1974. Production of multi polar spindles in canary grass following irradiation of dry seeds. J. Cytol. Genet. **788**: 184-187.

Preethi, K. C., Girija, K. and Ramadasan, K. 2005. Antitumor activity of *Ruta graveolens* extract and its possible mechanism of action. Amala Cancer Bull. **25**: 121 – 129.

Pritchard, A. J. and Court, R. D. 1968. The cytological effect of mimosin. Cytologia **33(1)**: 73-77.

Putul, M., Sunit, C. and Pritha, B. 2000 Neovascularization offers a new perspective to glutamine related therapy. Indian J. Exp. Biol. **38**: 88-90.

Qureshi, S., Shah, A. H., Ahmed, M. M., Rafatullah, S., Bibi, F. and Al-Bekairi, A. M. 1994. Effect of *Alpinia galanga* treatment on cytological and biochemical changes induced by cyclophosphamide in mice. Int. J. Pharm. **32(2)**: 171-177.

Raina, V. K., Srivastava, S. K. and Syama Sunder, K. V. 2002. The essential oil of *Alpinia galanga* from lower Himalayan region of India. Flavor Fragrance J. **17(5)**: 358-360.

Raj, S. A. and Rao,1972. Cytological studies in *Vicia faba* L. treated with lathyrogens. Cytologia **37(2)**: 245-246.

Ramsewak, R. S., Dewitt, D. L. and Nair, M. G. 2000. Cytotoxicity, antioxidant and anti-inflammatory activities of curcumins I-III from *Curcuma longa*. Phytomedicine **7(4)**: 303-308.

Ravishankar, M. N., Shrivastava, N., Padh, H. and Rajani, M. 2002. Evaluation of antioxidant activity of root bark of *Hemidesmus indicus* R. Br. (Anantmul). Phytomedicine **9**: 153 – 160.

Ray, M. and Barman, A. 1987. Effect of industrial waste on root meristematic cells of *Allium sativum*. Phytomorphology **37**: 359-364

Reitman, S. and Frankel, A. S. A. 1957. Colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. Am. J. Clin. Pathol. **28**: 56-63.

Rogers, A. E., Ziesel, S. H. and Croopmah, J. 1993. Diet and Carcinogenesis. Carcinogenesis **14**: 2205-2217.

Roy, J. 1998. Karyomorphometrical analysis and exploration of major essential oil constituents in Zingiberaceae. Ph. D. Thesis. Mahatma Gandhi University, Kottayam, Kerala.

Roy, J., Tessy, J. and Jose, J. 2001. Volatile essential oil constituents of *Alpinia smithiae*. Rev. Biol. Trop. **49(2)**: 509-512.

Ryeong, H. E., Park, S. and Yang, C. 2003. 7,8–Dihydroxyflavanone as an inhibitor for Jun-Fos-DNA complex formation and its cytotoxic effect on cultured human cancer cells. Nat. Prod. Res. **17(6)**: 431-436.

Sadaquat, A., Sotheeswaran, S., Tuiwawa, M. and Smith, R. M. 2002. Comparison of the composition of the essential oil of *Alpinia* and *Hedychium* species. J.Essetial Oil Res. **14(6)**: 409-411.

Saetung, A., Itharat, A., Dechsukum, C., Keawpradub, K., Wattana piromasakul, C. and Ratnasuwan, P. 2005. Cytotoxic activity of Thai medicinal plants for cancer treatment. Songklanakarin J. Sci. Technol. **27** (suple.2): 469-478.

Sang, H. P., Kim, G. J., Jeong, H. S. and Yum, S. K. 1996. Ar. Turmerone and beta-atlantone induce internucleosomal DNA fragmentation associated with programmed cell death in human myeloid leukemia HL-60 cells. Arch. Pharmacol. Res. **19(2)**: 91-94.

Sato, S. G. and Tanaka, R. 1972. Effects of gallic acid derivatives on mitotic cells. La Kromosoma. **89**: 2718.

Sax, K. 1940. Analysis of X-Ray induced chromosomal aberration in *Tradescantia*. Genetics. 25: 41-68.

Schneiderman, M. H., Dewey, W. C. and Highfield, D. P. 1971. Inhibition of DNA synthesis in synchronized Chinese hamster cell treated in GI with cycloheximide. Exp. Cell Res. **67**: 147-155.

Selvam, R., Subramanian, L., Gayathri, R. and Angayakanni, N. 1995. The antioxidant activity of turmeric (*Curcuma longa*). J. Ethnopharmacol. **47**: 59-67.

Shackelford, R. and Kaufmann, W. 2000. Oxidative stress and cell cycle: Check point function. Free Radical Biol. Med. **28**: 1387-1404.

Sharma, A. K. and Sharma, A. 1980. Chromosome Techniques. Theory and Practice 3rd ed. Butterworths and Co. Ltd, London.

Sharma, C. B. S. R. and Sahu, R. K. 1977. Cytogenetic hazards from agricultural chemicals. A preliminary study of the responses of root meristems to exotoxin from *Bacillus thuringiensis* a constituent of a microbial insecticide, 'Thuricide'. Mutat. Res. **46**: 19-26.

Shehab, A. S. 1979. Cytological effect of medicinal plants in Qatar. I. Mitotic effect of water extract of *Pulicaria crispa* on *Allium cepa*. Cytologia **44(3)**: 607.

Shehab, A. S. 1980. Cytological effect of medicinal plants in Qatar II. Mitotic effect of water extract of *Teucrium pilosum* on *Allium cepa*. Cytologia **45**: 57-64.

Shehab, A. S. and Adams, Z. M. 1983. Cytological effect of medicinal plants in Qatar III. Mitotic effect of water extract of *Anastatica heirochuntica* L. on *Allium cepa*. Cytologia **48(2)**: 343-348.

Shylesh, B. S. and Padikala, J. 2000. *In vitro* cytotoxic and anti tumor property of *Emilia sonchifolia* (L.) DC in mice. J. Ethano pharmacol. **73**: 495-500.

Sinha, R. K., Khokan, M. and Sinha, S. 1996. Sarasapogenin, a steroid drug for mitotic chromosome preparation in plant cells. Indian J. Exp. Biol. **34(4)**: 366-367.

Sirat, H. M. and Nordin, A. B. 1995. Chemical composition of the rhizome oil of *Alpinia conchigera*. J. Essential Oil Res. **7(2)**: 195-197.

Soliman, I. M. 2001. Genotoxicity testing of neem plant (*Azadirachta indica*) using *Allium cepa* chromosome aberration assay. Online J. Biol. Sci. **1(ii)**: 1021-1027.

Somasekhar, R. K. and Gowda, M. T. G. 1984. Effects of fungicide vitavax on *Allium cepa*. Cytologia **49**: 177-181.

Sreejayan, and Rao, M. N. A. 1996. Nitric oxide scavenging by curcuminoids. J. Pharm. Pharmacol. **49**: 105-107.

Srimal, R. C. and Dhavan, B. N. 1973. Pharmacology of curcumin, a non-steroid anti inflammatory agent. J. Pharmacol. **25**: 447-452

Stahelin, H. 1973 Activity of a new glycosidic lignan derivative related to podophyllotoxin in experimental tumours. Eur. J. Cancer **9**: 215-221.

Stephen, J. 1979. Cytological causes of spontaneous fruit abortion in *Haemanthus katherinae* Baker. Cytologia **44**: 805-812.

Stich, H. F. P., Lam, L. W., Korapatnik, J. and San, R. H. C. 1975. The search for relevant short term bioassay for chemical carcinogens. Can. J. Genet Cytol. **17**: 471-492.

Sudhakar, R., Ninge Gaida, K. N. and Venu, G. 2001. Mitotic abnormalities induced by silk dyeing industry effluents. Cytologia **66**: 236-239.

Surh Y. J. 1999. Molecular mechanism of chemopreventive effects of selected dietary and medicinal phenolic substances Mutat. Res. **428**: 305-327.

Sylvestre, M., Leganlt, J., Dufour, D. and Pichette, A. 2005. Chemical composition and anticancer activity of leaf essential oil of *Myrica gale* L. Phytomedicine **12(4)**: 299-304.

Sylvia, R., Lima, M., Valdir, F., Veiga, J., Herrock, B., Christs Angelo, C. P. and Patricoa, D. F. 2003. *In vivo* and *in vitro* studies on the anti cancer activity of *Copaifera multijuga* Hayne and its fractions. Phytotherapy Res. **17(9)**: 1048-1053.

Syu, W., Shen C. C., Don, M., Ou-Jun, C., Lee, G. H. and Sun-Chang, M. 1998. Cytotoxicity of curcuminoids and some novel compounds from *Curcuma zedoaria*. J. Nat. Prod. **61(12)**: 1531-1534.

Tajo, A. and Thoppil, J. E. 1998. Cytotoxic properties of *Ocimum americanum.* Bull. Pure. Appl. Sci. **17(2)**: 49-53.

Takada, Y., Akira, M. and Bharat, B. A. 2005. Zerumbone abolishes NFB and IB kinase activation leading to suppression of antiapoptotic and metastatic gene expression – upregulation of apoptosis and down regulation of invasion. Oncogene **24**: 6957-6969.

Talukdar, R., Sharma, A. and Geetha, T. 1985. Cytotoxic effects of dimethoate. Nucleus **28(3):** 243-259.

Tanaka, T., Masahito, S., Hiroyuki, K., Shin-Ichiro, Y., Yoshiaki, T., Akira, M., Ratu, S., Daisuke, T., Keiko, Y., Korchi, K., Hajime, O. and Hideki, M. 2001. Chemoprevention of azoxymethane-induced rat abberant crypt foci by dietary zerumbone isolated from *Zingiber zerumbet*. Life Sci. **69**: 1935-1945.

Tezuka, Y., Gewali, M. B., Mohammad, S. A., Banskota, A. H. and Kadota, S. 2001. Eleven novel diarylheptanoids and two unusual diarylheptanoid derivatives from the seeds of *Alpinia blepharocalyx.* J. Nat. Prod. **64(2)**: 208-213.

Thippeswamy, G. and Bharathi, P. S. 2006. *Curcuma aromatica* extract induces apoptosis and inhibits angiogenesis in Ehirlich Ascites Tumor cells *in vivo*. My Science **1(1):** 79-92.

Thomas, M. J. 2000. The role of free radicals and antioxidants. J. Nutr. **16(7/8):** 716-718.

Toshihiko, H. 1987. Cytotoxicity of carbol camphor on cultured mammalian cells. Shigaku **75**: 985-996.

Troll, W. and Wiesner, R. 1985. The role of oxygen radicals as a possible mechanism of tumor promotion. Annu. Rev. Pharmacol. Toxicol. **25**: 509-528.

Veronica, E. P. V., Marjori, L. C., Rosangela, D. O. T. and Mario, S. M. 2001. *Averrhoa carambola, Syzygium cumini* and *Cissus sicyoides.* Medicinal herbal tea effects on vegetal and animal test systems. Acta Sci. **23(2)**: 593-598.

Vimala, S., Norhanom, A. W. and Yadav., M. 1999. Antitumor promotor in Malaysian ginger rhizobia used in traditional medicine. Br. J. Cancer **80(1/2):** 110-116.

Wani, M. C., Taylor, H. L. and Wall, M. E. 1971. Plant antitumor agents. The Isolation and structure of taxol, a novel antileukemic and antitumor agent from *Taxus brevifolia.* J. Am. Chem. Soc. **93**: 2325-2327.

Wattenberg, L. W. 1985. Chemoprevention of cancer. Cancer. Res. **45**: 1-8.

Wattenberg, L. W. 1992. Inhibition of carcinogenesis by minor dietary constituents. Cancer Res. **52**: 2085-2091.

Williams, G. O. and Omoh, L. E. 1996. Mitotic effects of aqueous leaf extracts of *Cymbopogon citratus* in *Allium cepa* root tips. Cytobios **87**:161-168.

Wong Young, S. and Woo Young, P. 1993. Effect of plant growth regulators on mitotic chromosomes. Nucleus **36**: 109-113.

Wong, K. C., Lee, B. C., Lam, N. F. and Ibrahim, P. 2005. Essential oils of the rhizome of *Alpinia conchigera* and *Alpinia latilabris.* Flavour Fragrance J. **20(4):** 431-433.

Yadav, N. P., Zhihua, L. and Mohammed, M. R. 2003. A diaryl heptanoid from lesser galangal (*Alpinia officinarum*) inhibits proinflammatory mediators via inhibition of mitogen- activated protein kinase, p.44/42, and transcription factor nuclear factor-KB. J. Pharm. Exp. Ther. (JPET) **305**: 925-931.

Yadav, S., Amit, V. and Roopam, S. (2001). Cytotoxic potentiality of tomato fruit (*Lycopersicum esculentum*) extract on the root meristem of *Allium cepa*. Adv. Plant Sci. **14(1):** 237-242.

Yamada, Y., Kikuzaki, H. and Nakatani, N. 1992. Identification of antimicrobial gingerols from ginger (*Zingiber officinale* Roscoe) J. Antibact. Antifung. Agents **20**: 309-311.

Yuan, J., Gu, Z. L., Chou, W. H. and Kwok, C. Y. 1999. Elemene induces apoptosis and regulates expression of bcl-2 protein in human leukemia K_{562} cells. Acta Pharmacol. Sin. **20 (2)**: 103-106.

Zaeoung, S., Anuchit, P. and Niwat, K. 2005. Cytotoxic and free radical scavenging activities of zingiberaceous rhizomes. Songklanakarin J. Sci. Technol. **27(4)**: 799-812.

Zhang, L., Liu, W. S., Han, B. Q., Peng, Y. F. and Wang, D. F. 2006. Anti tumor activities of D-glucosamine and its derivatives. J. Zhejiang University Sci. **7(8)**: 608-614.

Zhao, Q., Hao, X. J., Chen, Y. Z. and Zou, C. 1995. Studies on diterpenoid constituents of *Hedychium forestii* and their cytotoxic activity. Acta Pharm. Sin. **30(2)**: 119-122.

Zhou, B. N., Baj, N. J., Glass, T. E., Malone, S., Werkhoven, M. C. M., Troon, F., Van, D., Wisse, J. H. and Kingston, D. G. I. 1997. Bioactive labdane diterpenoids from *Renealmia alpinia* collected in the Surinam rain forest. J. Nat. Prod. **60(12)**: 1287-1293.

NB5625