Functional Properties of Bioactive Molecules from Mango Ginger and its Application in Food

A Thesis Submitted to
The University of Mysore

For the award of the degree of
Doctor of Philosophy in
Biotechnology

By

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DECLARATION

I here by declare that, the thesis entitled **"Functional properties of bioactive molecules from mango ginger rhizome and its applications in food"** submitted to the University of Mysore, Mysore, for the award of the Degree of **Doctor of Philosophy** in the Faculty of Biotechnology is the result of work carried out by me under the guidance of **Dr. S.M. Aradhya,** Scientist, Department of Fruit and Vegetable Technology, Central Food Technological Research Institute, Mysore, during the period May 2003-August 2007.

I further declare that, the results of this thesis has not been submitted by me for award of any other degree/diploma to this or any other University

(Policegoudra R.S.)

Date:

Place: Mysore

Dr. S. M. Aradhya

Scientist, Research supervisor
Department of Fruit and Vegetable Technology

CERTIFICATE

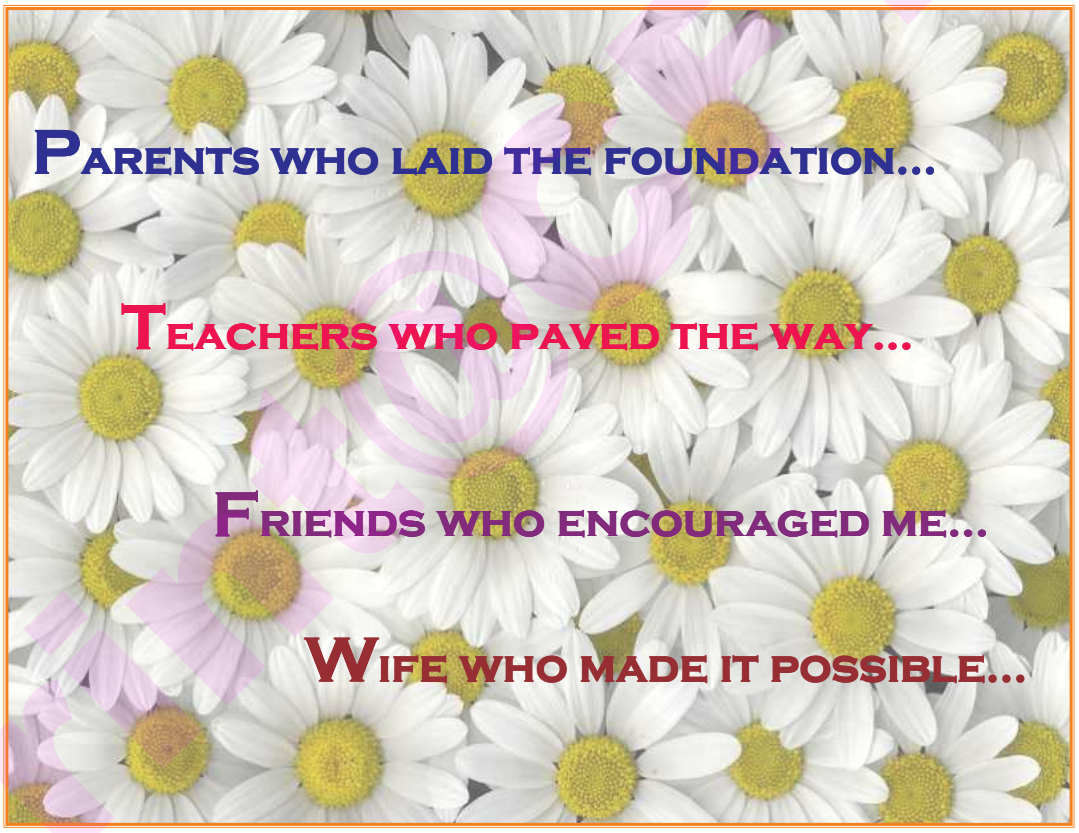
This is to certify that, the thesis entitled **"Functional properties of bioactive molecules from mango ginger rhizome and its applications in food"** submitted to the University of Mysore, Mysore, for the award of the Degree of **Doctor of Philosophy** in the Faculty of Biotechnology by **Mr. Policegoudra R.S.,** is the result of work carried out by him in the Department of Fruit and Vegetable Technology, Central Food Technological Research Institute, Mysore, under my guidance during the period May 2003 - August 2007.

(S.M. Aradhya)

Date:

Place: Mysore

Dedicated to my beloved…



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- Policegoudra R.S.

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Abbreviations

°C	Degree Celsius
µl	Microliter
µM	Micromolar
2D-HMQCT	Two dimensional heteronuclear multiple quantum coherence transfer spectroscopy
A-549	Human small cell lung carcinoma cell line
BHA	Butylated hydroxy anisole
BSA	Bovine serum albumin
cfu	Colony forming units
CTC_{50}	Cytotoxicity (kills 50% cells)
DPPH	1,1-Diphenyl-2-picrylhydrazyl
ESI	Electrospray ionization
g	Gram
Hep-2	Human larynx epithelial cancer cell line
HPLC	High performance liquid chromatography
IC_{50}	Inhibitory concentration (inhibits 50%)
Kg	Kilogram
LC	Liquid chromatography
MIC_{50}	Minimum inhibitory concentration (inhibits 50%)
min	Minutes
ml	Milliliter
mm	Millimeter
MS	Mass spectrometry
MTT	3-[4, 5-dimethylthiazole-2-yl]-2, 5-diphenyltetrazolium bromide
N	Normality
NMR	Nuclear magnetic resonance spectroscopy
pH	Negative logarithm of hydrogen ion concentation
SRB	Sulphorhodamine B
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
UV	Ultraviolet
Vero	Normal African green monkey kidney cell line
NBT	Nitroblue tetrazolium
NADH	Nicotinamide adenine dinucleotide (reduced form)
PMS	Phenazine methosulphate
OD	Optical density
v/v	Volume/volume
w/w	Weight/weight
EC_{50}	Effective concentration (50% effect)

Contents

General Introduction and Review of Literature

There are two ways to see life:
One is as though everything is a miracle.
The other as though nothing is a miracle.
-Albert Einstein

General Introduction

Eversince the prehistoric era, plants have been the basis for nearly all medicinal therapy until synthetic drugs were developed in the nineteenth century. In India, plant-derived extracts have been used in *Ayurveda* from the time immemorial, which indicate its safety and totalitarian approach. The World Health Organization [WHO] estimated that 80 % of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components. Plant based bioactive compounds are gaining attention due to their multi-functional, therapeutic properties and for overall safety.

There are increasing evidences to suggest that the free radicals induce oxidative damage to lipids, proteins and nucleic acids, which eventually cause atherosclerosis, ageing, cancer, diabetes, inflammation, AIDS and several degenerative diseases in humans [Halliwell, 1994; Maxwell, 1997]. Antioxidants that are rich in spices and other plant extracts serve as sources of nutraceuticals that alleviate the oxidative stress and therefore prevent or reduce the risk of degenerative diseases [Kitts *et al.,* 2000 and Fu and Ji, 2003]. Antioxidants from natural sources are preferred to use in food or medicine to replace synthetic ones, which are being restricted due to their carcinogenicity [Velioglu, *et al.,* 1998]. Hippocrate's famous dictum *"Let food be thy medicine and medicine be thy food"* has renewed interest in this context. Spices, which are essential ingredients of food, not only impart flavor but contribute copiously an array of bioactive molecules that prevents chronic diseases and promote health.

Among Spices, turmeric and ginger have been extensively studied. More number of both edible and wild spices are yet to be explored. Mango ginger is one such unique spice that remained untapped. Hence, a detail investigation on functional properties of bioactive molecules from mango ginger and its food applications was undertaken.

REVIEW OF LITERATURE

Plants contain a broad range of bioactive compounds such as lipids, carbohydrates, phenolics, terpenoids, carotenoids, anthocyanins, flavors and fragrances. Plant extracts with these bioactive molecules are widely used in the food, pharmaceutical and cosmetics industries. Extraction techniques have been widely investigated to obtain such valuable natural compounds from plants for commercialization [Wang and Weller, 2006]. Almost half of the best-selling pharmaceuticals are natural or closely related to natural products, which tell the tremendous potential for the identification of medicinally important bioactive compounds from these sources. It has been estimated that only a small percentage of compounds from biological sources have been investigated for this purpose [Strege, 1999].

There is increasing interest both in the industry and in scientific research for spices and aromatic herbs because of their strong antimicrobial and antioxidant properties, which exceed many currently used synthetic antioxidants. Spices and herbs render their antioxidant components as preservative agents in food [Calucci *et al.,* 2003]. These properties are due to many substances, including vitamins, flavonoids, terpenoids, carotenoids, phytoestrogens, minerals, and others.

Extensive review of literature has indicated an accumulation of voluminous literature on bioactive compounds and their functional properties in various spices, fruits, vegetables, tubers and herbs. The present review encompasses an up to-date published literature from various sources on different aspects mango ginger. However, relevant earlier works from ginger, turmeric and other plant sources have been copiously cited for critical evaluation and meaningful conclusion of results.

Importance of Zingiberaceae

The family Zingiberaceae, commonly known as ginger family, is one of the economically important families [Aminul, 2004] and also the largest family in the order Zingiberales with about 53 genera and more than 1200 species [Kress *et al.,* 2002]. The members of Zingiberaceae are distributed mostly in tropical and subtropical areas with the centre of distribution in South-East Asia. The genus *Curcuma* L. belongs to the family Zingiberaceae, which is composed of 70-80 species of rhizomatous annual or perennial herbs [Purseglove, 1974: Aminul, 2004]. The genus is originated in the Indo-Malayan

region, and widely distributed in the tropics of Asia to Africa and Australia [Sasikumar, 2005]. The plants belong to the genus *Curcuma* are well known for their multiple uses as spices, medicines, cosmetics, dyes, flavourings, starch and ornamentals. The extensive work has been carried out on *Curcuma longa* and *Zingber offcinale*. But, *Curcuma amada* is an untapped medicinal plant in the ginger family.

The present genus name *Curcuma* was coined by Linnaeus in his *Species Plantarum* in 1753. Probably the word derives from the Arabic word *'Kurkum'* that means yellow colour [Salvi *et al.,* 2000; Shirgurkar, *et al.,* 2001]. *Curcuma amada* Roxb., commonly known as mango ginger, is a member of *Zingiberaceae.* The taxonomical hierarchy of mango ginger is as follows;

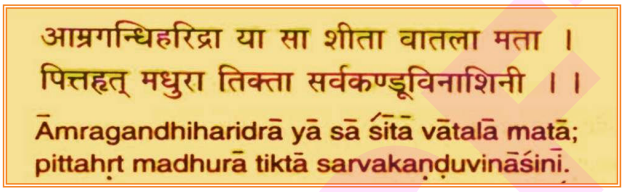
Kingdom: Plantae
Super division: Spermatophyta
Division: Magnoliophyta
Class: Monocotyledonae
Order: Zingiberales
Family: Zingiberaceae
Genus: *Curcuma*
Species: *C. amada* Roxb.

C. amada is a unique spice having morphological resemblance with ginger [*Zingiber officinale*] but imparts raw mango [*Mangifera indica*] flavour.

Mango Ginger

- Since time immemorial....

Medicinal treatise of *Ayurveda* datesback to pre-historic Vedic era, which is the anceient testimony for use of plants as medicine. Accordingly, the medicinal properties of mango ginger are depicted in the following Sankrit shloka;


आम्रगन्धिहरिद्रा या सा शीता वातला मता ।
पित्तहृत् मधुरा तिक्ता सर्वकण्डूविनाशिनी ।।
Āmragandhiharidrā yā sā śītā vātalā matā;
pittahṛt madhurā tiktā sarvakaṇḍūvināśinī.

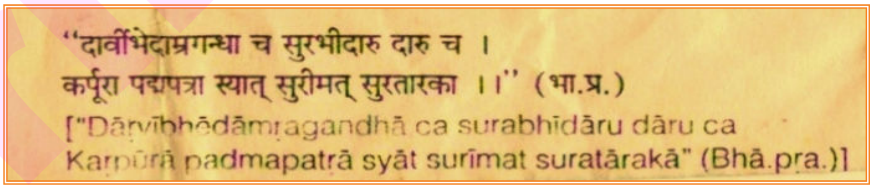
*Raw mango flavored ginger has cooling effect to the body. It aggravates **Vata***;*

*It also pacifies deranged **Pitta****, cures all types of itching and skin diseases.*

* *Vata- an Ayurvedic principle necessary to co-ordinate the function of the nervous system.*

***Pitta- an Ayurvedic principle which uses bile, to direct digestion and hence metabolism into the venous system.*

The following Sankrit shloka explains the synonyms of mango ginger;


"दार्वीभेदाम्रगन्धा च सुरभीदारु दारु च ।
कर्पूरा पद्मपत्रा स्यात् सुरीमत् सुरतारका ।।" (भा.प्र.)
["Dārvībhēdāmragandhā ca surabhīdāru dāru ca
Karpūrā padmapatrā syāt surīmat suratārakā" (Bhā.pra.)]

Darvibheda, Amragandha, Surabhidaru,

Karpura padmapatra, Surimat and Surataraka.

Table R1: Vernacular names of mango ginger

State	Language	Vernacular Name
West Bengal	Bengali	Amada
Karnataka	Kannada	Mavinakayi shunti
Uttar Pradesh	Hindi	Ama-haldi
Kerala	Malayalam	Mannayinci
Tamilnad	Tamil	Mangai inji
Andra Pradesh	Telugu	Mamidi allamu

The vernacular names of mango ginger that prevail in different parts of India are given in **table R1.** Mango ginger is also distributed in China, Bangladesh, Myanmar, Thailand, Burma, Japan and Australia.

Fig. R1: Mango ginger plant

Mango ginger is a perennial, rhizomatous, aromatic herb belongs to the family Zingiberaceae. The plant grows to a height of one meter **[Fig. R1]**. The leaves are long, oblong, lanceolate, radical, sheathed, petiolate, in tufts. Each plant bears 5 to 6 pairs of leaves.

Mango ginger rhizomes **[Fig. R2]** are fleshy, buff coloured, 5-10 cm long, 2-5 cm in diameter, demarcated into nodes and internodes. At the nodes scaly leaves are arranged circularly giving the appearance of growth rings with scars on the surface. The rhizomes are branched, branching is sympodial. The rhizomes emulate raw mango flavour and taste pungent. When fully matured, each plant yields 1.5-2 kg of rhizomes.

Fig. R2: Mango ginger rhizomes

Uses of mango ginger

The main use of mango ginger rhizome is in the manufacture of pickles, as a source of raw mango flavour in culinary preparations. The extract of mango ginger is used as appetizer, alexteric, antipyretic, aphrodisiac, etc **[Table R2]** in Ayurveda, the oldest system of medicine in India. According to Unani, Greek system of medicine, it is diuretic, emollient, expectorant, antipyretic, appetizer, etc. [CSIR, 1950; Kirtikar and Basu, 1984; Warrier, *et al.,* 1994; Hussain, *et al.,* 1992].

Table R2: Therapeutic properties of mango ginger in *Ayurveda* and *Unani* medicinal systems

Ayurveda	Unani
Alexteric	Antipyretic
Antipyretic	Appetizer
Aphrodisiac	Diarrhea
Appetizer	Diuretic
Asthma	Emollient
Biliousness	Expectorant
Bronchitis	Lumbago
Hiccough	Maturant
Inflammation	Scabies
Itching	Stomatitis
Laxative	
Skin diseases	

Current status

Extensive review of literature on mango ginger revealed that, there are no reports on functional properties of bioactive compounds. Much attention has been focused on essential oil/ volatile oil and to understand the compounds that are responsible for mango flavour. Presence of car-3-ene and *cis*-ocimene was found to be responsible for mango flavour. In addition the α-pinene, car-3-ene and *cis*-ocimene were also reported from essential oils of mango ginger rhizome [Dutt and Tayal, 1941; Achyut and Bandyopadhyaya 1984; Choudhury, *et al.,* 1999; Srivastava, *et al.,* 2001; Singh, *et al.,* 2003; Mustafa, *et al., 2005*]. The volatile oil is composed of 68 compounds [Rao, *et al.,* 1989]. The acetone extract of mango ginger is composed of colourless oil, curcumin, phytosterol and azulenogenic oil containing pinene, camphor, 1-β- and 1-α- curcumene and *ar*-turmerone [Jain, *et al.,* 1964].

Mango ginger is used medicinally as a coolant, aromatic and astringent and to promote digestion. A rhizome paste has traditionally been used for healing of wounds, cuts and itching [Srivastava, *et al.,* 2006]. The external use of the rhizome paste for sprains and skin diseases is also an old practice [Gupta, *et al.,* 1999]. The rhizome has carminative properties, as well as being useful as a stomachic [Hussain, *et al.,* 1992]. A whole plant paste with crushed long peppers [*Piper longum*] is reported to be effective for the treatment of piles, and a decoction of rhizome with common salt is an effective treatment

for colds and coughs [Kambaska, 2006]. The *C. amada* rhizome is considered good as a stomachic because it has bitter, aromatic, cooling, astringent and carminative qualities [CSIR, 1950] and also used to improve blood quality [Kapoor, 1990]. Chemical constituents like demethoxy curcumin, diaryl heptanoid curcumin and bis-demethoxy curcumin were identified by HPLC. However, their functional properties are not been reported [Gupta, *et al.,* 1999]. Mango ginger is used therapeutically as a carminative and stomachic, and topically for contusions and sprains [Kirtikar and Basu, 1935; Nadkarni, 1954; Rao, *et al.,* 1989]. Apart from the above reports, the important functional properties of mango ginger rhizomes have been given in **table R3.**

Table R3: Consolidated report of important functional properties of mango ginger rhizome

Sl. No.	Functional properties	Source	Reference
1	Antioxidant activity	Methanol extract	Niranjan, *et al.,* 2003 ; Chirangini, *et al.,* 2004
2	Antibacterial activity	Water and organic extract	Rao and Nigam, 1970; Rao and Joseph, 1971; Kindra and Narayana, 1978; Ghosh, *et al.,* 1980; Chandarana, 2005
3	Antifungal activity	Volatile oils	Rao and Joseph, 1971; Ghosh, *et al.,* 1980; Singh, *et al.,* 2002
4	Anti-inflammatory	Ethanol extract	Mujumdar, *et al.,* 2000
5	Antimicrobial activity	Essential oil	Banerjee, *et al.,* 1982
6	Brine-shrimp lethal activity	Water extract	Krishnaraju, *et al.,* 2006
7	CNS depressant and analgesic activity	Ethanol extract	Mujumdar, *et al.,* 2004
8	Enterokinase inhibitory activity	Water extract	Bhat, *et al.,* 1981
9	Hyportriglyceridemic activity	Mango ginger powder	Pachauri and Mukherjee, 1970; Srinivasan, *et al.,* 1992 and 1993
10	Insecticidal activity	Essential oils	Ahmad and Ahmad, 1992
11	Trypsin inhibitor activity	Water extract	Sumathi, *et al.,* 1975

Extensive review of literature available from various sources indicated that there are no reports neither on the isolation, characterization of bioactive compounds nor their functional properties from mango ginger rhizomes.

Scope of the Present Investigation

Mango ginger rhizome is a unique spice profusely used in *Ayurveda* and *Unani* system of medicines to cure both infectious and degenerative diseases. Lack of earlier reports on isolation and characterization of bioactive molecules, their physiological role and biochemical changes during developmental stages and also during postharvest storage offered sufficient scope to undertake this research work. These aspects which I believe have commercial application. Understanding the physiological role of bioactive compounds during development of rhizome is essential. It may provide an opportunity to standardize the stage of physiological maturity, which is critical to obtain quality raw material for design and development of products of health benefits. Similar studies on bioactive molecules during storage provide an insight into the storage quality and extension of shelf-life of the rhizome under various conditions. Root crops are rich source of different types of starch. True to this, starch is a major storage component in mango ginger rhizome. Whether it also endowed with inimitable property? This natural scientific inquisitiveness was also ardently attended by undertaking detailed investigation on characterization of starch from mango ginger rhizome.

The results of scientific pursuit carried out in this regard have been presented in five chapters for the convenience of reading; [1] functional properties of mango ginger extracts, [2] isolation and characterization of bioactive compounds and their bioactive properties [3] physiological role of bioactive compounds during development of mango ginger rhizomes [4] functional properties and biochemical changes in mango ginger rhizomes during storage [5] functional properties of starch - a major storage component of mango ginger rhizomes. Reprints of publications are furnished after bibliography.

Chapter -1

Functional Properties of Mango Ginger Extracts

Research is to see what everybody else has seen,
And to think what nobody else has thought
– Albert Szent-Gyorgyi

INTRODUCTION

Ayurveda, *Unani* and other traditional medicines have gained a momentum due to their totalitarian approach. Potential application of mango ginger rhizome extracts in *Ayurveda*, *Unani* and other traditional medicines to reduce oxidative stress and as a source of natural antimicrobial along with other health benefits is well documented. Further, infectious and degenerative diseases are becoming more persistence due increasing resistance of microorganisms to the existing synthetic drugs. In this contest, natural, multifunctional, stable, non-toxic and natural bioactive compounds from plants may prefer panacea for disease causing microbes. Fruits, vegetables, spices, tubers and roots are rich in phytochemicals that provide protection against various chronic and degenerative diseases and also impart other health benefits [Wildman, 2001].

A systematic review of literature on research work on mango ginger has revealed that, earlier reports have concentrated only on volatile constituents and water extract of mango ginger rhizome. Surprisingly, there are no reports on isolation and characterization of bioactive compounds. Water extract was not encouraged due to its inadequacy to extract all types of bioactive compounds that are soluble in lipids or sparingly soluble in water [Zhang and Lewis, 1997]. Hence, we have carried out sequential extraction of dried mango ginger powder with the solvents of increasing polarity. All the extracts were tested for an array of functional properties like- antibacterial activity, antioxidant activity, platelet-aggregation inhibitory activity and cytotoxicity. The details of the work carried out are presented in this chapter.

MATERIALS AND METHODS

Plant material

Fresh and healthy mango ginger rhizomes were procured from the local market, Mysore, India. Rhizomes were washed, sliced and dried in a hot air oven at 50°C for 72 hrs and powdered to 100-120 meshes in an apex grinder [Apex Constructions, London].

Preparation of extracts

Sequential extraction was carried out using solvents of different polarity [from non-polar to polar]. Sequential extraction was employed to resolve the compounds of different polarity effectively and completely. About 100 g of dry mango ginger powder was sequentially extracted using n-hexane, followed by chloroform, ethyl acetate, acetone, methanol and water at room temperature [25±2°C], at normal atmospheric pressure, by shaking at 100 rpm for 48 hrs**.** Each extract was filtered and concentrated by using rotary evaporator [Buchi Rotavapor R-124, Switzerland]. The concentrated extracts were freeze-dried and stored in refrigerator till used.

Determination of Phenolics

Total phenolic content was determined with the modified method of Taga, *et al.,* [1984]. In brief, 100 µl of test sample was mixed with 2 ml of 2 % sodium carbonate solution. After 3 min, 100 µl of 50 % of Folin-Ciocalteau's phenol reagent was added to the mixture. After 30 min of incubation at room temperature, absorbance was measured at 750 nm against a blank. Total phenolic content was calculated on the basis of the calibration curve of gallic acid and expressed as gallic acid equivalents.

ANTIBACTERIAL ACTIVITY

Bacterial strains and inoculum preparation

The antibacterial activity was tested against *Pseudomonas aeruginosa, Escherichia coli, Salmonella typhi, Klebsiella pneumoniae, Enterobacter aerogenes, Proteus mirabilis, Yersinia enterocolitica, Micrococcus luteus, Staphylococcus aureus, Enterococcus fecalis, Bacillus subtilis, Bacillus cereus* and *Listeria monocytogenes.* The above bacterial strains isolated from clinical samples were obtained from the Department of Microbiology,

Mysore Medical College, Mysore, India. Their cultural characteristics and morphological features were reconfirmed and also subjected to standard biochemical tests [Krieg and Holt 1984; Sneath *et al.,* 1986] before experimentation. The test organisms were maintained on nutrient agar slants.

Agar-well diffusion method

In vitro antibacterial activity was determined by agar-well diffusion method [Mukherjee *et al.,* 1995]. The overnight bacterial culture was centrifuged at 8000 rpm for 10 minutes at 4°C. The supernatant was discarded and bacterial cells were resuspended in the saline to make suspension 10^5 CFU ml^{-1} and used for the assay. The plating was carried out by transferring bacterial suspension [10^5 CFU ml^{-1}] to sterile Petri plate and mixed with molten Nutrient agar medium [Hi-Media Laboratories Limited, Mumbai, India] and allowed to solidify. About 75 µl of the sample [2 mg ml^{-1}] was placed in the wells and allowed to diffuse for 2 h. Plates were incubated at 37°C for 48 h and the activity was determined by measuring the diameter of inhibition zones. Solvent control and amoxicillin [Galpha Lab. Mumbai, India] were also maintained. The assay was carried out in triplicate.

Minimum inhibitory concentration [MIC]

The minimum inhibitory concentration was determined according to the method described by Jones *et al.,* [1985]. Different concentrations [20 ppm to 300 ppm] of hexane, chloroform, ethyl acetate, acetone and methanol extracts and 100 µl of the bacterial suspension [10^5 CFU ml^{-1}] was placed aseptically in 10 ml of nutrient broth separately and incubated for 24 h at 37°C. The growth was observed both visually and by measuring O.D. at 600 nm at regular intervals followed by pour plating as described above. The lowest concentration of test sample showing no visible growth was recorded as the minimum inhibitory concentration. Triplicate sets of tubes were maintained for each concentration of test sample.

Determination of minimum bactericidal concentration [MBC]

Minimum bactericidal concentration [MIC] was determined according to the method of Smith-Palmer *et al.,* [1998]. Test tubes containing nutrient broth with different concentrations of hexane, chloroform, ethyl acetate, acetone and methanol extracts were

inoculated with 100 µl of the bacterial suspension [10^5 CFU ml^{-1}]. Inoculated tubes were incubated for 24 h at 37°C and growth was observed both visually and by measuring O.D. at 600 nm. About 100 µl from the tubes not showing growths were plated on nutrient agar as described above. Minimum bactericidal concentration is the concentration at which bacteria failed to grow in nutrient broth and nutrient agar inoculated with 100 µl suspension. Triplicate sets of tubes were maintained for each concentration of test sample.

ANTIOXIDANT ACTIVITY

DPPH free radical scavenging activity

DPPH [1, 1-diphenyl-2-picrylhydrazyl] radical scavenging activity was determined according to the method described earlier [Blois, 1958; Bondet *et al.,* 1997; Moon and Terao, 1998]. The test samples [10- 100 µl] were mixed with 0.8 ml of Tris-HCl buffer [pH 7.4] to which 1 ml of DPPH [500 µM in ethanol] was added. The mixture was shaken vigorously and left to stand for 30 min. Absorbance of the resulting solution was measured at 517 nm in a UV-Visible Spectrophotometer [UV-160A, Shimadzu co. Japan]. The radical scavenging activity was measured as a decrease in the absorbance of DPPH. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Radical scavenging potential was expressed as EC_{50} value, which represents the sample concentration at which 50 % of the DPPH radicals scavenged.

Superoxide radical scavenging activity

The superoxide scavenging ability was assessed according to the method of Nishikimi, *et al.,* [1972] with slight modifications. The reaction mixture contained NBT [0.1 mM] and NADH [0.1 mM] with or without sample to be assayed in a total volume of 1 ml of Tris-HCl buffer [0.02 M, pH 8.3]. The reaction was started by adding PMS [10 µM] to the mixture, and change in the absorbance was recorded at 560 nm every 30 seconds for 2 min. The percent inhibition was calculated against a control without test sample. Radical scavenging potential was expressed as EC_{50} value, which represents the sample concentration at which 50 % of the radicals scavenged.

Lipid peroxidation inhibitory activity

Lipid peroxidation inhibitory activity was determined according to the method described earlier [Duh and Yen, 1997]. In brief, lecithin [3 mg/ ml phosphate buffer, pH 7.4] was sonicated in dr. Hielscher Gmb_H, UP 50H ultraschallprozessor [DrHielscher GmbH, Teltow, Berlin, Germany]. The test samples [100 µl] were added to 1ml of liposome mixture, control was without test sample. Lipid peroxidation was induced by adding 10 µl $FeCl_3$ [400 mM] and 10 µl L-ascorbic acid [400 mM]. After incubation for 1 hour at 37°C, the reaction was stopped by adding 2 ml of 0.25 N HCl containing 15 % TCA and 0.375 % TBA and the reaction mixture was boiled for 15 min. then cooled, centrifuged and absorbance of the supernatant was measured at 532 nm. Inhibitory activity was expressed as EC_{50} value, which is sample concentration inhibited 50 % of lipid peroxidation.

Metal chelating activity

The chelating of ferrous ions by the test sample was estimated by the method described earlier [Decker and Welch, 1990; Dinis *et al.,* 1994]. Briefly, the test samples at different concentrations were added to a solution of 2mM $FeCl_2$ [0.05 ml]. The reaction was initiated by the addition of 5mM ferrozine [0.2 ml] and the mixture was vigorously shaken and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the mixture was read at 562nm against a blank. EDTA was used as positive control. Results were expressed as EC_{50} value, which represents the sample concentration at which 50 % of metal chelation occurred.

Total reducing power

The reducing power was quantified by the method described earlier by Yen and Chen [1995] with minor modifications. Reaction mixture, containing test samples at different concentrations [10-100 µl] in phosphate buffer [0.2 M, pH 6.6], was incubated with potassium ferricyanide [1 % w/v] at 50°C for 20 min. The reaction was terminated by the addition of TCA solution [10 % w/v] and the mixture was centrifuged at 3000 rpm for 20 min. The supernatant was mixed with distilled water and ferric chloride [0.1 % w/v] solution and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

PLATELET-AGGREGATION INHIBITORY ACTIVITY

Platelet preparation

Blood samples were taken from healthy volunteers who assured not to have taken any drugs during the 2 weeks prior to the blood sampling. Blood was collected into buffered sodium citrate [3.8 % w/v] pH 6.5 as the anticoagulant at a ratio of 9:1 v/v and used within 3 hr of collection. Platelet-rich plasma [PRP] was obtained by centrifugation of the citrated blood at 1100 rpm for 20 min. the residual blood was again centrifuged at 2500 rpm for 20 min to obtain the homologous Platelet Poor Plasma [PPP]. Platelet count was adjusted to 1.6 x 10^7 platelets per µl of PRP.

Platelet-aggregation assay

Aggregation was measured turbidimetrically at 37°C with constant stirring at 1000 rpm in a Chronolog Dual Channel Aggregometer. About 0.45 ml of PRP was kept stirred at 1200 rpm at 37°C, and aggregation was induced by collagen [10 µM] and. The change in turbidity was recorded with reference to PPP using an omniscribe recorder for at least 5 min. The slope was calculated and it was used as control.

Similarly, 100-500 µM of the mango ginger extracts and isolated bioactive compounds were added to PRP, incubated for five min after which collagen [10 µM], was added. Platelet aggregation was recorded using an omniscribe recorder for 5 min. The slope was calculated. The difference in the slope between the control and the treated was expressed as percent inhibition of platelet aggregation by mango ginger extracts.

CYTOTOXICITY OF MANGO GINGER EXTRACTS

Chemicals

Sulforhodamine B [SRB], 3-[4, 5-dimethyl thiazol-2-yl]-5-diphenyl tetrazolium bromide [MTT], New Born Calf Serum [NBCS] were obtained from Sigma Aldrich Co, St Louis, USA., Phosphate Buffered Saline [PBS], Dulbecco's Modified Eagle's medium [DMEM] and antibiotics from Hi-Media Laboratories Ltd., Mumbai., Trichloro acetic acid [TCA] and tris buffer from SD fine chemicals Pvt. Ltd., Boisar, India., 25 cm^2 and 75 cm^2 tissue culture flasks, 96 well microtitre plates were procured from Tarson India Pvt. Ltd., Kolkata, India, DMSO, glacial acetic acid and propanol from E-Merck Ltd., Mumbai, India.

Preparation of test solutions

For cytotoxicity studies, each extract was weighed separately, dissolved in distilled dimethyl sulphoxide [DMSO] and volume was made up to 10 ml with DMEM, pH 7.4, supplemented with 2 % inactivated NBCS [maintenance medium] to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration and stored at -20°C till use. Serial two fold dilution of the extracts was prepared from the stock solution to obtain lower concentrations.

Cell lines and culture medium

Vero [normal African Green Monkey Kidney] cell culture was procured from National Centre for Cell Sciences [NCCS], Pune, India., A-549 [human small cell lung carcinoma] cells from Christian Medical College, Vellore, India. Stock cells of Vero and A-549 cell lines were cultured in DMEM medium supplemented with 10 % inactivated NBCS, penicillin [100 IU/ml], streptomycin [100 µg/ml] and amphotericin B [5 µg/ml] in a humidified atmosphere of 5 % CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution [0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS]. The stock cultures were grown in 25 cm^2 flat bottles and all experiments were carried out in either 96 well microtitre plates.

Determination of mitochondrial synthesis by MTT assay

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The cleavage of MTT to a blue formazan derivative by living cells is clearly a very effective principle on which the assay is based. The principle involved is the cleavage of tetrazolium salt 3-[4, 5 dimethyl thiazole-2-yl]-2,5-diphenyl tetrazolium bromide [MTT] into a blue coloured product [formazan] by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used [Francis and Rita, 1986].

Procedure

The monolayer cell culture was trypsinized and cell count adjusted to 1.0 x 10^5 cells/ml using DMEM medium containing 10 % NBCS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension [approximately 10,000 cells] was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different extract concentrations were added to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5 % CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h. After 72 h, the extract solutions in the wells were discarded and 50 µl of MTT in DMEM-PR [Dulbecco's Modified Eagle's medium without phenol red, 2 mg/ml] was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5 % CO_2 atmosphere. The supernatant was removed and 50 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and CTC_{50} [concentration of drug or test extract needed to inhibit cell growth by 50 %] values were generated from the dose-response curves for each cell line. The pattern of all the cell lines as a group is used to rank compounds as toxic or non-toxic.

$$\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100 \right)$$

Determination of total cell protein content by Sulforhodamine B [SRB] assay

SRB is a bright pink aminoxanthine dye with two sulfonic groups. Under mild acidic conditions, SRB binds to protein basic amino acid residues in trichloro acetic acid [TCA] fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of at least two orders of magnitude. Colour development in SRB assay is rapid, stable and visible. The developed colour can be measured over a broad range of visible wavelength in either a spectrophotometer or a 96 well plate reader. When TCA-fixed and SRB stained samples are air-dried, they can be stored indefinitely without deterioration [Philip *et al.,* 1990].

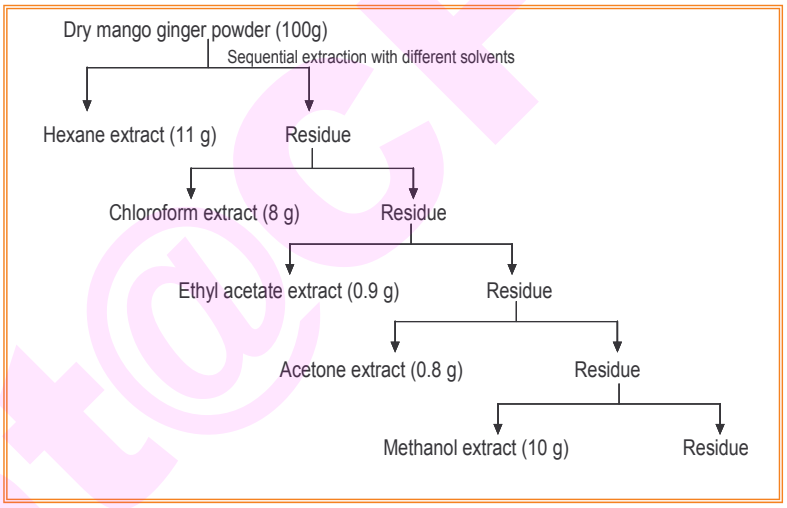
Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM medium containing 10 % NBCS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension [approximately 10,000 cells] was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different extract concentrations were added to the cells in microtitre plates. The plates were then incubated at 37°C for 3 days in 5 % CO_2 atmosphere, microscopic examination was carried out and observations were recorded every 24 h. After 72 h, 25 µl of 50 % trichloro acetic acid was added to the wells gently in such a way that it forms a thin layer over the extract to form an over all concentration of 10 %. The plates were incubated at 4°C for 1 h. The plates were flicked and washed five times with water to remove traces of medium, extract and serum, and air-dried. They were stained with SRB [0.4 % prepared in 1 % acetic acid] for 30 min. The unbound dye was then removed by rapidly washing four times with 1 % acetic acid. The plates were then air-dried. Tris base [10 mM, 100 µl] was then added to the wells to solubilise the dye. The plates were shaken vigorously for 5 min. The absorbance was measured using microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the same formula used for MTT assay. CTC_{50} values were calculated.

Results and Discussion

Sequential extraction of 100 g of mango ginger powder using hexane, chloroform, ethyl acetate, acetone and methanol yielded 11 g, 8 g, 0.9 g, 0.8 g and 10 g of extract respectively [**Fig. 1.1].** This kind of extraction method will be helpful to extract wide range of [both non-polar and polar] bioactive molecules in a plant material. Each extract was tested for the antibacterial activity, antioxidant activities, platelet-aggregation inhibitory activity and cytotoxicity.

Fig. 1.1: Schematic representation of sequential extraction of mango ginger rhizome



Solvent V/S active components

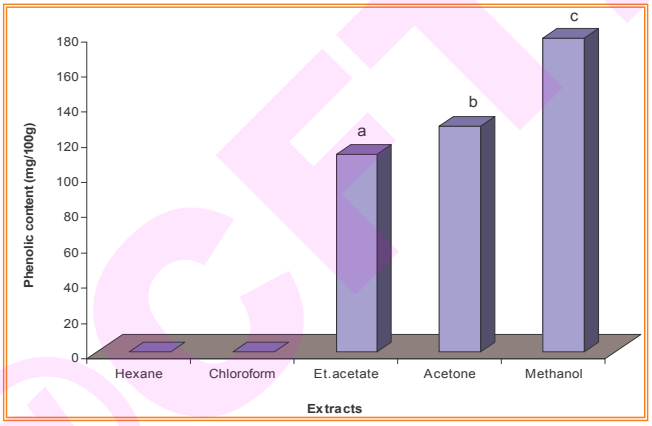
The extraction procedure depends upon the type of bioactive component to be extracted. Polar components such as phenolic acids and glycosides of many flavonoids are generally extracted using water, alcohols or a mixture of water and alcohols. For flavonoids and most carotenoids, non-polar solvents are used [Tsao and Deng, 2004]. It has been indicated that methanol is the best solvent for extraction of polyphenols, lactones, phenones, quassinoids, flavones, saponons and some terpenoids. It has also been indicated that acetone/water mixtures are more useful for extracting polyphenols from proteic matrices, since they appear to degrade the polyphenols-protein complexes [Cowan, 1999; Suhaj, 2006].

PHENOLIC CONTENT IN MANGO GINGER EXTRACTS

Among five mango ginger extracts, the methanol extract showed high phenolic content [178±7 mg] followed by acetone [128±5 mg], ethyl acetate [112±7 mg] extract **[Fig. 1.2].** The phenolic compounds were not detected in hexane, chloroform extracts. The concentrations of phenolics in the extracts were expressed as mg /100 g.

Plant phenolics present in the fruit and vegetables have received considerable attention because of their potential antioxidant activity [Lopez-Velez, *et al.,* 2003]. Phenolic compounds are the major contributors of antioxidant activity in vegetable juices [Gardner, *et al.,* 2000]. Phenolic compounds are effective hydrogen donors, which make them good antioxidant [Rice-Evans, *et al.,* 1995].

Fig. 1.2: Phenolic content in mango ginger extracts



* Values expressed are means ± SD of three replicates.
Values with different letters [a, b and c] differ significantly at P <0.05

The antioxidant activity was independent of phenolic content. Polar extracts exhibited potential DPPH radical scavenging activity, superoxide radical scavenging activity, lipid peroxidation-inhibitory activity and metal chelating activity, but the non-polar extracts showed potential lipid peroxidation-inhibitory activity and metal chelating activity. However, it is known that non-phenolic antioxidants could also contribute to the antioxidant activity of extracts [Mariko, *et al.,* 2005]. Antioxidant-rich plant extracts serve as sources of nutraceuticals that alleviate the oxidative stress and therefore prevent or slow down the degenerative diseases [Ames *et al.,* 1993; Cao *et al.,* 1996; Kitts *et al.,* 2000; Fu and Ji, 2003]. The broad range of antioxidant activity of the mango ginger extracts indicates the potential of the rhizomes as a source of natural antioxidants or nutraceuticals with potential application to reduce oxidative stress with consequent health benefits.

ANTIBACTERIAL ACTIVITY OF MANGO GINGER EXTRACTS

Among the five extracts of mango ginger, chloroform extract showed antibacterial activity against seven out of 13 bacteria screened viz. *B. cereus, B. subtilis, M. luteus, S. aureus, L. monocytogenes, E. fecalis and S. typhi*. Hexane extract, similar to chloroform extract, showed inhibition to all the bacteria except *S. typhi*. Ethyl acetate extract showed inhibitory effect as shown by chloroform extract, except *S. typhi* and *L. monocytogenes*. Acetone extract inhibited *M. luteus, L. monocytogenes* while methanol extract showed inhibition only against *E. faecalis*. The chloroform extract of mango ginger exhibited highest antibacterial activity against wide range of bacteria, when compared to other solvent extracts **[Table 1.1].** However, *E. coli*, *E. aerogenes, K. pneumoniae, P. aeruginosa, P. mirabilis* and *Y. enterocolitica* were not inhibited by any of the solvent extracts of mango ginger.

Table 1.1: Minimum inhibitory concentrations [MIC] for different extracts of mango ginger

Bacteria	MIC [in ppm] * for mango ginger extracts				
	Hexane extract	Chloroform extract	Ethyl acetate extract	Acetone extract	Methanol extract
P. aeruginosa	--	--	--	--	--
E. coli	--	--	--	--	--
S. typhi	--	180 [a]	--	--	--
K. pneumoniae	--	--	--	--	--
E. aerogenes	--	--	--	--	--
P. mirabilis	--	--	--	--	--
Y. enterocolitica	--	--	--	--	--
M. luteus	80 [a]	80 [a]	100 [b]	200 [c]	--
S. aureus	120 [c]	80 [a]	100 [b]	--	--
E. fecalis	180 [b]	140 [a]	140 [a]	--	220 [c]
B. cereus	160 [c]	60 [a]	80 [b]	--	--
B. subtilis	120 [b]	60 [a]	60 [a]	--	--
L. monocytogenes	80 [a]	100 [b]	--	140 [c]	--

* Each value represents mean of three different observations.
Mean values with different superscripts [a, b and c] differ significantly at P<0.05

Different solvent extracts of mango ginger showed MIC values ranged from 60-220 ppm **[Table 1.1].** The high antibacterial activity i.e. low MIC for wide range of bacteria was exhibited by chloroform extract. Chloroform extract was very effective against *B. subtilis, B. cereus* with MIC of 60 ppm and also inhibited the growth of *M. luteus* and *S. aureus* at 80 ppm, while *S. typhi, E. fecalis* and *L. monocytogenes* were completely inhibited at 180, 140 and 100 ppm respectively. In contrast, lack of outer polysaccharides layer in Gram-positive bacteria may be responsible for more permeable to

amphipathic compounds [Cowan 1999]. However, *E. coli*, *E. aerogenes, K. pneumoniae, P. aeruginosa, P. mirabilis* and *Y. enterocolitica,* were not inhibited by the entire range of solvent extracts of mango ginger **[Table 1.1].** High resistance of Gram-negative bacteria to mango ginger extracts may be due to the presence of their outer layer composed of lipo-polysaccharides.

The results indicated differential activity between polar and non-polar solvent extracts of mango ginger. It appeared that with the increase in polarity of the solvent of mango ginger extracts, there was a decrease in potential of antibacterial activity and decrease in range. According to Cowan [1999], polar extracts are less effective against microbes than non-polar extracts. It may be due to the presence of polysaccharides, polypeptides and lectins that are more effective as inhibitors of pathogen adsorption and would not be identified in the screening techniques commonly used.

ANTIOXIDANT ACTIVITY OF MANGO GINGER EXTRACTS

To understand nature and function of antioxidant activity of the extracts, various assays like DPPH radical scavenging activity, superoxide radical scavenging activity, lipid peroxidation inhibitory activity, metal chelating activity and total reducing power were tested. BHA was used as a standard for antioxidant assay.

DPPH radical scavenging activity

Except hexane and chloroform extracts all mango ginger extracts showed DPPH scavenging activity. Ethyl acetate extract exhibited potential radical scavenging activity with very low IC_{50} [77±5]. While acetone and methanol extracts showed less activity with high IC_{50} values of 146 and 140 respectively **[Table 1.2].** The high antioxidant activity of ethyl acetate extract may be due to the cumulative effect of its potential antioxidant compounds along with phenolics**.** Phenolics are very important bioactive constituents of mango ginger rhizome and tuber crops which are known for their radical scavenging ability due to their hydroxyl groups [Hatano, *et al.,* 1980]. Even though the concentration of phenolic compound is less in ethyl extract compared to other extracts of mango ginger rhizome **[Fig. 1.2],** it may be having other potential radical scavenging bioactive compounds. DPPH radical scavenging activity may be attributed to the presence of hydrogen-donating ability of -OH and $-CH_3$ groups in extracts/ compounds [Chen and Ho, 1995; Nikolaos, *et al.,* 2003].

Superoxide radical scavenging activity

Among five extracts of mango ginger, only ethyl acetate and acetone extracts exhibited superoxide radical scavenging activity. Ethyl acetate extract scavenged superoxide radicals significantly with low IC_{50} value of 77±5 µg **[Table 1.2].** Superoxide anion plays an important role in the formation of reactive oxygen species [ROS] such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA [Dahl and Richardson, 1978; Halliwell and Gutteridge, 1989; Pietta, 2000].

Table I.2: Antioxidant activity of mango ginger extracts

EXTRACTS	IC_{50} [µg/ml] *			
	DPPH scavenging activity	Superoxide scavenging activity	Lipid-peroxidation inhibitory	Metal chelating activity
Hexane	--	--	94 ± 3 ^{c}	218 ± 4 ^{c}
Chloroform	--	--	80 ± 4 ^{a}	142 ± 4 ^{a}
Ethyl acetate	77± 5 ^{a}	146 ± 6 ^{a}	91 ± 2 ^{b}	168 ± 3 ^{b}
Acetone	146± 3 ^{b}	288 ± 3 ^{b}	95 ± 1 ^{c}	233 ± 5 ^{d}
Methanol	140± 4 ^{b}	--	178 ± 7 ^{d}	268 ± 5 ^{e}

* Values expressed are means ± SD of three replicates.
Values with different superscripts [$^{a, b}$ and ^{c}] differ significantly at $P<0.05$

Lipid peroxidation inhibitory [LPI] activity

All the mango ginger extracts showed potential lipid peroxidation inhibitory activity. The chloroform extract was found to be more potential inhibitor of lipid peroxidation with an IC_{50} value of 80±4 µg. Hexane, ethyl acetate and acetone extracts showed potential LPI activity with IC_{50} value ranged from 91-95 µg **[Table 1.2].** Even though the phenolic compounds were absent in chloroform extract **[Fig. 1.2]** the LPI activity may be due to the presence of terpenoids and other non-polar compounds. Lipid peroxidation inhibitory activity was mainly depends upon the solubility, hydrophobicity of the compounds present in the respective extracts. Lipid peroxidation causes destabilization and disintegration of the cell membrane, leading to liver injury, atherosclerosis, kidney damage, aging, and susceptibility to cancer [Rice-Evans and Burdon, 1993].

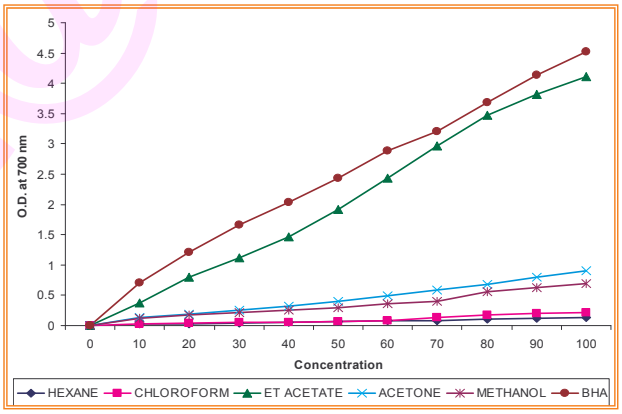
Metal chelating activity

All mango ginger extracts showed metal chelating activity **[Table 1.2].** Ethyl acetate and acetone extracts were found to be potential metal chelator with low IC_{50} value of 142 ± 4 and 168 ± 3 µg respectively. It was reported that the structures containing two or more of the following functional groups: -OH, -SH, -COOH, $-O_3H_2$, C=O, -NR2, -S- and -O- in a favorable structure-function configuration can show metal chelating activity [Lindsay, 1996; Yuan, *et al.,* 2005]. Hence, Mango ginger extracts may be potential source of compounds having above functional groups. Since ferrous ions are the most effective proxidant in the food system [Yamaguchi, *et al.,* 1988] the high chelating abilities of mango ginger extracts would be beneficial. Iron can stimulate lipid peroxidation by Fenton reaction, and also accelerates peroxidation by decomposing lipid hydro peroxides into peroxyl and alkoxyl [Halliwell, 1991; Gulcin, *et al.,* 2003].

Total reducing power

Acetone and ethyl acetate extracts showed high reducing power compared to hexane, chloroform, methanol and water extracts. All the extracts exhibited concentration dependent activity **[Fig. 1.3].** The presence of compounds with -OH groups in the extracts may be responsible for reduction power. The reducing capacity of extracts may serve as a significant indicator of its antioxidant capacity [Meir, *et al.,* 1995].

Fig. 1.3: Total reducing power of mango ginger extracts [a]



[a] concentration of each test sample was 2 mg/ ml

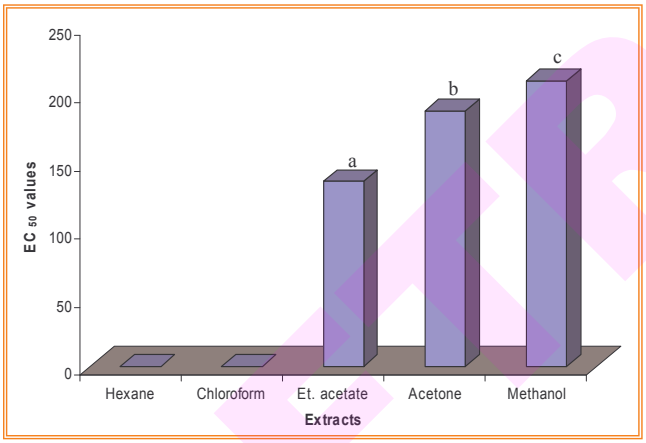
Platelet-aggregation inhibitory activity

Platelets readily aggregate in response to a variety of endogenous substances and they can initiate thrombus formation, leading to ischemic diseases. In addition, the interactions between platelets and blood vessel walls are important in the development of thrombosis and cardiovascular diseases [Ross, 1978; Hirsh, 1987; Dinerman and Mehta,

1990]. Therefore, the inhibition of platelet function represents a promising approach for the prevention of thrombosis.

Platelet aggregation inhibitory activity of ethyl acetate extract [IC_{50} = 136±6 µg] and acetone extract [IC_{50} = 188±7 µg] was very high followed by methanol [IC_{50} = 210 µg extract. Hexane and chloroform extracts did not show activity **[Fig. 1.4].** The high platelet-aggregation inhibitory activity of ethyl acetate, acetone and methanol extracts appears to be correlated with high phenolic content and concentration dependent **[Fig. 1.4].**

Fig. 1.4: Platelet-aggregation inhibitory activity of mango ginger extracts



* Values expressed are means of three replicates.
Values with different letters [a, b and c] differ significantly at P<0.05

CYTOTOXICITY OF MANGO GINGER EXTRACTS

All the five extracts of mango ginger showed moderate cytotoxicity **[Table 1.3]** towards both normal and cancer cell cultures tested. All the extracts showed comparatively more toxicity towards cancer cells when compared to normal cells which is a good indicator of anticancer property of extracts. Among the five extracts tested, the ethyl acetate extract showed more toxicity with CTC_{50} values ranging between 52 to 65 µg/ml concentration, followed by chloroform [between 90-106 µg/ml], hexane [95-110 µg/ml], acetone [132-140 µg/ml] and methanol [395-423 µg/ml] concentration. The cytotoxicity results of different extracts of mango ginger indicate that the extracts are not toxic towards the cells.

Table 1.3: Cytotoxicity of mango ginger extracts

EXTRACTS	CTC_{50} [µg/ml] *			
	Vero		A-549	
	MTT	SRB	MTT	SRB
Hexane	106± 1 ^{b}	110 ± 2 ^{b}	95 ± 2 ^{b}	100 ± 1 ^{b}
Chloroform	102± 2 ^{b}	106 ± 3 ^{b}	90 ± 1 ^{b}	96 ± 2 ^{b}
Ethyl acetate	65± 1 ^{a}	62 ± 1 ^{a}	52 ± 1 ^{a}	57 ± 1 ^{a}
Acetone	137± 1 ^{c}	140 ± 3 ^{c}	132 ± 3 ^{c}	138 ± 2 ^{c}
Methanol	408± 4 ^{e}	423 ± 3 ^{e}	395 ± 5 ^{d}	397 ± 5 ^{d}

* Values expressed are means ± SD of three replicates
Values with different superscripts [$^{a, b}$ and ^{c}] differ significantly at P<0.05

Conclusion

In the present investigation, screening of mango ginger extracts for different functional properties has revealed that, they are potential inhibitors of bacteria and also scavengers of free radicals, metal chelator and lipid peroxidation-inhibitors. Mango ginger extracts also showed platelet-aggregation inhibitory activity and cytotoxicity properties. The highest antibacterial and antioxidant activities exhibited by mango ginger chloroform extract prompted us for further purification and characterization of bioactive compounds from this extract.

Chapter - 2

Isolation and Characterization of Bioactive Compounds and their Functional Properties

Water is H_2O, hydrogen two parts, oxygen one, but
There is also a third thing, that makes it water
And nobody knows what that is

– *D. H. Lawrence*

INTRODUCTION

Screening of extracts for biological activities played a strategic role in the isolation and identification of bioactive molecules. Different extracts of mango ginger rhizome exhibited various bioactive properties like antibacterial activity, antioxidant activity, platelet-aggregation inhibitory activity and cytotoxicity. The current strategy to extract rhizome with different solvents of increasing polarity have yielded interesting results. Apparently, the chloroform extract exhibited highest antioxidant, antimicrobial activities. It also showed platelet-aggregation inhibitory activity and cytotoxicity properties. This provides the deep insight into the use of extracts for therapeutic purpose and totalitarian approach emphasized in traditional medicine. Hence, chloroform extract was selected for the isolation, purification and characterization of bioactive molecules. The more inquisitiveness and additional impetus behind selection of chloroform extract owe to the fact that, it displayed potential bioactive properties. In order to obtain their bioactive constituents, the first step is the extraction of the dried and powdered plant material, which contains chemically different classes of compounds. The search for bioactive components with multiple bioactivities from plant source has gained increasing importance in recent time, due to growing worldwide concern about alarming increase in the rate of infection by antibiotic-resistant microorganisms and carcinogenicity of synthetic compounds [Velioglu, *et al.,* 1998; Salvat *et al.,* 2004]. Natural products continue to provide greater structural diversity than standard combinatorial chemistry and they offer major opportunities for finding novel bioactive compounds with potential bioactivities. This was achieved by repeated bioactivity-guided fractionation, followed by elucidation of structure using appropriate chromatographic and spectroscopic techniques.

MATERIALS AND METHODS

Isolation of Bioactive compounds from Chloroform extract of mango ginger

1. Isolation of antibacterial compound

Fractionation of the chloroform extract

Activated silica gel [60-120 mesh] was packed on to a glass column [450 x 40 mm] using n-hexane solvent. For large scale isolation of antibacterial compound, about 15 g of crude chloroform extract was loaded and eluted stepwise with 500 ml of hexane, 2000 ml of hexane: chloroform [75: 25 to 0: 100, v/v], 2000 ml of chloroform: ethyl acetate [75: 25 to 0: 100, v/v], 2000 ml of ethyl acetate: acetone [75: 25 to 0: 100, v/v] and 1500 ml of acetone: methanol [75: 25 to 0: 100, v/v]. About 82 fractions measuring 100 ml each were collected and concentrated by using the rotary evaporator.

Thin Layer Chromatography [TLC]

An aliquot of all the concentrated fractions were loaded on the activated silica gel TLC plates [20 x 20 cm]. The plates were developed using hexane: chloroform [80:20], chloroform: ethyl acetate [90:10] and ethyl acetate: methanol [90:10] solvents. The spots were located by exposing the plate to iodine fumes. Fractions having same number of spots with similar R_f values on TLC plate were pooled into five fractions [Fr.1- Fr.5]. All the five fractions were tested for antibacterial activity as described below.

Further purification of bioactive fraction three [Fr.3]

Since fraction three [Fr.3] obtained from first step column chromatography showed high antibacterial activity, it was selected for further purification. About 3.5 g of bioactive fraction three [Fr.3] was further purified using silica gel [60-120 mesh] column [450 x 20 mm]. The column was eluted stepwise with 100 ml of hexane, 200 ml of hexane: chloroform [90: 10 to 0: 100, v/v], 800 ml of chloroform: ethyl acetate [90: 10 to 0: 100, v/v], 600 ml of ethyl acetate: acetone [90: 10 to 0: 100, v/v] and 400 ml of acetone: methanol [90: 10 to 0: 100, v/v]. About 21 fractions measuring 100 ml each were collected

and concentrated on a rotary evaporator. An aliquot of all the fractions were loaded on the TLC plate, fractions having similar R_f values were pooled into four sub-fractions [Fr.3.1-Fr.3.4]. These four sub-fractions were tested for antibacterial activity.

Sub-fraction two [Fr.3.2] obtained from second step chromatography showed high antibacterial activity, hence selected for further purification. About 800 mg of bioactive sub-fraction two [Fr.3.2] was further purified on a silica gel [100-200 mesh] column [600 x 15mm]. The column was eluted stepwise with 100 ml of hexane: chloroform [90: 10 to 0: 100, v/v], 400 ml of chloroform: ethyl acetate [95: 05 to 0: 100 v/v] and 200 ml of ethyl acetate: acetone [95: 05 to 0: 100, v/v]. About 28 fractions measuring 25 ml each were collected and concentrated. Fractions having similar R_f values on TLC plate were pooled and numbered [Fr.1``-Fr.3``]. Among these, sub-fraction two [Fr.3.2.2] obtained from third chromatographic step showed a single spot on TLC. This pure compound was subjected to various spectroscopic techniques for elucidation of the structure.

High performance liquid chromatography [HPLC]

The purified compound was tested for its purity using HPLC, using LC-10AT liquid chromatograph [Shimadzu, Singapore] equipped with C-18 column [300 x 4.6 mm, 5 µ Thermo Hypersil] and methanol: water [60: 40] as a mobile phase with a flow rate of one ml min^{-1}. UV detection was carried out with a diode array detector [Shimadzu, Singapore].

2. Isolation of antibacterial compound

Further purification of bioactive fraction two [Fr.2]

Since fraction two [Fr.2] obtained from first step column chromatography also showed high antibacterial activity, it was selected for further purification. Bioactive fraction two [Fr.2] was further purified using silica gel [60-120 mesh] column [450 x 20 mm]. The column was eluted stepwise with 100 ml of hexane, 200 ml of hexane: chloroform [95:5, 75:25, 50:50, v/v], 300 ml of hexane: ethyl acetate [95: 5, 75:25, 0: 100, v/v and 100 ml of methanol [0:100, v/v]. About 33 fractions measuring 100 ml each were collected and concentrated on a rotary evaporator. An aliquot of all the fractions were loaded on the TLC plate, fractions having similar R_f values were pooled into eight sub-fractions [Fr.2.1- Fr.2.8]. These eight sub-fractions were tested for antibacterial activity.

Sub-fraction four [Fr.2.4] obtained from second step chromatography showed high antibacterial activity, hence selected for further purification. About 1.2 g of bioactive sub-fraction four [Fr.2.4] was further purified on a silica gel [100-200 mesh] column [600 x 15mm]. The column was eluted stepwise with 200 ml of hexane: chloroform [75:25, v/v] and 200 ml of hexane: acetone [95:05, v/v]. About 21 fractions measuring 25 ml each were collected and concentrated. Fractions having similar R_f values on TLC plate were pooled into five sub-fractions [Fr.2.4.1-Fr.2.4.5]. Among these, sub-fraction five [Fr.2.4.5] obtained from third step chromatography showed a single spot in TLC profile. This pure compound was subjected to various spectroscopic techniques for elucidation of the structure.

High performance liquid chromatography [HPLC]

The isolated compound [Fr.2.4.5] was filtered and freeze dried before using the sample for HPLC analysis using LC-10AT liquid chromatograph [Shimadzu, Singapore] equipped with C-18 column [300 x 4.6 mm, 5 µ Thermo Hypersil]. The gradient programme used for mobile phase with methanol: water as follows; 0 min, 25:75, v/v; 5 min, 40:60, v/v; 10 min, 50:50, v/v; 20 min, 70:30, v/v; 40 min, 100:0, v/v, with a flow rate of one ml min^{-1}. UV detection was carried out with a diode array detector [Shimadzu, Singapore].

3. Isolation of antioxidant compound

Further purification of bioactive fraction five [Fr.5]

Since fraction five [Fr.5] obtained from first step column chromatography showed high antioxidant activity, it was selected for further purification. About 2.1 g of bioactive fraction five [Fr.5] was further purified using silica gel [60-120 mesh] column [450 x 20 mm]. The column was eluted step wise with 100 ml of hexane, 200 ml of hexane: chloroform [90: 10 to 0:100, v/v], 500 ml of chloroform: ethyl acetate [90:10 to 0:100, v/v], 1000 ml of ethyl acetate: acetone [90:10 to 0:100, v/v] and 1000 ml of acetone: methanol [90:10 to 0:100, v/v]. Twenty-eight fractions measuring 100 ml each were collected and concentrated on a rotary evaporator. An aliquot of all the fractions were loaded on the TLC. Fractions having same number of spots with similar R_f values were

pooled into four sub-fractions [Fr.5.1- Fr.5.4]. These four sub-fractions were tested for the antioxidant activity.

Sub-fraction four [Fr.5.4] obtained from second column chromatography step showed high antioxidant activity, hence selected for further purification. About 520 mg of bioactive sub-fraction four [Fr. 5.4] was further purified on a silica gel [100-200 mesh] column [600 x 5 mm]. The column was eluted step wise with 100 ml of hexane: chloroform [90:10 to 0:100, v/v], 100 ml of chloroform: ethyl acetate [95:05 to 0:100, v/v], 500 ml of ethyl acetate: acetone [90:05 to 0:100 v/v] and 500 ml of acetone: methanol [95:05 to 0:100, v/v]. About 23 fractions measuring 50 ml each were collected and concentrated. Fractions having same number of spots with similar R_f values on TLC plate were pooled into three sub-fractions [Fr.5.4.1-Fr.5.4.3]. Among these, sub-fraction two [Fr.5.4.2] obtained from third step column chromatography showed a single spot on TLC. This pure compound was subjected to various spectroscopic techniques for elucidation of the structure.

High performance liquid chromatography [HPLC]

The purified fraction was tested for its purity using HPLC, using LC-10AT liquid chromatograph [Shimadzu, Singapore] equipped with C-18 column [300 x 4.6 mm, 5 µ Thermo Hypersil] and acetonitrile: water [60: 40] as a mobile phase with a flow rate of 1 ml/min. Diode array [Shimadzu, Singapore] was used as a detector.

Characterization of Bioactive Compounds

UV spectrophotometry

UV-Visible spectrum of the isolated compound was recorded on a Shimadzu UV-160A instrument [Shimadzu, Singapore] at room temperature. About 1 mg of isolated compound dissolved in 20 ml of chloroform was used to record the spectrum [from 200-800 nm].

IR spectrometry

IR spectrum of isolated compound was recorded on a Perkin-Elmer FT-IR Spectrometer [Spectrum 2000] at room temperature. About 1 mg of isolated compound

dissolved in 10 ml of DMSO was used to record the spectrum [frequencies between 4000 and 400 cm^{-1}].

Liquid chromatography- Mass spectrometry [LC-MS]

Mass spectrum of the isolated compound was recorded on instrument HP 1100 MSD series [Palo Alto, CA] by electro spray ionization [ESI] technique with a flow rate of 0.2 ml min^{-1} on C-18 column and total run time of 40 min. Diode array was used as a detector. About 1 mg of isolated compound dissolved in five ml of methanol was used for recording the spectrum.

Two-dimensional Heteronuclear Multiple Quantum Coherence Transfer [2D-HMQCT] NMR Spectroscopy

NMR spectra were recorded on a Bruker DRX 500 NMR instrument [Rheinstetten, Germany] operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C at room temperature. A region from 0-12 ppm for ^{1}H and 0-200 ppm for ^{13}C was employed. Signals were referred to internal standard tetramethylsilane. About 45 mg of isolated compound dissolved in 0.75 ml of $CDCl_3$ was used for recording the spectra.

BIOACTIVE PROPERTIES OF ISOLATED COMPOUNDS

Following bioactive properties were determined according to the methods described in chapter 1

Antioxidant activity

DPPH radical scavenging activity, superoxide radical scavenging activity, lipid peroxidation-inhibitory activity, metal chelating activity and total reducing power

Antibacterial activity

Minimum inhibitory concentration and minimum bactericidal concentration

Platelet-aggregation inhibitory activity

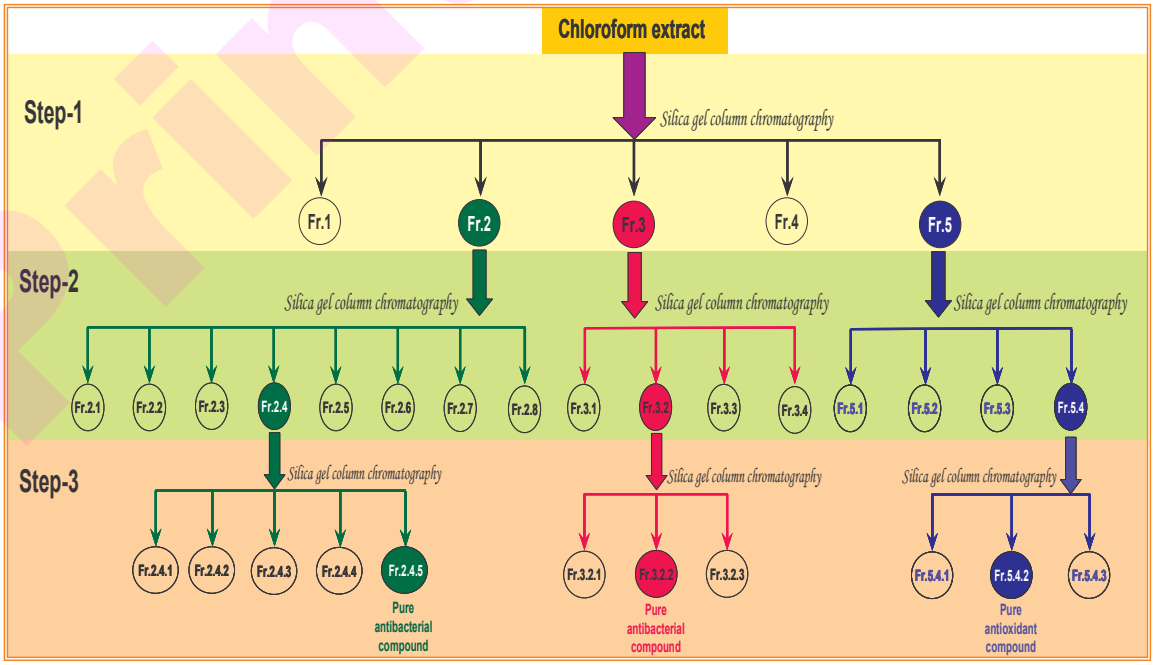
Cytotoxicity

Results and Discussion

1. Purification of antibacterial compound from Chloroform extract of mango ginger

Fractionation of chloroform extract using silica gel column chromatography has yielded about 82 fractions. These fractions having same number of spots with similar R_f values on TLC plate were pooled into five fractions [Fr.1- Fr.5] **[Fig. 2.1]**. All the five-pooled fractions were tested for antibacterial activity. Since fraction three [Fr.3] obtained from first step column chromatography showed high antibacterial activity **[Table 2.1]**, it was selected for further purification. Further purification of bioactive fraction three [Fr.3] yielded 21 fractions. Fractions having similar R_f values were pooled into four sub-fractions [Fr.3.1- Fr.3.4]. These four sub-fractions were tested for antibacterial activity. Sub-fraction two [Fr.3.2] obtained from the second step column chromatography **[Table 2.2]** that showed high antibacterial activity, hence, it was selected for further purification. Further purification active sub-fraction two [Fr.3.2] yielded 28 fractions. Fractions having similar R_f values on TLC plate were pooled into three sub-fractions [Fr.3.2.1-Fr.3.2.3]. Among

Fig 2.1: Schematic representation for isolation of antibacterial and antioxidant compounds from chloroform extract of mango ginger



these, sub-fraction two [Fr.3.2.2] obtained from third step chromatography **[Fig. 2.1]** showed a single spot on TLC. This pure compound was subjected to various spectroscopic techniques for the elucidation of the structure.

Table 2.1: Antibacterial activity of different fractions of chloroform extract

Bacteria	Inhibition zone [mm] * exhibited by five fractions obtained from chloroform extract				
	Fr. 1$^{\Psi}$	Fr. 2	Fr. 3	Fr. 4	Fr. 5
P. aeruginosa	--	--	--	--	--
E. coli	--	--	--	--	--
S. typhi	--	--	--	--	--
K. pneumoniae	--	--	--	--	--
E. aerogenes	--	--	--	--	--
P. mirabilis	--	--	--	--	--
Y. enterocolitica	--	--	--	--	--
M. luteus	15 ± 0.8^{b}	13 ± 0.5^{a}	17 ± 1.0^{c}	12 ± 0.3^{a}	--
S. aureus	--	--	15 ± 0.5^{a}	--	--
E. fecalis	12 ± 0.2^{a}	12 ± 0.6^{a}	14 ± 0.5^{b}	--	--
B. cereus	12 ± 0.5^{a}	13 ± 0.4^{a}	16 ± 0.6^{b}	--	--
B. subtilis	13 ± 0.3^{a}	14 ± 0.8^{a}	16 ± 0.7^{b}	--	--
L. monocytogenes	--	14 ± 0.7^{a}	15 ± 0.9^{a}	--	13 ± 0.8^{a}

* Each value represents mean of three different observations. Mean values with different superscripts [$^{a, b}$ and ^{c}] differ significantly at $P<0.05$.
$^{\Psi}$ Five fractions [Fr.1- Fr.5] were obtained from the chloroform extract by silica gel column chromatography.

Table 2.2: Antibacterial activity of fractions obtained from second step column chromatography

Bacteria	Inhibition zone [mm] * exhibited by four fractions [Fr.3.1-Fr.3.4] obtained by second step chromatography of fraction three [Fr. 3]			
	Fr. 3.1 $^{\Psi}$	Fr.3. 2	Fr. 3.3	Fr. 3.4
P. aeruginosa	--	--	--	--
E. coli	--	--	--	--
S. typhi	--	--	--	--
K. pneumoniae	--	--	--	--
E. aerogenes	--	--	--	--
P. mirabilis	--	--	--	--
Y. enterocolitica	--	--	--	--
M. luteus	14 ± 0.6^{a}	18 ± 1.0^{c}	15 ± 0.8^{b}	14 ± 0.5^{b}
S. aureus	14 ± 0.8^{a}	16 ± 0.3^{b}	--	13 ± 0.4^{a}
E. fecalis	--	13 ± 0.5^{a}	13 ± 0.3^{a}	12 ± 0.7^{a}
B. cereus	12 ± 0.4^{a}	16 ± 0.5^{b}	13 ± 0.5^{a}	--
B. subtilis	12 ± 0.9^{a}	16 ± 0.6^{b}	14 ± 0.7^{a}	--
L. monocytogenes	--	15 ± 0.8^{a}	--	--

* Each value represents mean of three different observations. Mean values with different superscripts [$^{a, b}$ and ^{c}] differ significantly at $P<0.05$.
$^{\Psi}$ Four fractions [Fr.3.1- Fr.3.4] were obtained by second step chromatography of "active fraction" [Fr.3].

ELUCIDATION OF STRUCTURE OF ANTIBACTERIAL COMPOUND

The pure compound was subjected to various spectroscopic analysis viz. UV, IR, LC-MS and 2D-HMQCT NMR to deduce the structure. The compound exhibited UV maxima at 242 nm corresponding to π- π* transition of C=C double bonds. IR spectral data showed O-H stretching at 3442 cm^{-1}, alkyl stretching at 2995 cm^{-1} and carbonyl stretching at 1670 cm^{-1} indicating the presence of -OH and olefinic carbonyl groups. LC-MS data showed parent molecular ion peak at 498 and other major M/e fragments at 219 and 279 **[Table 2.3].**

Table 2.3: Spectral data of the isolated compounds

Spectra	AMADALDEHYDE			DIFUROCUMENONOL			AMADANNULEN		
UV	242 nm			242 nm			242 nm		
IR	2964 cm^{-1} [alkyl stretching] 1720 cm^{-1} [carbonyl stretching] 2360 and 2336 cm^{-1} [C≡C groups]			3442 cm^{-1} [-OH stretching] 2995 cm^{-1} [Alkyl -CH stretching] 1670 cm^{-1} [C=O stretching] 1437 cm^{-1} and 1055 cm^{-1}			3403 cm^{-1} [OH stretching] 2996 cm^{-1} [Alkyl -CH stretching] 1657 cm^{-1} [C=O stretching] 1427 cm^{-1} [C-H bending] 1202 cm^{-1} [C-O stretching]		
MASS	930 [M^{+1}], m/z 686 and 187			498 [M^{+4}], *M/z* 279 and 219			377 [M^{+1}]		
NMR	**Signal**	**^{13}C [ppm]**	**^{1}H [ppm]**	**Signal**	**^{13}C [ppm]**	**^{1}H [ppm]**	**Signal**	**^{13}C [ppm]**	**^{1}H [ppm]**
	1	193.6	9.40	1-CH_3	19.0	1.48	1-C	133.4	--
	2-6	14.0-42.0	0.6-2.5	5-CH_3	14.5	0.65	2-CH_2	42.7	1.85
	7, 10	55.1, 56.1	--	10-CH_3	21.8	0.83	3-CH	34.6	1.40
	8, 9	67.9	--	14- CH_3	14.4	0.75	4-CH_2	29.1	1.35
	11-16	14.0-42.0	0.6-2.5	17 -CH_3	20.2	2.10	5-CH_2	21.5	1.30
	17, 20	130.6	6.73	21- CH_3	14.4	0.72	6-CH_2	24.4	1.30
	18,19	128.5	4.41, 4.88	9-CH_2	38.3	2.40	7-CH_2	22.8	1.35
	21-53	14.0-42.0	0.6-2.5	2-CH	55.5	1.15	8-CH	29.3	1.40
	54	57.8	3.39	6-CH	144.0	7.05	9-CH_2	39.7	1.85
	55-62	14.0-42.0	0.6-2.5	10-CH	29.5	1.25	10-C	133.1	--
	63	10.6	0.76	11-CH	136.1	4.45	11-CH_2	42.2	2.10
	OCH_2CH_3	57.8	3.39	12-CH	115.5	6.75	12-CH	58.1	3.80
	OCH_2CH_3	13.7	0.85	13-CH	56.5	3.95	13-CH_2	42.4	2.10
				17-CH	38.0	2.40	14-CH_3	19.0	0.85
				20-CH	144.0	7.10	15-CH	31.7	1.28
				23-CH	50.8	3.50	16-CH_2	29.6	1.25
				25-CH	65.5	4.55	17-CH	27.1	1.35
				3-CO	197.8	--	18-CH_2	33.7	1.40
				C-1	43.1	--	19-CH	39.0	2.33
				C-4	b	--	20-CH_2	33.2	1.40
				C-5	b	--	21-CO	175.4	--
				C-8	b	--	22-CH_2	60.1	4.15
				C-14	42.0	--	23-CH_3	14.1	0.90
				C-15	97.2	--	24-CH_3	20.2	0.85
				C-16	b	--			
				C-18	142.1	--			
				C-21	b	--			
				C-22	106.9	--			
				C-24	116.2	--			

Two-dimensional HMQCT spectrum showed as many as three CH_3 groups as singlet. Other three CH_3 signals were observed as doublets indicated that they were attached to CH carbons. The corresponding ^{13}C signals for the remaining six CH_3 groups were also observed. The region between 1.1 ppm and 2.4 ppm indicated quite a lot of CH and CH_2 signals with complex multiple splitting. The region between 3.5 ppm and 4.5 ppm in ^{1}H spectrum showed CH signals attached to OH groups along with corresponding ^{13}C signal. A carbonyl signals at 197.8 ppm was also observed. Some quaternary carbons and aromatic carbons in the region 97.2 ppm to 116.2 ppm were observed. Olefinic carbon signals were observed at 136.1 ppm. A furan signal at 144.1 ppm was also observed **[Table 2.3]**.

Based on all these spectral data the structure was deduced to be a probable precursor of difurocumenone and designated it as a "difurocumenonol" [13, 15, 23, 25-tetrahydroxy-1, 5, 10, 14, 17, 21-hexamethyl-7, 19-dioxahexacyclo [13.9.1.0 $^{2, 14}$.0 $^{4, 8}$.0 $^{16, 24}$.0 $^{18, 22}$] pentacosa-4 [8], 5, 11, 16 [24], 18 [22], 20-hexaen-3-one] **[Fig 2.2].** The repeated antibacterial activity-guided fractionation of chloroform extract by silica gel column chromatography yielded pure compound. The structure of the isolated compound was deduced as "difurocumenonol" after extensive analysis of spectroscopic data. This is the first antibacterial compound isolated and characterized from mango ginger rhizome.

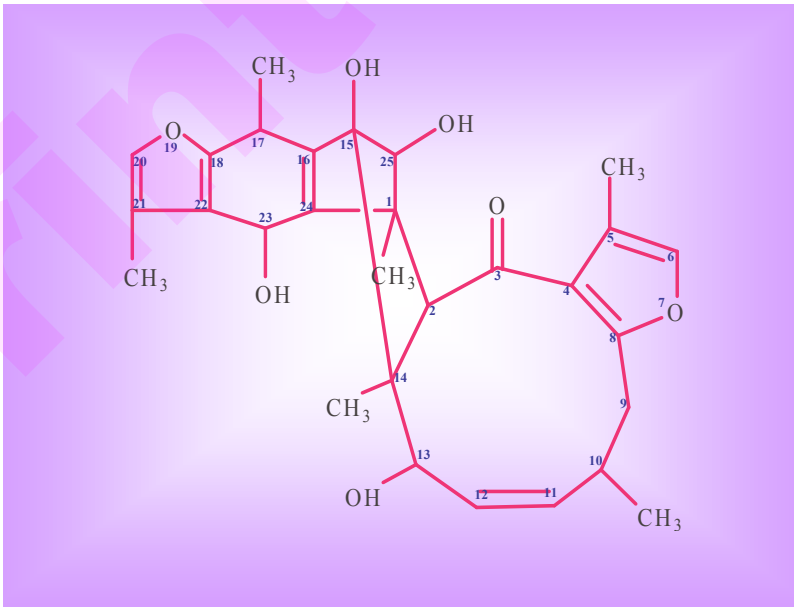


Fig. 2.2: Structure of Difurocumenonol

2. PURIFICATION OF ANTIBACTERIAL COMPOUND FROM CHLOROFORM EXTRACT OF MANGO GINGER

The fraction two [Fr.2] obtained from chloroform extract also showed high antibacterial activity **[Table 2.4]**, it was selected for further purification to obtain antibacterial compound.

Table 2.4: Antibacterial activity of eight sub-fractions obtained from second step column chromatography

Bacteria	Inhibition zone [mm] * exhibited by eight sub-fractions [Fr.2.1-Fr.2.8] obtained from second step chromatography							
	Fr. 2.1	Fr.2. 2	Fr. 2.3	Fr. 2.4	Fr. 2.5	Fr. 2.6	Fr. 2.7	Fr. 2.8
P. aeruginosa	--	--	--	--	--	--	--	--
E. coli	--	--	--	--	--	--	--	--
S. typhi	--	--	--	--	--	13 ± 0.4	--	--
K. pneumoniae	--	--	--	16 ± 0.9	--	--	--	--
E. aerogenes	--	--	--	--	--	--	13 ± 0.9	--
P. mirabilis	--	--	--	13 ± 0.8	--	14 ± 1.0	--	13 ± 0.4
Y. enterocolitica	--	--	--	--	--	--	--	--
M. luteus	12 ± 0.9	11 ± 1.3	18 ± 0.8	20 ± 0.8	15 ± 0.7	--	--	--
S. aureus	11 ± 1.3	12 ± 0.5	--	--	--	14 ± 0.9	--	--
E. fecalis	--	--	13 ± 0.8	16 ± 0.9	13 ± 0.6	--	14 ± 0.8	14 ± 0.8
B. cereus	13 ± 0.7	13 ± 0.7	16 ± 1.0	18 ± 1.0	15 ± 1.0	17 ± 0.6	13 ± 1.4	
B. subtilis	13 ± 0.5	14 ± 0.9	16 ± 0.9	19 ± 1.0	14 ± 0.9	16 ± 0.8	14 ± 0.5	
L. monocytogenes	--	--	--	15 ± 0.4	--	--	--	12 ± 0.5

* Each value represents mean of three different observations.
Ψ Eight fractions [Fr.2.1- Fr.2.8] were obtained by second step chromatography of "active fraction" [Fr.2].

Further purification of bioactive fraction two [Fr.2] yielded 33 fractions. Fractions having similar R_f values were pooled into eight sub-fractions [Fr.2.1- Fr.2.8]. These eight sub-fractions were tested for antibacterial activity. Sub-fraction four [Fr.2.4] obtained from the second step column chromatography **[Table 2.4]** showed high antibacterial activity, hence, it was selected for

Table 2.5: Antibacterial activity of five sub-fractions obtained from third step column chromatography

Bacteria	Inhibition zone [mm] * exhibited by five sub-fractions [Fr.2.4.1-Fr.2.4.5] obtained from third step chromatography				
	Fr.2.4.1	Fr.2.4.2	Fr.2.4.3	Fr.2.4.4	Fr.2.4.5
P. aeruginosa	17 ± 0.8	--	--	--	18 ± 0.8
E. coli	--	--	--	--	--
S. typhi	--	--	--	--	19 ± 0.8
K. pneumoniae	--	17 ± 0.8	--	16 ± 0.9	20 ± 0.8
E. aerogenes	16 ± 0.6	--	--	--	20 ± 0.8
P. mirabilis	--	--	--	13 ± 0.8	--
Y. enterocolitica	18 ± 0.7	--	--	--	17 ± 0.6
M. luteus	15 ± 0.6	12 ± 1.3	17 ± 0.6	20 ± 0.8	21± 0.8
S. aureus	13 ± 1.1	14 ± 0.5	--	--	15 ± 0.7
E. fecalis	--	--	14 ± 0.5	16 ± 0.9	14 ± 0.6
B. cereus	16 ± 0.7	15 ± 0.7	15 ± 1.3	18 ± 1.0	16 ± 1.0
B. subtilis	14 ± 0.5	17± 0.9	15 ± 0.7	19 ± 1.0	17 ± 0.9
L. monocytogenes	--	--	--	15 ± 0.4	20 ± 0.8

* Each value represents mean of three different observations.
Ψ Five fractions [Fr.2.4.1- Fr2.4.5] were obtained by second step chromatography of "active fraction" [Fr2.4].

further purification. Further purification of active sub-fraction four [Fr.2.4] yielded 21 fractions. Fractions having similar R_f values on TLC plate were pooled into five sub-fractions [Fr.2.4.1-Fr.2.4.5]. Among these, sub-fraction five [Fr.2.4.5] obtained from third step chromatography showed high antibacterial activity **[Table 2.5]** and a single spot on TLC. This pure compound was subjected to various spectroscopic techniques for elucidation of the structure.

ELUCIDATION OF STRUCTURE OF ANTIBACTERIAL COMPOUND

The structure of the isolated bioactive compound was deduced by analyzing UV, IR, MS and NMR spectral data. The compound exhibited UV λ maxima at 242 nm **[Table 2.3]** corresponding to π- π* transition indicating the presence of double and triple bonds. The IR spectra showed the alkyl stretching at ν 2964 cm^{-1} and carbonyl stretching at ν 1720 cm^{-1} indicating the presence of C-H and C=O groups respectively **[Table 2.3]**. The transmittance peaks at ν 2360 and 2336 cm^{-1} indicated the presence of C≡C groups. One-dimensional ^{1}H and ^{13}C NMR spectra gave the clear indication of the structure of the compound **[Table 2.3]**. The spectrum indicated the presence of two methyl groups at δ 0.76 and 0.85 ppm. One methyl signal was assigned for the methyl group located at the terminal end. The compound showed the presence of many CH_2 groups in the region of δ 0.6-2.5 ppm. The signal at δ 9.40 [s] ppm indicated the presence of aldehyde proton. The signals at δ 4.41, 4.88 and 6.7 ppm indicated the presence of allylic olefinic group [-CH=CH-CH=CH-]. The signals at δ 0.85 and 3.39 showed the presence of ethoxy group [-HC-O-CH_2CH_3]. The mass spectrum **[Table 2.3]** showed the M^+ ion at *m/z* 930 and other two ions at *m/z* values 686 and 187 indicating the cleavage at allylic olefininc and at ethoxy groups respectively. The corresponding ^{13}C signals were also observed in carbon NMR spectrum. The signal at δ 193.6 was assigned to carbon [C-1] of aldehyde group. The signals at δ 128.5 and 130.6 were assigned to carbons of allylic olefinic group at C-17 to 20. The signals at δ 13.7 and 57.8 were assigned to the methyl carbon and C-O-C in the ethoxy group. Several methylene carbons were observed in the region of δ 14.0-42.0. In addition, four methine carbon signals [C-7 to C-10] were observed at δ 55.1, 56.1 and 67.9 ppm indicating two C≡C groups. The terminal methyl carbon was observed at δ 10.6 ppm.

All these spectral characters indicated that, the molecule is aliphatic in nature with an empirical formula of $C_{65}H_{118}O_2$. Hence, based on the spectral data the structure was deduced and named as amadaldehyde [**Fig. 2.3**].

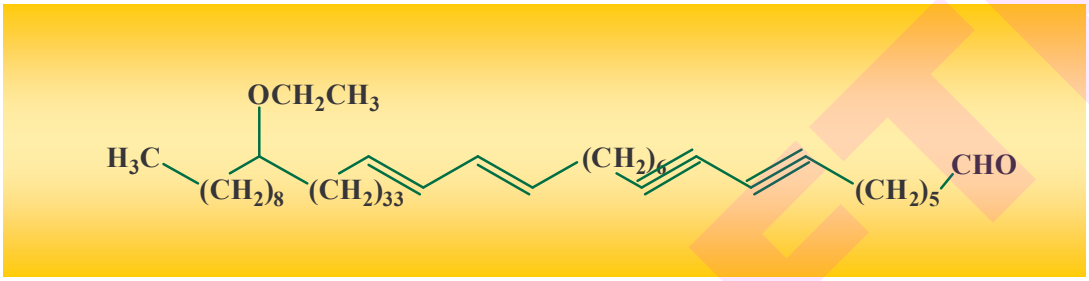
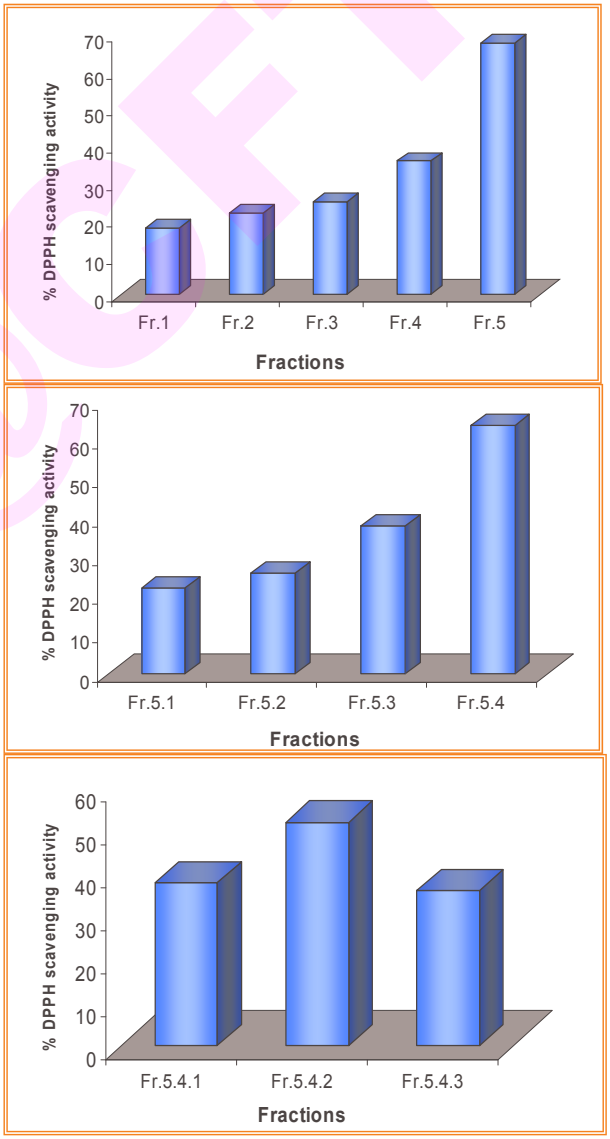


Fig. 2.3 : Structure of amadaldehyde

2. Purification of antioxidant compound from Chloroform extract of mango ginger

Mango ginger extracts were screened for antioxidant properties namely- DPPH radical scavenging activity, superoxide radical scavenging activity, lipid peroxidation-inhibitory activity, metal chelating activity and total reducing power. Chloroform extract exhibited promising lipid peroxidation inhibitory activity and moderate metal chelating activity. Hence, it was selected for the isolation of antioxidant compound. Fractionation of chloroform extract yielded five fractions [Fr.1-Fr.5]. Among these, fifth fraction [Fr.5] showed 68 % DPPH scavenging activity, while, Fr.1, 2, 3 and Fr. 4 showed 18 %, 22%, 25 % and 36 % DPPH scavenging activity [at 2 mg/ml] respectively **[Fig. 1.4].** Further purification of the fifth fraction [Fr.5] yielded four sub-fractions [Fr.5.1- Fr.5.4], wherein fourth sub-fraction [Fr.5.4] exhibited high DPPH scavenging activity [64 %], while Fr.5.1, Fr. 5.2 and Fr.5.3 showed 22 %, 26 % and 38 % DPPH scavenging activity respectively [at 2 mg/ml] **[Fig. 2.4].** The active sub-fraction four [Fr.5.4] was further purified using silica gel [100-200 mesh] column chromatography and yielded three fractions [Fr.5.4.1-Fr.5.4.3]. Among these, fraction two [Fr.5.4.2] showed high DPPH scavenging activity [52 %], while Fr. 5.4.1 and Fr. 5.4.3 exhibited 38 % and 36 % DPPH radical scavenging activity respectively [at 2 mg/ml] **[Fig.**

Fig 2.4: DPPH radical-scavenging activity of different fractions obtained from chloroform extract by three steps of column chromatography



* each value represents mean of three different observations at a concentration of 2 mg/ml

2.4]. The second fraction [Fr.2``] showed a single spot on TLC. The purity of the isolated compound was confirmed by HPLC. The schematic representation for the isolation of antioxidant compound is given in **figure 1.5.** The isolated compound was subjected to spectroscopic analysis to elucidate the structure.

Elucidation of structure of antioxidant compound

The structure of bioactive compound was elucidated after analyzing the data obtained by various spectroscopic techniques. The molecular weight was determined using LC-MS. Mass spectrum showed the parent molecular ion *m/z* at 377. Elemental analysis [VARIO EL III CHNS Elementar] revealed that the compound consists of 74.22 % of carbon and 10.10 % of hydrogen. The molecular formula of the compound was found to be $C_{24}H_{40}O_3$. The UV maxima were observed at 242 nm, which indicated the presence of double bond. Identification of specific functional groups was carried out using IR spectra. The O-H stretching observed at 3403 cm^{-1} is the characteristic of hydrogen bonded -OH group. The stretching at 2996 cm^{-1} and 1657 cm^{-1} can be attributed to C-H and C=O stretching vibrations respectively. The alkyl C-H bending vibration has been observed at 1427 cm^{-1}. The C-O stretching vibration at 1202 cm^{-1} indicates the presence of ester moiety **[Table 2.3].**

A Two Dimensional Heteronuclear Quantum Coherence Transfer [2D-HMQCT] NMR spectrum was recorded along with 1-Dimensional ^{1}H and ^{13}C NMR spectra, which gave clear indication of carbon skeleton of the compound **[Table 2.3].** No peaks were detected for aromatic moiety both in ^{1}H and ^{13}C NMR spectra. The $-CH_3$ protons showed peaks in the region 0.8-0.9 ppm. There are three $-CH_3$ groups and the corresponding ^{13}C peaks were detected between 14-20 ppm. The cyclic $-CH_2$ and -CH protons exhibited peaks in the range 1.2-1.7 ppm and the corresponding ^{13}C peaks have been observed between 21-34 ppm. The four $-CH_2$ protons of cyclopentyl ring showed doublet at 2.1 ppm indicating clearly both the $-CH_2$ groups are attached to -CH carbon. The -CH group attached to -OH moiety showed ^{1}H peak at 3.8 ppm and the corresponding ^{13}C peak at 58 ppm. The quartet at 4.15 ppm exhibited by ethyl $-CH_2$ group indicates that it has been attached to $-CH_3$ group. The corresponding ^{13}C peak has been observed at 60 ppm. Further, the ^{13}C signals at 178 ppm and 133 ppm clearly confirms the presence of -CO and C=C groups. Based on these spectral data, the probable structure of the compound was deduced **[Fig. 2.5]** and tentatively named it as 'amadannulen'. The compound has been

reported for the first time from mango ginger rhizome. The bioactive compounds that have ten-member carbon ring structures have been reported from *Euphorbia semiperfoliata* [Giovanni, *et al.,* 1998], *Mikania micrantha* [Hongjuan, *et al.,* 2004], *Neurolaena oaxacana* [Claus, *et al.,* 1999], *Elephantopus tomentosus* [Toshimitsu, *et al.,* 1999] and gorgonian corals [Ping-Jyun, *et al.,* 2004]. The conformation of the ten-membered ring in this type of compounds has been defined by the endocyclic torsion angles by earlier reports [Toshimitsu, *et al.,* 1999].

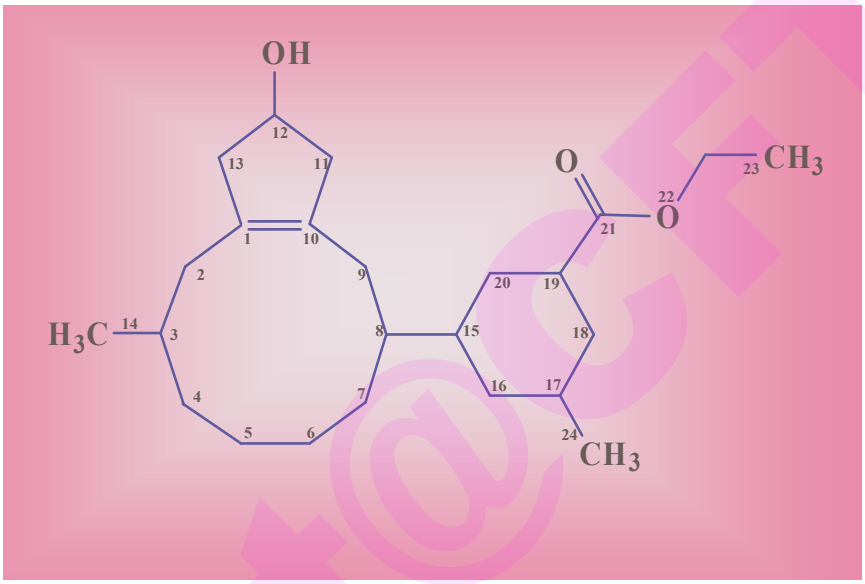


Fig. 2.5: Structure of amadannulen

BIOACTIVE PROPERTIES OF ISOLATED COMPOUNDS

BIOACTIVE PROPERTIES OF DIFUROCUMENONOL

ANTIOXIDANT ACTIVITY

DPPH radical scavenging activity

Difurocumenonol showed DPPH radical scavenging activity with an IC_{50} of 180µg/ml **[Table 2.6].** The antioxidant activity of this compound may be attributed to the presence of -OH and C=O groups, as reported in structurally similar type of compounds [Chen and Ho, 1995; Nikolaos, *et al.,* 2003].

Table 2.6: Antioxidant activity of difurocumenonol

Antioxidant activity	EC_{50} value [µg/ml]	
	Difurocumenonol	BHA
DPPH radical scavenging activity	180 ± 3 [b]	5 ± 0 [a]
Superoxide radical scavenging activity	114 ± 3 [a]	258 ± 2 [b]
Lipid peroxidation inhibitory activity	61 ± 2 [a]	94 ± 1 [b]
Metal chelating activity	196 ± 2 [a]	N.D.

Each value represents mean of three different observations ± S.D.

Superoxide radical scavenging activity

Difurocumenonol showed superoxide radical scavenging activity with an IC_{50} 114 µg/ml **[Table 2.6].** The activity was higher than that of BHA [IC_{50}=258 ± 2 µg/ml]. Superoxide radicals are generated during the normal physiological process mainly in mitochondria. Although superoxide anion is by itself a weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as singlet oxygen both of which contribute to the oxidative stress [Dahl and Richardson, 1978; Meyer and Isaksen, 1995]. Therefore superoxide radical scavenging by antioxidants has physiological implications.

Lipid peroxidation-inhibitory activity

Difurocumenonol showed lipid peroxidation inhibitory activity with an IC_{50} of 61 µg/ml **[Table 2.6].**. Lipid peroxidation is a free radical mediated propagation of oxidative insult to polyunsaturated fatty acids involving several types of free radicals. Its termination occurs in biological system through enzymatic means or by radical scavenging activity by antioxidants [Heim *et al.*, 2002].

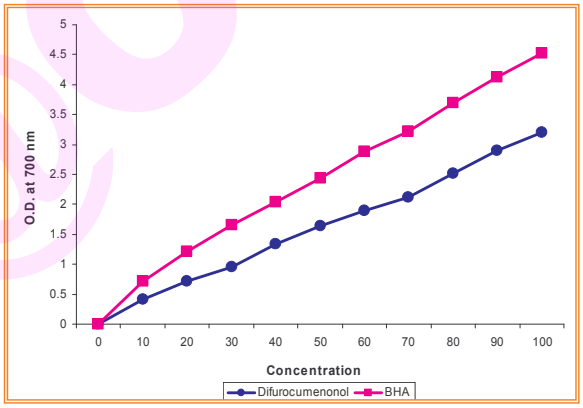
Metal chelating activity

Difurocumenonol showed metal chelating activity with an IC_{50} of 196 µg/ml **[Table 2.6].** Iron is known to generate free radicals through the Fenton and Haber-Weiss reaction [Halliwell and Gutteridge, 1990]. Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the catalyzing transition metal in lipid peroxidation [Duh *et al.,* 1999]. Hence, difurocumenonol can play a very important role as a natural antioxidant.

Total reducing power

The reducing capacity of a compound from Fe^{3+}/ferricyanide complex to the ferrous form may serve as a significant indicator of its antioxidant capacity [Meir *et al.,* 1995]. **Figure 2.6** shows the reductive capability of difurocumenonol compared with BHA. A gradual increase in reducing power with increasing concentration of difurocumenonol was observed. The result revealed that the difurocumenonol is a potential electron donor and also could react with free radicals, converting them to more stable products and terminating the radical chain reaction.

Fig 2.6: Total reducing power of difurocumenonol [a] and BHA [b]



[a] concentration of test sample was 2 mg/ ml
[b] concentration of test sample was 1 mg/ ml

ANTIBACTERIAL ACTIVITY

Minimum inhibitory concentration

Difurocumenonol is a novel compound exhibited an interesting antibacterial activity. It was more effective against wide spectrum of bacteria viz. *P. aeruginosa, S. typhi, K. pneumoniae, E. aerogenes, Y. enterocolitica, M. luteus, S. aureus, E. fecalis, B. cereus, B. subtilis* and *L. monocytogenes*. Difurocumenonol showed antibacterial activity against *B. cereus, B. subtilis,* and *M. luteus* with MIC 40, 40 and 60 ppm respectively. In addition, difurocumenonol also inhibited the growth of five Gram-negative bacteria viz. *P.*

aeruginosa, *S. typhi*, *K. pneumoniae, E. aerogenes,* and *Y. enterocolitica* with MIC of 100, 80, 160, 120 and 60 ppm respectively **[Table 2.7].** However, these were not inhibited by the chloroform extract. High antibacterial activity of difurocumenonol for wide range of bacteria may be attributed to its structural components. It possesses four-hydroxyl, six-methyl and one-carbonyl groups along with two furan rings.

Difurocumenonol by virtue of possessing two furan rings, which are aromatic in nature, thus possesses units, which are capable of exhibiting delocalization of electrons, a feature that has been proposed to be responsible for increased antibacterial activity [Ultee *et al.* 2002]. These may account for the enhanced activity of difurocumenonol compared to its source extract. The bioactivity of difurocumenonol may be similar to several other compounds like curcumin, capsaicin, caffeic acid, carvacrol, eugenol and menthol [Apisariyakul *et al.* 1995; Cichewicz *et al.* 1996; Ali-shtayeh *et al.* 1997; Cowan, 1999].

Table 2.7: Antibacterial activity of difurocumenonol

Bacteria	Difurocumenonol	
	MIC [ppm] *	Bactericidal activity
P. aeruginosa	100	NO
E. coli	--	NO
S. typhi	80	NO
K. pneumoniae	160	NO
E. aerogenes	120	NO
P. mirabilis	--	NO
Y. enterocolitica	60	YES
M. luteus	60	YES
S. aureus	60	YES
E. fecalis	120	NO
B. cereus	40	YES
B. subtilis	40	YES
L. monocytogenes	40	YES

* Each value represents mean of three different observations. The MIC values also represent the bactericidal concentrations for all the bacteria

In addition, presence of hydroxyl groups in plant derivatives has been associated with many biological activities [David 1995; Halliwell *et al.* 1995; Tess *et al.* 1999; Laurence *et al.* 2001; George *et al.* 2002; Adewole *et al.* 2004; Sara 2004]. Hydroxyl group may be actively responsible for depletion of ATP dependent metabolic functions, ultimately leading to cell death [Ultee *et al.* 2002]. Further, presence of oxygen function in the framework of the compound increases the antibacterial properties [Naigre *et al.* 1996]. Further investigations are in progress to test the mode and site of action of the difurocumenonol.

Determination of bactericidal effect

Difurocumenonol was found to be bactericidal against wide range of bacteria tested **[Table 2.7].** Minimum Bactericidal Concentration [MBC] ranged from 40 to 60 ppm for Gram-positive bacteria [*M. luteus, S. aureus, B. cereus, B. subtilis,* and *L. monocytogenes*].

Interestingly difurocumenonol also showed bactericidal activity against only one of the six Gram-negative bacteria [*Y. enterocolitica*] at 60 ppm. It appeared that effective MIC also represents the effective bactericidal concentration for the bacteria tested.

Platelet-aggregation inhibitory activity

Regulation of platelet activity by using specific pharmacological agents has proven to be a successful strategy for the prevention of thrombosis. Antiplatelet agents, such as aspirin, dipyridamole, thienopyridines, and platelet glycoprotein IIb/IIIa antagonists have amply demonstrated their utility in preventing and treating coronary artery thrombosis [Van De Graa and Steinhubl, 2000; Calverley, 2001] Difurocumenonol exhibited platelet-aggregation inhibitory activity with an IC_{50} of 135 µg/ ml in the presence of ADP as an agonist. Results obtained by the incubation of human or animal platelets with isolated polyphenols suggest that the platelet-aggregation inhibitory properties may be attributed to the inhibition of TxA2 formation [You, *et al.,* 1999], thromboxane receptor antagonism [Hubbart *et al.,* 2003], protein kinase C activation [Ganet-Payrastre *et al.,* 1999] and phosphoinositide synthesis.

Cytotoxicity

Difurocumenonol showed less cytotoxicity against A-549 cell line when compared to Vero cell lines with CTC_{50} of 120 µg and 145 µg/ ml respectively, in MTT assay. Similar results were obtained in SRB assay, wherein, difurocumenonol exhibited cytotoxicity with CTC_{50} of 128 µg and 148 µg/ ml respectively. The mode of action on cytotoxicity is not well established, but there are reports that protein binding ability, in particular membrane protein of cell lines may affects the cell growth and its viability [Damianaki *et al.,* 2000].

BIOACTIVE PROPERTIES OF AMADALDEHYDE

ANTIOXIDANT ACTIVITY

DPPH radical scavenging activity

Amadaldehyde showed DPPH radical scavenging activity with an IC_{50} of 170µg/ml **[Table 2.8].** The antioxidant activity of this compound may be attributed to the presence of -OH and C=O groups, as reported in structurally similar type of compounds [Chen and Ho, 1995; Nikolaos, *et al.,* 2003].

Table 2.8: Antioxidant activity of amadaldehyde

Antioxidant activity	EC_{50} value [µg/ml]	
	Amadaldehyde	BHA
DPPH radical scavenging activity	170 ± 3 [b]	5 ± 0 [a]
Superoxide radical scavenging activity	120 ± 3 [a]	258 ± 2 [b]
Lipid peroxidation inhibitory activity	92 ± 2 [a]	94 ± 1 [a]
Metal chelating activity	158 ± 2 [a]	N.D.

* Each value represents mean of three different observations ± S.D.
Mean values with different superscripts [a, b and c] differ significantly at $P < 0.05$

Superoxide radical scavenging activity

Amadaldehyde showed superoxide radical scavenging activity with an IC_{50} 120 µg/ml **[Table 2.8].** The activity was higher than that of BHA [IC_{50}=258 ± 2 µg/ml]. Superoxide radicals are generated during the normal physiological process mainly in mitochondria. Although superoxide anion is by itself a weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as singlet oxygen both of which contribute to the oxidative stress [Dahl and Richardson, 1978; Meyer and Isaksen, 1995]. Therefore superoxide radical scavenging by antioxidants has physiological implications.

Lipid peroxidation-inhibitory activity

Lipid peroxidation is a free radical mediated propagation of oxidative insult to polyunsaturated fatty acids involving several types of free radicals. Its termination occurs in biological system through enzymatic means or by radical scavenging activity by antioxidants [Heim *et al.*, 2002]. Amadaldehyde showed lipid peroxidation inhibitory activity with an IC_{50} of 92 µg/ml **[Table 2.8].** Hydroxyl radical and perferryl ion are highly reactive, and act as the actual initiating species for cellular lipid peroxidation [Fridovich, 1989]

Metal chelating activity

Amadaldehyde showed metal chelating activity with an IC_{50} of 158 µg/ml **[Table 2.8].** Iron is known to generate free radicals through the Fenton and Haber-Weiss reaction [Halliwell and Gutteridge, 1990]. Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Metal ion chelating capacity plays a significant role in antioxidant mechanism since it reduces the concentration of the catalyzing transition metal in lipid peroxidation [Duh *et al.,* 1999]. Hence, amadaldehyde can play a very important role as a natural antioxidant.

BIOACTIVE PROPERTIES OF AMADANNULEN

ANTIOXIDANT ACTIVITY

DPPH radical scavenging activity

Amadannulen showed moderate DPPH radical scavenging activity [EC_{50}= 178 µg/ml] compared to BHA [EC_{50}= 5 µg/ml] **[Table 2.9].** Interestingly, no activity was observed in the chloroform extract. A linear increase in free radical scavenging ability of amadannulen was observed with its increasing concentration. DPPH radical scavenging by antioxidants has been attributed to their hydrogen-donating ability of -OH and -CH_3 groups.

Table 2.9: Antioxidant activity of amadannulen

Antioxidant activity	EC_{50} value [µg/ml]*	
	Amadannulen	BHA
DPPH radical scavenging activity	178 ± 2 ^{b}	5 ± 0 ^{a}
Superoxide radical scavenging activity	132 ± 4 ^{a}	258 ± 2 ^{b}
Lipid peroxidation inhibitory activity	65 ± 3 ^{a}	94 ± 1 ^{b}
Metal chelating activity	216 ± 2 ^{b}	N.D. ^{b}

* Each value represents mean of three different observations ± S.D.
Mean values with different superscripts [$^{a, b}$ and ^{c}] differ significantly at $P < 0.05$

Superoxide scavenging activity

Amadannulen exhibited significant superoxide radical scavenging activity **[Table 2.9],** with an EC_{50} of 132 µg/ ml compared to BHA [EC_{50} of 258 µg/ ml]. Superoxide anion plays an important role in the formation of reactive oxygen species [ROS] such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA [Dahl and Richardson, 1978; Halliwell and Gutteridge, 1989].

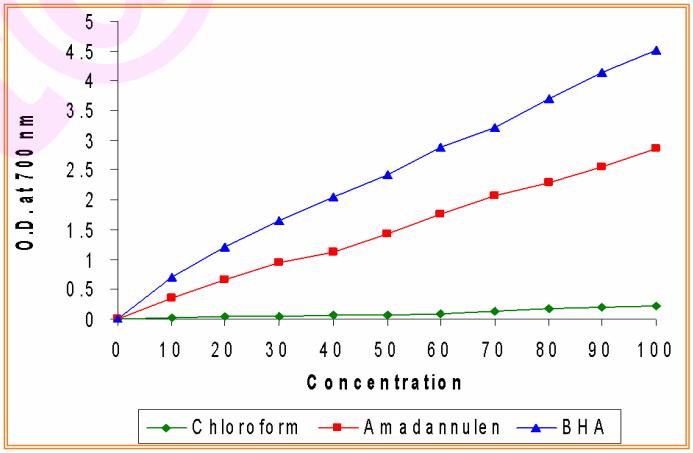
Lipid peroxidation Inhibitory activity

Lipid peroxidation causes destabilization and disintegration of the cell membrane, leading to liver injury, atherosclerosis, kidney damage, aging, and susceptibility to cancer [Rice-Evans and Burdon, 1993]. Highest lipid peroxidation inhibitory activity was observed in amadannulen with EC_{50} of 80 µg/ml compared to chloroform extract and BHA with EC_{50} of 65 µg/ml and 94 µg/ml respectively [**Table 2.9].** Lipid peroxidation inhibitory activity was mainly attributed to the number of hydroxyl groups, solubility and hydrophobicity of the compounds [Sopheak and Betty, 2002]. Presence of methyl groups in amadannulen may be responsible for high lipid peroxidation inhibitory activity.

Metal chelating activity

Amadannulen showed metal chelating activity with EC_{50} of 216 µg/ml compared to chloroform extract with EC_{50} of 142 µg/ ml **[Table 2.9]**. It was reported that the structures containing two or more of the following functional groups: -OH, -SH, -COOH, $-PO_3H_2$, C=O, -NR2, -S- and -O- in a favorable structure-function configuration is responsible for metal chelating activity [Lindsay, 1996; Yuan *et al.,* 2005]. Amadannulen, which is having both -OH and C=O functional groups can contribute for the metal chelating activity. Ferrous ions can stimulate lipid peroxidation by Fenton reaction, and also accelerates peroxidation by decomposing lipid hydro peroxides into peroxyl and alkoxyl [Halliwell, 1991; Gulcin *et al.,* 2003]. Since these are the most effective proxidants in the food system [Yamaguchi *et al.,* 1988] the high chelating ability of amadannulen may be beneficial.

Fig.2.7: Total reducing power of amadannulen [a], chloroform extract [c] and BHA [b]



[a, c] concentration of test sample was 2 mg/ ml
[b] concentration of test sample was 1 mg/ ml

Total reducing power

The reducing capacity of a compound from Fe^{3+} /ferricyanide complex to the ferrous form may serve as a significant indicator of its antioxidant capacity [Meir *et al.,* 1995]. **Fig. 2.7** shows the reductive capability of amadannulen and chloroform extract compared with BHA. A linear increase in reducing power with increasing concentration of amadannulen was observed.

ANTIBACTERIAL ACTIVITY

Minimum inhibitory concentration

Amadannulen exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria. It was more effective against wide spectrum of bacteria viz. *P. aeruginosa, S. typhi, K. pneumoniae, E. aerogenes, Y. enterocolitica, M. luteus, S. aureus, E. fecalis, B. cereus, B. subtilis* and *L. monocytogenes*. The most striking increase in activity was observed against *B. cereus, B. subtilis,* and *M. luteus* with MIC of 70, 60 and 100 ppm respectively. It also inhibited the growth of five Gram-negative bacteria viz. *P. aeruginosa*, *S. typhi*, *K. pneumoniae, E. aerogenes,* and *Y. enterocolitica* **[Table 2.10].**

Table 2.10: Antibacterial activity of amadannulen

Bacteria	Amadannulen	
	MIC [ppm] *	MBC [ppm] *
P. aeruginosa	180	--
E. coli	--	--
S. typhi	140	--
K. pneumoniae	190	--
E. aerogenes	220	--
P. mirabilis	--	--
Y. enterocolitica	90	--
M. luteus	100	180
S. aureus	80	--
E. fecalis	170	--
B. cereus	70	120
B. subtilis	60	130
L. monocytogenes	80	--

* Each value represents mean of three different observations.

Determination of bactericidal effect

Amadannulen was found to be bactericidal against *M. luteus, B. cereus, B. subtilis*] **[Table 2.10]** with Minimum Bactericidal Concentration [MBC] of 180, 120 and 130 ppm respectively. It did not show bactericidal activity against other bacterial strains tested even at higher concentrations. Amadannulen was bacteriostatic for most of the strains.

Recently, investigations have shown that the site of action of cyclic hydrocarbons, [including terpene hydrocarbons] is at the cell membrane [Sikkema, *et al.,* 1995]. Terpenoid compounds were shown to permeabilize the membranes, making them swell.

This inhibits respiratory enzymes, which led to a partial dissipation of the pH gradient and electrical potential, which are crucial to the energy system in a cell [Sikkema, *et al.,* 1992 and 1994].

The effect of several terpenoids on microbes has also been studied. Most of the terpenoids tested were found to inhibit oxygen uptake and oxidative phosphorylation. It was suggested that the free -OH group may be a key to their activity [Knobloch, *et al.,* 1985]. The mode of action of several terpenoids has been studied, but the actual structure-activity relationships of the terpenoids are not well understood. It is known that carbonylation of terpenoids increases their bacteriostatic activity but not necessarily their bactericidal activity [Naigre, *et al.,* 1996].

Platelet-aggregation inhibitory activity

Amadannulen also showed potential platelet-aggregation inhibitory activity with an IC_{50} of 123 µg/ ml in the presence of ADP as an agonist.

Cytotoxicity

Amadannulen exhibited cytotoxicity against A-549 cell line when compared to Vero cell lines with CTC_{50} of 141 µg and 163 µg/ ml respectively, in MTT assay. Similar results were obtained in SRB assay, wherein, amadannulen exhibited cytotoxicity with CTC_{50} of 144 µg and 160 µg/ ml respectively.

Conclusion

Three bioactive compounds were successfully isolated and characterized from chloroform extract of mango ginger rhizome. Difurocumenonol, amadaldehyde and amadannulen are novel bioactive compounds not reported previously from any other sources. All the three bioactive compounds exhibited high antibacterial and multi system antioxidant activities. Among these, difurocumenonol and amadannulen have also showed potential platelet-aggregation inhibitory activity and less toxicity. Difurocumenonol was used as a biomarker for the determination of quality index and physiological maturity of mango ginger rhizome during development. The details are furnished in the next chapter.

Chapter - 3

Physiological Role of Bioactive Compounds during Development of Mango Ginger Rhizomes

All great truths begin as blasphemies
– George Bernard Shaw

INTRODUCTION

Participation of bioactive compounds in an array teleological functions as precursors in imparting characteristic flavour, color, defense intermediaries and health benefiting factors in fruits, vegetables, and rhizomes were well documented [Tholl, 2006]. Temporal variation in the concentration of bioactive molecules is regulated by a complex interaction between intrinsic plant factors and external factors, both abiotic and biotic [Herms and Mattson, 1992; Beckman, 2000; Booij-James *et al.,* 2000]. Interestingly, they showed site and cells specificity in accumulation, as a function of maturity [Kause *et al.,* 1999; Samanani, 2002]. Lack of such studies in Mango ginger is apparent despite its pharmaceutical importance and exotic mango flavour.

Coordinated biochemical alterations during development and growth mango ginger plant determine the quality of rhizomes in terms of maturity, peak accumulation of bioactive compounds in rhizome. Extensive review of literature showed lack of any maturity index for harvest. This is critical in agriculture to know when to harvest the plant. Successful identification of three bioactive compounds in mango ginger gave an impetus to test teleological role of these bioactive compounds to define the maturity of rhizome for harvest with preferred nutritional or nutraceutical or pharmaceutical quality. Among the isolated compounds Difurocumenonol exhibited the highest antimicrobial and antioxidant activity along with other functional property. Since it is highly stable and easy to estimate accumulation pattern of difurocumenonol and phenolics along with changes in solubles and storage components as a function of physiological maturity in mango ginger were studied. A time course study of these changes from the time of planting to harvest, which ranged from 0 to 240 days at a time interval of 30 days, were carried out. The details were presented in this chapter.

Materials and Methods

Sample collection

The rhizomes were collected from commercial plot near Hassan, Karnataka, India. The first sampling time [60 days after planting] was conducted when the rhizomes initiated their growth. Subsequently, the samples were collected at 90, 120, 150, 180, 210 and 240 days after planting. Each sample was prepared from rhizomes obtained from five mango ginger plants that were harvested randomly from five different beds. All the biochemical analysis and other experiments were carried out in triplicates.

Chemical composition of mango ginger

Sample preparation

About 500 g of mango ginger rhizomes were sliced, homogenized, and squeezed in two-layered muslin cloth, to extract the complete juice. The juice was centrifuged at 8,000 rpm for 20 min at 4°C and used to determine pH, titrable acidity, total soluble solids [TSS], sugar content, protein content, and phenolic content and antioxidant activities.

The pulp [residue] left after the extraction of juice is still a rich source of bound phenolic compounds. Hence, the pulp was homogenized with 80% methanol to extract the phenolics completely. The extraction was repeated till it became colorless. The methanol extract was filtered, and evaporated using a rotary evaporator. The extract was dissolved and diluted to a final volume of 100 mL with 80% methanol. The mixture was centrifuged at 8000 rpm at refrigerated temperature [4°C] for 20 min and used for determination of total phenolic content, DPPH radical scavenging activity and total reducing power.

Extraction and quantification of difurocumenonol by HPLC

To study the accumulation and quantification of difurocumenonol, the fresh rhizomes [10 g] were homogenized with chloroform till they became colorless. The extract was filtered and concentrated using a rotary evaporator and freeze dried before using the sample for HPLC analysis. Difurocumenonol [the isolated compound] and chloroform extracts obtained during different developmental stages were tested using a LC-10AT liquid chromatograph [Shimadzu, Singapore] equipped with 300 x 4.6 mm i.d., 5 μ, Thermo Hypersil C-18 column [Bellefonte, PA, USA]. The gradient programme used for mobile phase was, methanol: water, as follows; 0 min, 25:75, v/v; 5 min, 40:60, v/v;

10 min, 50:50, v/v; 20 min, 70:30, v/v; 40 min, 90:10, v/v; 60 min, 100:0, v/v; with a flow rate of 1 ml/ min. UV detection was carried out with a SPD-M10A VP diode array detector [Shimadzu, Singapore], operated at 240 nm

Determination of phenolics

The total phenolic content in mango ginger juice as well as pulp was determined with the modified method of Taga *et al.* [1984]. In brief, 100 µL of sample was mixed with 2 mL of 2% aqueous sodium carbonate solution. After 3 min, 100 µL of 50% Folin-Ciocalteau phenol reagent was added to the mixture. After 30 min of incubation at room temperature, absorbance was measured at 750 nm against a blank. Total phenolic content was calculated on the basis of the standard curve of gallic acid.

DPPH radical scavenging activity and Total reducing power

The details of the methodologies have been described in materials and methods of chapter 1

Reducing sugars, total sugars and total protein content

Reducing sugars and total sugars were determined by using the method as described by Ranganna [2001]. The total protein content was determined by the Bradford method [1976], using bovine serum albumin [BSA; Sigma Chemical, St. Louis, USA] as a standard protein.

pH, titrable acidity and total soluble solids

pH of the fresh juice was measured using Control Dynamics pH meter calibrated with standard buffer pH 7. Titrable acidity was determined by AOAC [1990] method. The total soluble solids [TSS] were determined by a digital refractometer [ATAGO RX-5000, ATAGO, Japan] calibrated with distilled water. Mango ginger juice was passed through Whatman No.1 filter paper using vacuum before analysis.

Statistical analysis

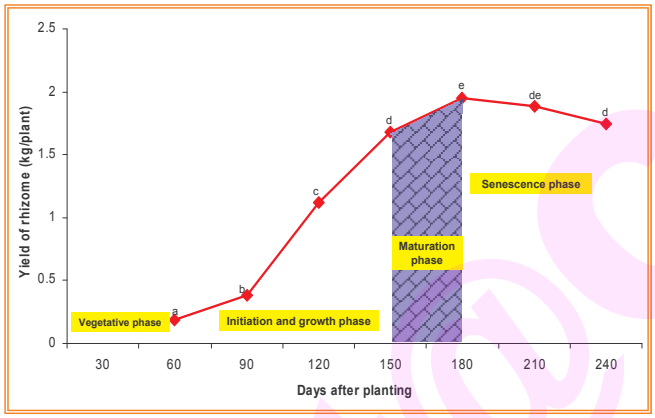
The data was subjected to Duncan's Multiple Range Test [DMRT] to determine significant differences [$P < 0.05$].

Results and Discussion

Developmental stages and yield of mango ginger

Synthesis and accumulation of bioactive compounds along with other soluble and storage components were investigated during developmental stages of mango ginger rhizome from 60 to 240 days. Four distinct phases of growth and development in mango ginger plant were defined, namely; [1] vegetative growth phase; [2] rhizome initiation and growth phase; [3] maturation phase; and [4] senescence phase **[Fig. 3.1].** Active vegetative growth extends up to 60 days from planting. The events were evident by the formation of six to eight pairs of green leaves. Initiation of rhizomes was observed little earlier than 60 days from planting. The yield of rhizomes increased rapidly after 90 days and highest yield was recorded on 180 days after planting. A conspicuous drying of leaves after 150 days and falling of leaves after 180 days gives a visual marker for maturity of mango ginger rhizomes. The maturation of rhizomes was characterized by the increase in size, weight and yield that may be due to rapid accumulation of bioactive and storage components like starch, proteins and phenolics. In contrast, total sugar and reducing sugar contents decreased. The decline in all these components after 180 days heralds the onset of the senescence phase.

Fig. 3.1: Yield of mango ginger rhizome during developmental stages

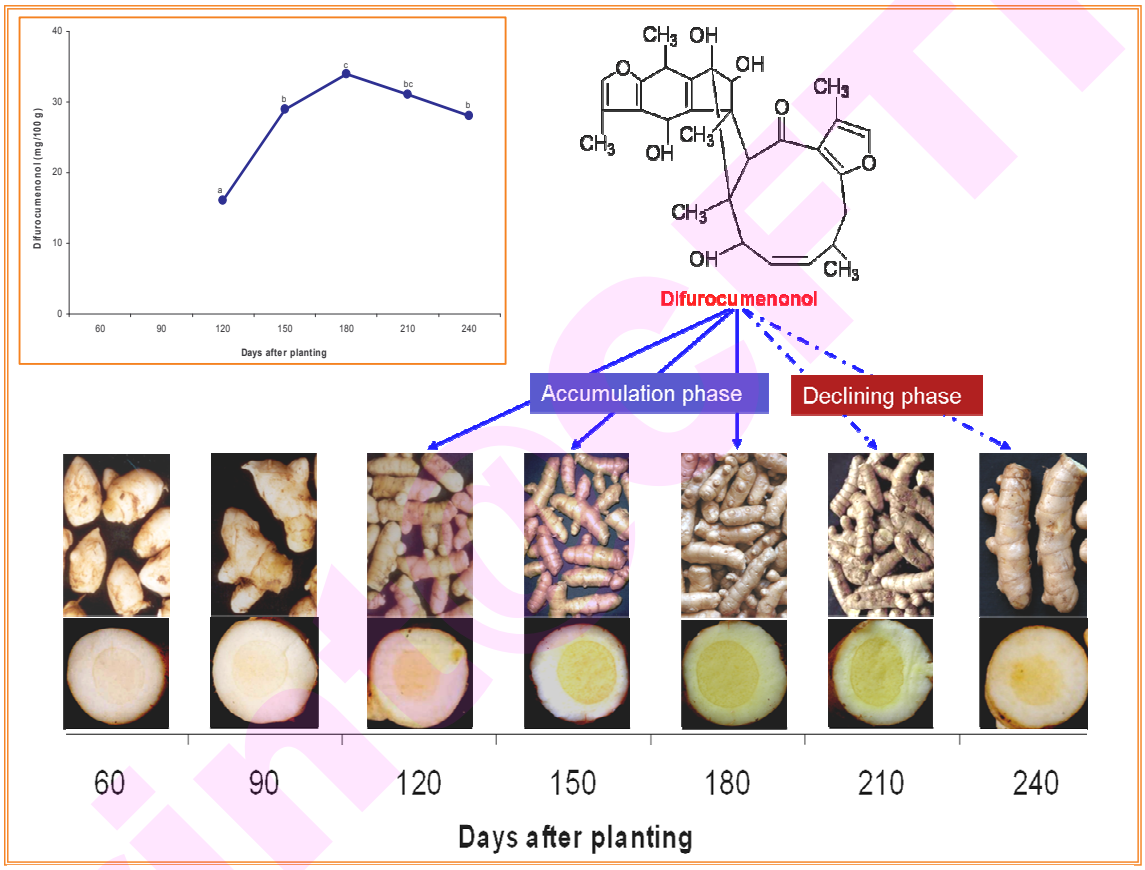


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Differentiation of rhizome

The internal tissue of rhizome remained undistinguished as white, juicy mass of cells until 90 days of planting **[Fig. 3.2].** Differentiation of central, circular, yellow colored pith that was demarked from the surrounding white cortex tissue was observed after 120

Fig. 3.2: Different stages of growth and development of mango ginger rhizomes with transverse section showing internal color. Inset: Concentration of difurocumenonol during developmental stages of mango ginger rhizome



Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

days. The ratio between pith and cortex during growth period remained constant and measured [1:1, w/w]. With the onset of maturity, a significant increase in pith [2:1, w/w] was observed. In addition, change in color of pith from yellow to greenish yellow was exhibited. Dispersion of yellow color to cortex region was also visible **[Fig. 3.2].** Coincidently, these changes were in accordance with physiological maturity of rhizomes. Thus, they could be identified as visual maturity indices.

Accumulation of difurocumenonol

Difurocumenonol **[Fig. 3.2]** - a terpenoid compound, has been reported to be an antimicrobial compound against a wide range of microbes [Policegoudra *et al.,* 2007a]. Accumulation of difurocumenonol in mango ginger rhizomes during developmental stages [60 to 240 days] was carried out using HPLC. Newly initiated rhizomes contained no difurocumenonol and it was first observed in 120 days old rhizomes. The highest concentration of difurocumenonol [34 mg/100 g] was noticed in 180 days old rhizomes **[Fig. 3.2].** The synthesis and accumulation of this compound in the developing rhizome during growth and maturation of rhizome is essential to counteract bacteria and fungi. The terpenoids play a major role in contributing to flavor components and defense intermediates in plants [Aharoni, *et al.,* 2004]. Participation of difurocumenonol in synthesis of flavor components in mango ginger may be responsible for its decrease in concentration during senescence. The tissue-specific biosynthesis and accumulation of difurocumenonol has yet to be identified. It is interesting to note that the pattern of synthesis, accumulation and degradation of difurocumenonol is in accordance with growth, maturation and senescence of the rhizome. Hence, it has been identified as nutraceutical marker to determine the physiological maturity of rhizome and as a harvest index.

Physiological role of difurocumenonol

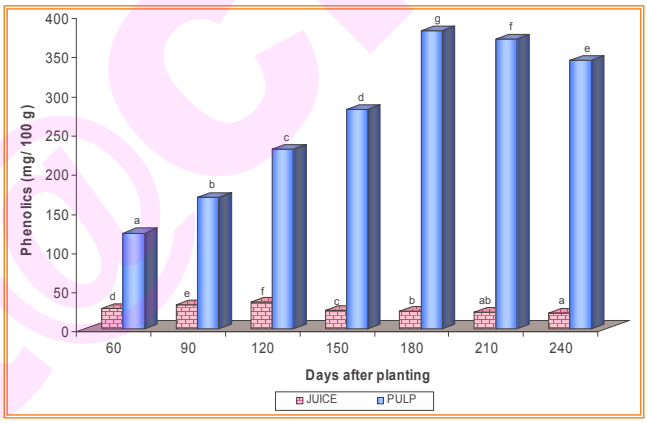
It may be a biological inevitability for mango ginger rhizome to develop compounds of multifunctional activity to counteract the diversified underground abiotic and biotic challenges. Difurocumenonol proved to be one such compound with multifunctional properties found in mango ginger. Antimicrobial nature of difurocumenonol [Policegoudra *et al.,* 2007a] can effectively thwart the constant challenges posed by underground pathogens. In addition, it possesses a wide range of antioxidant activities and other bioactive properties. High antioxidant property provides stability against auto oxidation. Thus difurocumenonol ensures prolonged antimicrobial activity during growth, senescence and dormancy period of rhizome. High lipid peroxidation inhibitory activity and metal chelating activity of difurocumenonol may act as competitive inhibitor for sprouting, due to its high oxygen demand. Thus, it may offer physiological protection to tide over dormancy. The terpenoids play a major role in contributing to flavor components and defense intermediates in plants [Aharoni, *et al.,* 2004]. Difurocumenonol being a terpenoid compound, its derivatives may participate as

metabolic intermediaries in synthesis of flavour during distress storage conditions and during senescence depending upon the physiological needs. Thus, it may be responsible for the persistence of mango flavour in rhizome even after shriveling beyond the level of commercial acceptability. The role of difurocumenonol derivatives in various other physiological activities is interesting and worth investigating.

Phenolic content

The phenolic content was quantified in mango ginger at different developmental phases. Accumulation of phenolics occurred immediately after the initiation of rhizome formation, with an initial concentration of 122 mg/100 g and 26 mg/100 g in pulp and juice respectively **[Fig. 3.3].** The abundance of phenolics on the 120th day in juice and 180th day in pulp is attributed to increase in weight of rhizomes during growth and maturation phase of the rhizome. The concentration of phenolics in pulp was ten times higher than that of phenolics in juice, at all the stages of development of rhizome. High content of phenolics may be an essential component for defense against various pathogens that are constantly challenging the underground rhizome, as evident from the antimicrobial activity of mango ginger extracts containing phenolics with other bioactive compounds such as difurocumenonol and amadannulen that were isolated from mango ginger rhizome [Policegoudra *et al.,* 2007a, 2007b]. Phenolics from various plant sources and their contribution to antimicrobial and other biochemical responses are well documented [Benner, 1993; Bennett and Wallsgrove, 1994]. It appears that peak accumulation of phenolics in pulp on the 180th day may herald the onset of senescence in mango ginger rhizome. Decrease in phenolics may be attributed to strengthening of the plant cell walls by polymerization into lignans and lignins [Randhir and Shetty, 2005]. Therefore, the synthesis and accumulation

Fig. 3.3: Changes in phenolic content in mango ginger rhizome during development



Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

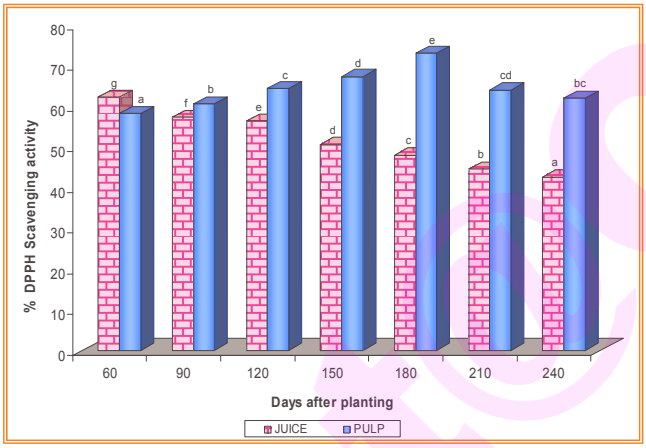
pattern of phenolics may be used as an indicator to differentiate the physiological maturity and quality of rhizome in mango ginger.

ANTIOXIDANT ACTIVITY

DPPH radical scavenging activity

Mango ginger juice exhibited a gradual decrease in DPPH radical scavenging activity until 240 days during developmental stages. In contrast, mango ginger pulp showed a gradual increase in DPPH radical scavenging activity, which was highest on 180 days of development and decreased thereafter until 240 days **[Fig. 3.4].** DPPH radical scavenging activity of pulp has been attributed to the concentration of difurocumenonol and total phenolics. Antioxidant activity of amadannulen and mango ginger extracts and phenolics were reported in mango ginger and also in other vegetables [Chen and Ho, 1995; Nenadis *et al.,* 2003; Policegoudra *et al.,* 2007b]. In addition, terpenoids also have medicinal properties such as anti-carcinogenic, antimalarial, anti-ulcer, hepatoprotective, antioxidant and antimicrobial activity [Rodriguez-Concepcion, 2004; Policegoudra *et al.,* 2007a]. An increase of antioxidant activity associated with accumulation of bioactive compounds like phenolics and difurocumenonol at 180 days could be a better method to determine the optimum physiological maturity to harvest mango ginger rhizomes, rather than conventional harvest from 200 to 240 days after planting.

Fig. 3.4: Changes in DPPH radical scavenging activity of mango ginger rhizome during development

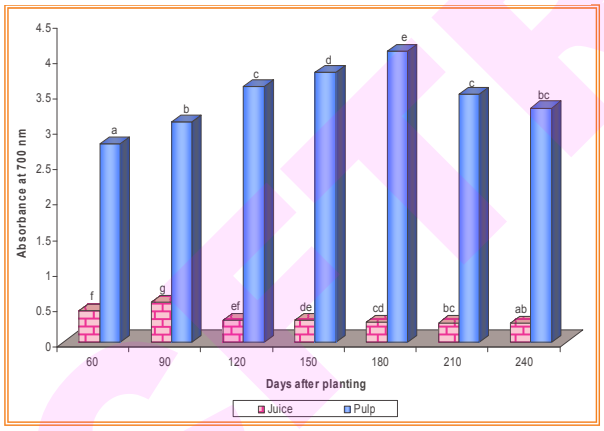


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Total reducing power

The reducing power of pulp increased with rate of growth and development and recorded the highest concentration after 180 days. There was an initial spurt in reducing power of mango ginger juice up to 90 days and decrease thereafter **[Fig. 3.5].** The reducing capacity of samples from ferricyanide complex to the ferrous form may serve as a significant indicator of its antioxidant capacity [Meir, *et al.,* 1995]. The reducing power of mango ginger pulp was almost ten times higher than that of juice. This may be attributed to the presence of high concentration of difurocumenonol, amadannulen and phenolics in the pulp of mango ginger as reported earlier [Policegoudra *et al.,* 2007a and 2007b].

Fig. 3.5: Changes in total reducing power of mango ginger during development

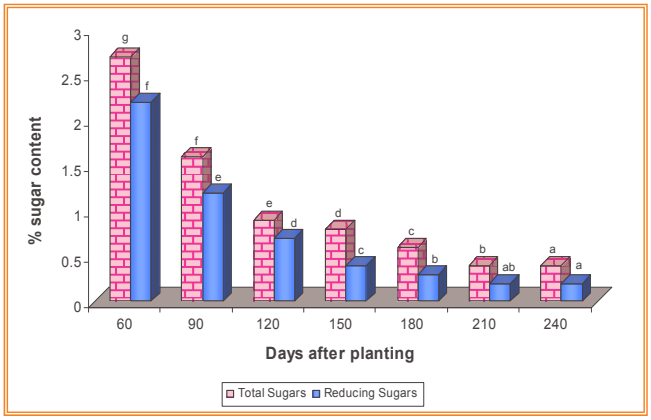


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

Total sugars and reducing sugars

Total sugars and reducing sugars decreased gradually as the rhizome attained maturity **[Fig. 3.6].** There was a steep decline in sugar during the growth phase of rhizome, because it acts as an active source for supply of energy. Reduction of total and reducing sugars was very significant on 180th day of harvest. The decrease during the maturation phase of rhizome may be attributed to formation of storage components such as starch and its derivatives. Structure, biochemical components and functional properties of mango

Fig. 3.6: Changes in total sugar and reducing sugar content in mango ginger rhizome during development



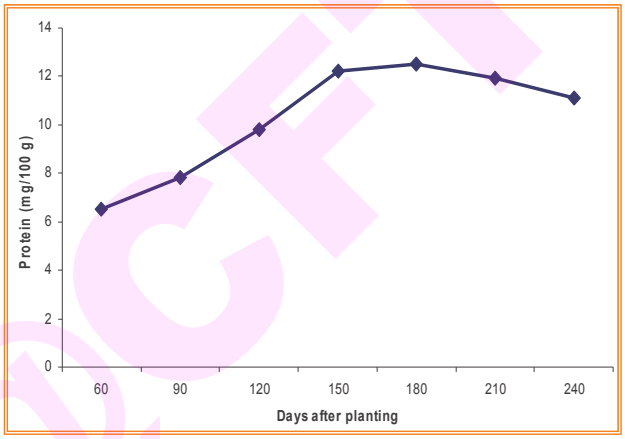
Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

ginger starch is well documented [Policegoudra and Aradhya, 2007]. The starch is essential to protect the energy resource of rhizome during the dormant stage. It may acts as a store house of sugars that are necessary for the development of shoot, as in other tubers [Lewis, 1994].

Total protein content

The protein concentration increased gradually during growth and maturation period. The concentration ranged from 6.5 to 11.1 mg/100g, with a peak accumulation period at 180 days **[Fig. 3.7].** Increase in concentration of protein is in accordance with increase in phenolics and difurocumenonol. The accumulation of storage proteins were reported in tubers like potato, sweet potato, yam, taro, cassava, where the major role was to act as stores of nitrogen, sulfur and carbon, that are required to survive periods of adverse conditions and to provide nutrients for shoot formation [Shewry, 2003]. The storage proteins also exhibit biological activities that are consistent with a role in protecting the tubers against pests, pathogens and also abiotic stresses as antioxidants and enzyme inhibitors [Shewry, 2003]. Protein abundance decreased during the senescence phase **[Fig. 3.7].** Protein synthesis and accumulation is complex phenomenon governed by the physiological and abiotic factors during developmental stages of the rhizome.

Fig. 3.7: Changes in total protein content in mango ginger rhizome during development



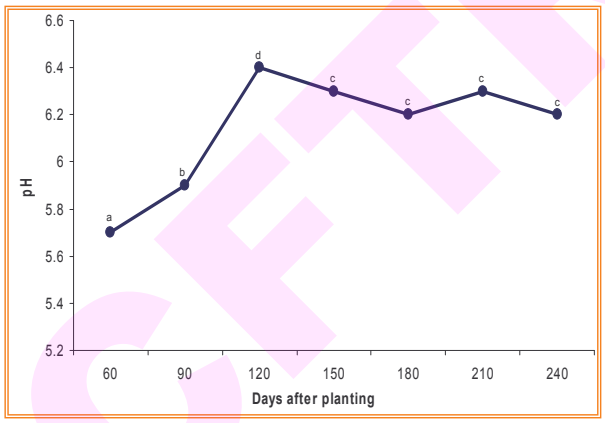
Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

pH, titrable acidity and total soluble solids

The pH of mango ginger juice increased gradually with advance in maturity and was highest on the 120th day. Later it remained constant throughout with a pH range of 6.2 to 6.4 [**Fig. 3.8**].

A gradual increase in percentage of total solubles with increase in growth and advancement of maturity of mango ginger rhizome was noticed until 120 days. The trend was reversed recording a decrease up to 240 days because the solubles, which mainly contains sugars, form a source of energy for various physiological functions that varied with different developmental stages of mango ginger rhizome.

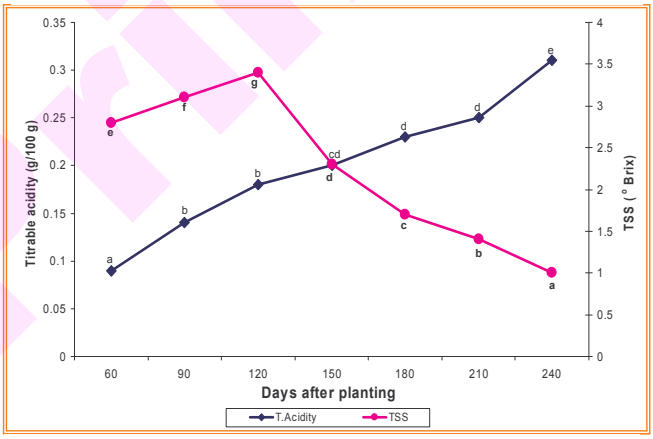
Fig. 3.8: Changes in pH of mango ginger rhizome during development



Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

In contrast, titrable acidity of mango ginger gradually increased throughout the growth and developmental phases of mango ginger rhizome **[Fig. 3.9].** The increased or decreased concentration of pH and titrable acidity along with TSS appears to be governed by the variation in composition of cellular metabolites and their functions. It is interesting to note that the point of intersection between total soluble solids and acidity coincides with 150 days **[Fig. 3.9]** of developmental stages of mango ginger. This point of may terminate the growth phase and initiate the onset of maturation.

Fig. 3.9: Changes in total soluble solids and titrable acidity in mango ginger rhizome during development



Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at p < 0.05.

Maturity markers for mango ginger

The present study clearly indicated that the synthesis and accumulation pattern of difurocumenonol, phenolics and protein concentrations served as bioactive markers to determine the physiological maturity for harvest of the mango ginger rhizomes. Difurocumenonol was first observed in 120 day old rhizomes after planting, while phenolics and protein accumulation were detected after 60 days after planting. However, they follow a similar pattern of accumulation as a function of growth and maturation of the rhizome. During the growth phase there was a gradual increase in concentration of phenolics, difurocumenonol and total proteins, while their accumulation was maximum after 180 days. High concentration of soluble and storage components along with bioactive compounds is of paramount important, since their concentrations depleted with delay in harvest after 180 days, which indicates the onset of senescence phase. The distinct patterns of these biochemical markers were associated with conspicuous display of drying and detachment of leaves from the rhizome. This provides a visual clue for maturation of rhizome in mango ginger plant. The various maturity indices displayed on 180 days **[Table 3.1]** from planting were found to be optimum for harvest, compared with the conventional harvest ranges from 200 to 240 days.

Table 3.1 Maturity standards for harvest of mango ginger rhizome

Sl No.	Maturity markers	Index
	Biochemical	
1	✓ Phenolic content [mg/ 100 g]	380
2	✓ Difurocumenonol concentration [mg/ 100 g]	34
3	✓ Protein content [mg/ 100 g]	12
	Morphological	
4	✓ Drying and detachment of leaves [days]	180
	Characteristics of rhizome	
5	✓ Size of rhizome; a] *Length [cm]*	12-13
	b] *Diameter [cm]*	3-4
6	✓ Lemon yellow pigmentation of pith region [days]	180
7	✓ Pith and cortex ratio of rhizome	2:1

Conclusion

For the first time, a new set of optimum maturity indices for harvest of mango ginger rhizome has been established, based on the synthesis and accumulation pattern of difurocumenonol and phenolics during development of mango ginger rhizome. Its maturation is also directly influenced by coordinated alterations of several biochemical factors. Interestingly, they demonstrated a distinct pattern of accumulation governed by the physiological maturity of the rhizome during development. The importance of these patterns highlighted in the present investigation which I believe has commercial application.

Chapter - 4

Functional Properties and Biochemical Changes in Mango ginger Rhizomes during Storage

If I have seen further than others,
It is by standing upon the shoulders of giants.
-Isaac Newton

INTRODUCTION

Underground storage organ vegetables generally are subject to a number of physiological disorders that limits the ability of postharvest technology to maintain freshness and quality. Root vegetables are also susceptible to high water loss, a major limitation for postharvest storage [Burton, 1982], and various bacteria and fungi that are present in the soil cause decay during storage [Brecht, 2003]. Postharvest losses of vegetables have been estimated to be as high as 25-50 % due to poor postharvest handling and storage temperature management [Nunes and Emond, 2003]. Postharvest deterioration of mango ginger includes physiological loss of water, and shriveling followed by sprouting. The antioxidant components of rhizome are highly susceptible to postharvest physiological stress, temperature and duration of storage. Tropical and subtropical root vegetables are also susceptible to chilling injury below 7°C, which is accompanied by a loss of flavour and sprouting [Ravi and Aked, 1996].

Temperature is the most critical factor that alleviates or aggravates the physiological and bioactivity in mango ginger rhizome after harvest. In areas in which cold storage facilities are not available, it is a common practice to rebury the rhizomes or leave them unharvested. Because of high medicinal properties and its importance in the food industry as a source of raw mango flavour and for its nutraceutical properties, there is a need to retain quality after harvest. Hence, storage studies of freshly harvested rhizomes were planned to evaluate the antioxidant activity and biochemical quality changes as a function of storage temperature and time. This investigation also aimed to find out optimum storage temperature to extend the shelf life of mango ginger rhizome. The details of the work carried out presented in this chapter.

MATERIALS AND METHODS

Storage conditions and sample size

Mango ginger rhizomes were harvested fresh from the commercial farm at Sultan's Bathery, Kerala, India. They were immediately transferred to laboratory. The rhizomes were washed, air-dried and stored in plastic mesh basket at three different temperatures; [1] Room Temperature [RT] [25 ± 1°C]; [2] Low temperature [LT] [14 ± 1°C]; [3] Chilling temperature [CT] [4 ± 1°C], with three replicates of 8 kg each. The samples were analyzed periodically at different time intervals: 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 days. All analysis was carried out in triplicates.

Weight loss, chilling injury and sprouting

Weight loss was measured at different time intervals using an electronic scale. Weight loss during postharvest storage was determined by subtracting sample weights from their previous recorded weights and presented as % of weight loss compared to initial weight. The rhizomes stored at CT were subjected to analysis of susceptibility to chilling injury which is characterized by surface lesions, discoloration, water-soaked tissue, internal browning, loss of mango flavor, and softening of tissue. Similarly, the rhizomes stored at RT were observed for initiation and elongation of sprouts throughout the storage period.

BIOCHEMICAL COMPOSITION

Sample preparation

About 500 g of rhizomes stored at RT, LT and CT were sliced and homogenized and squeezed in two-layered muslin cloth, to extract the complete juice. The juice was centrifuged at 8000 rpm for 20 min at 4°C and used to determine pH, titrable acidity, TSS, sugar content, protein content, phenolic content and antioxidant activity.

The pulp [residue] left after the extraction of juice is still a rich source of bound phenolic compounds. Hence, the pulp was homogenized with 80 % methanol to extract the phenolics completely. The extraction was repeated till it became colourless. The methanolic extract was filtered, and evaporated using a rotary evaporator [Buchi Rotavapor R-124, Switzerland]. The extract was dissolved and diluted to a final volume of 100 ml with 80 % methanol. The mixture was centrifuged at 8000 rpm at refrigerated

temperature [4°C] for 20 min and used for determination of total phenolic content and DPPH radical scavenging activity.

Determination of phenolics

The total phenolic content in mango ginger juice as well as pulp was determined using a modification of the modified method of Taga *et al.* [1984]. In brief, 100 µl of sample was mixed with 2 ml of 2 % aqueous sodium carbonate solution. After 3 min, 100 µl of 50 % Folin-Ciocalteau's phenol reagent was added to the mixture. After 30 min of incubation at room temperature, absorbance was measured at 750 nm against a blank. Total phenolic content was calculated on the basis of a standard curve of gallic acid.

DPPH radical scavenging activity

DPPH [1, 1-diphenyl-2-picrylhydrazyl] radical scavenging activity was determined according to a method previously described [Blois, 1958; Bondet *et al.,* 1997]. The test samples [100 µl] were mixed with 0.8 ml of Tris-HCl buffer [pH 7.4] to which 1 ml of DPPH [250 µM in ethanol] was added. The mixture was shaken vigorously and left to stand for 30 min. Absorbance of the resulting solution was measured at 517 nm in a UV-Visible Spectrophotometer [UV-160A, Shimadzu co. Japan]. The radical scavenging activity was measured as a decrease in the absorbance of DPPH. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity.

pH, titrable acidity and total soluble solids

pH of the fresh juice was measured using a Control Dynamics pH meter calibrated with standard buffer pH 7. Titrable acidity was determined by the AOAC [1990] method. The total soluble solids [TSS] were determined by a digital refractometer [ATAGO RX-5000, ATAGO, Japan] calibrated with distilled water. Mango ginger juice was passed through a filter paper Whatman No.1 using vacuum before analysis.

Reducing sugars, total sugars and total protein contents

Reducing sugars and total sugars were determined by using the method as described by Ranganna [2001]. The total protein content was determined by the Bradford method [1976], using bovine serum albumin [BSA; Sigma Chemical, St. Louis, USA] as a standard protein.

Extraction and quantification of difurocumenonol by HPLC

To study the concentration of difurocumenonol, the fresh rhizomes [10 g] were homogenized with chloroform till they became colorless. The extract was filtered and concentrated using a rotary evaporator and freeze dried before using the sample for HPLC analysis. Difurocumenonol [the isolated compound] and chloroform extracts obtained from mango ginger rhizomes stored at different temperatures at different storage time were tested using a LC-10AT liquid chromatograph [Shimadzu, Singapore] equipped with 300 x 4.6 mm i.d., 5 μ, Thermo Hypersil C-18 column [Bellefonte, PA, USA]. The gradient programme used for mobile phase was, methanol: water, as follows; 0 min, 25:75, v/v; 5 min, 40:60, v/v; 10 min, 50:50, v/v; 20 min, 70:30, v/v; 40 min, 90:10, v/v; 60 min, 100:0, v/v; with a flow rate of 1 mL/min. UV detection was carried out with a SPD-M10A VP diode array detector [Shimadzu, Singapore], operated at 240 nm.

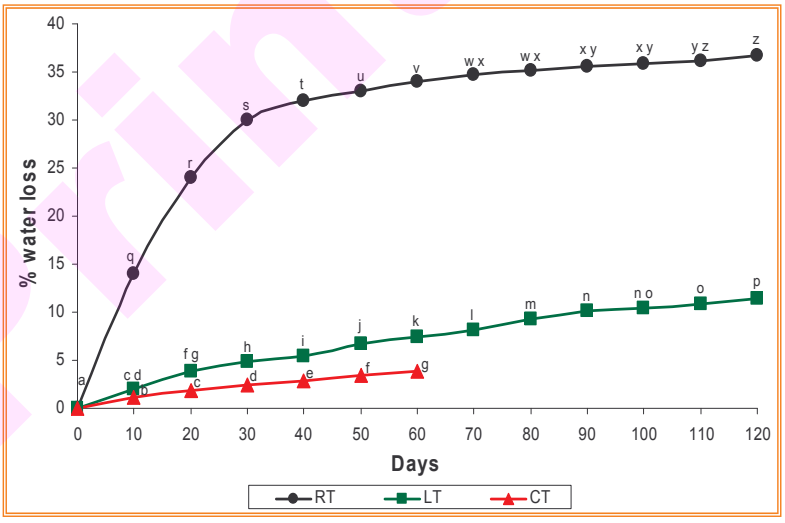
Results and Discussion

Harvest causes an artificial interruption of the rhizome's natural life cycle, where water can no longer be taken up from the soil, while biochemical changes and antioxidant activity are difficult to interrupt. The physiological changes and antioxidant activity of rhizomes at three different temperatures, Room temperature [RT], low temperature [LT] and chilling temperature [CT] were studied. The rhizomes stored at RT and LT were analyzed periodically for biochemical changes and antioxidant activity up to 120 days, and CT stored rhizomes only for 60 days because of spoilage due to chilling injury.

Water loss, sprouting and chilling injury

The cut portion of the mango ginger rhizome forms the main avenue for increased loss of water due to increased rate of respiration and transpiration that are governed directly by storage temperature and time. Water loss was higher in the rhizomes stored at room temperature than at low temperature **[Fig. 4.1].**

Fig. 4.1: Changes in physiological loss of water in mango ginger rhizomes during storage



Each value is a mean of three different observations. Values showed by different letters for each line are significantly different at $p < 0.05$.

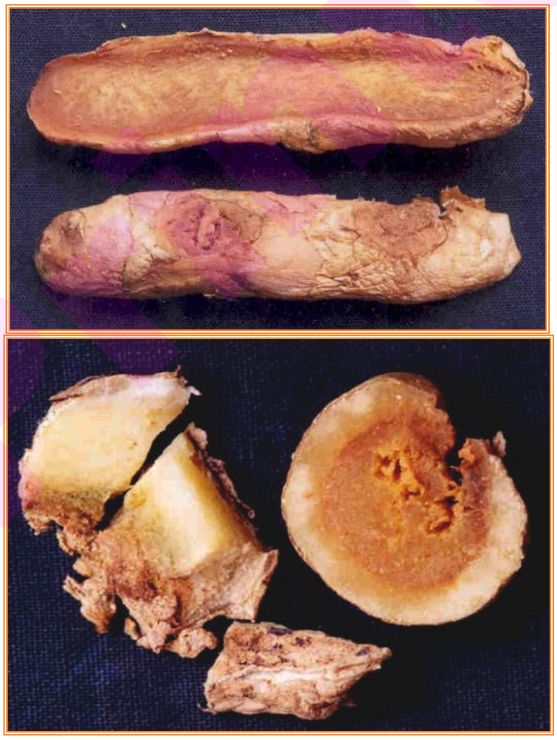
Rapid loss of water at a rate of 1 % per day occurred in rhizomes during the initial 30 days, and later at a rate of 0.07 % until 120 days of storage at RT. About 33 to 36 % of water loss on the 50th day of storage from the time of harvest coincides with manifestation of visible shriveling of the rhizomes. Further water loss of 3 to 4 % leads to a commercially objectionable level of shriveling and rhizomes become unsalable. Many vegetables

become unsalable and manifest shriveling after loosing 7-20 % of their weight [Ben-Yehoshua and Rodov, 2003]. Rhizomes stored at 14°C and 4°C lost about 8 and 2 g/ kg/month respectively. The pivotal physiological role of water in mango ginger is maintenance of rhizome turgidity, freshness and quality as is the case for most perishables [Herppich *et al.,* 2000].

The initiation of sprouting was observed on the 80th day in rhizomes stored at RT **[Fig. 4.2].** There was a significant increase in protein content prior to sprouting of rhizome at RT, which may be due to *de novo* synthesis of proteins. Increase in proteins and nucleic acids prior to sprouting were observed in many vegetables [Macdonald and Osborne, 1988]. Protein synthesis required for sprouting, while nucleic acid synthesis for sprout elongation [Madison and Rappaport, 1968; Alam *et al.,* 1994].

Fig. 4.2: Sprouted mango ginger rhizomes

Fig 4.3: Chilling injury in mango ginger rhizomes

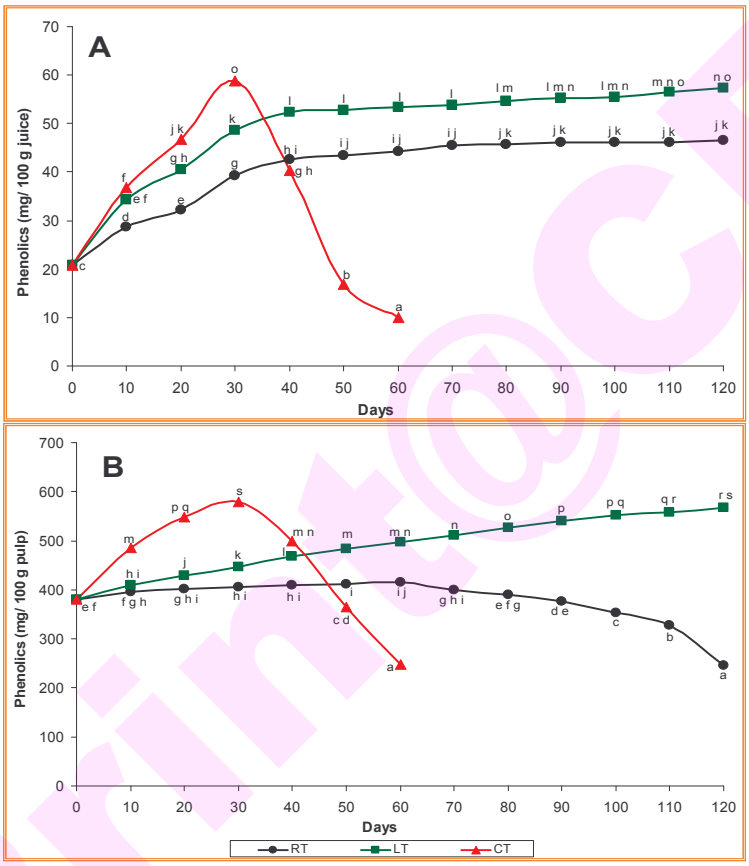


Rhizomes stored at CT failed to sprout due to chilling injury. The potential symptoms of chilling injury were water-soaked lesions with tissue softening and browning **[Fig. 4.3].** The injured tissue eventually lost its characteristic mango flavour followed by off-odours. Until recently, it was believed that chilling injury involved membrane modification and changes in proteins [Hausman *et al.,* 2000] and increased permeability and ion leakage [Murata, 1990; Saltveit, 2002]. Extensive studies have demonstrated that ion leakage occurs from chilling injured, yet living cells [Palta, 1990]. Hence, rhizomes could not be stored for more than 30 days at CT.

Total phenolics

The total phenolic content in fresh juice was 20.8 mg/ 100 g. It showed an increasing trend both in RT and LT stored rhizomes. Low temperature favored significant accumulation of total phenolics throughout the storage period. The chilling temperature induced the highest concentration of 58.8 mg/100 g of phenolics within a short duration of 30 days, but this declined sharply to 10 mg/100 g on the 60th day **[Fig. 4.4A].**

Fig. 4.4: Changes in total phenolic content in mango ginger juice [2A] and aqueous methanol extract of mango ginger pulp [2B] during storage



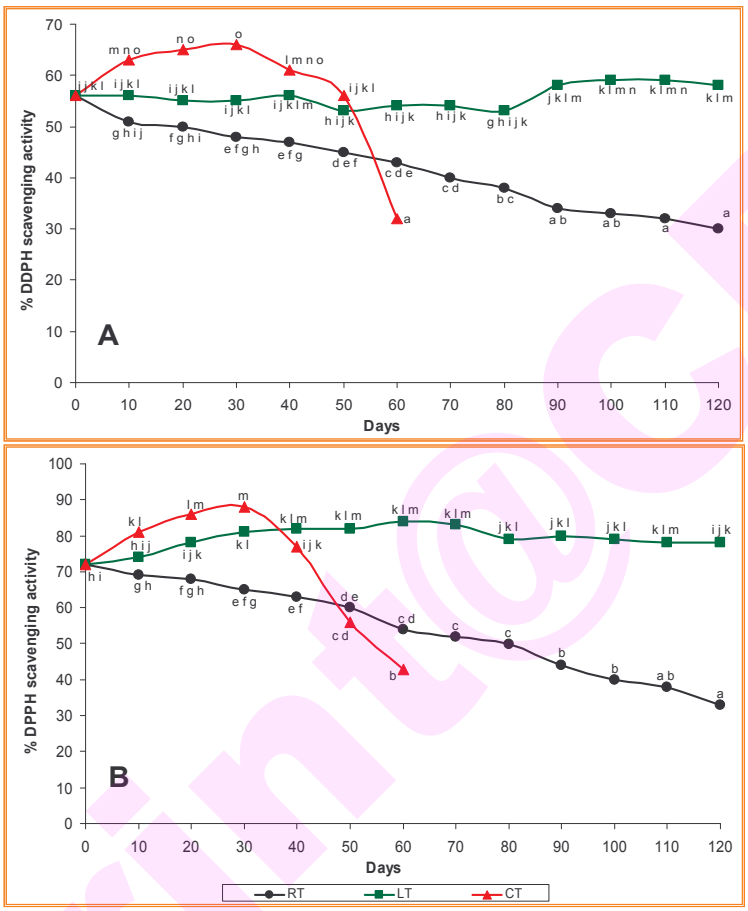
Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

The phenolic content of rhizome pulp was 20 times higher than that of the juice. It remained unchanged until the 60th day but decreased with extended time of storage at RT. Low temperature favored the accumulation of phenolics and it increased from 380 to 568 mg/ 100 g for a storage period of 120 days. Chilling temperature resulted in an increase in phenolics in rhizome pulp for a period of 30 days **[Fig. 4.4B].** The accumulation of phenolics in rhizomes stored at CT could inhibit or slow down sprouting because of their affinity towards oxygen [Cvirkova, *et al.,* 1994]. Several secondary metabolites, including phenolics accumulate to mediate the chilling temperature and other stresses [Christie *et al.,* 1994]. Subsequent browning of chilling injured rhizome tissues may be due to the interaction between phenols and polyphenol oxidase which are generally found in separate compartments in the cell [Crisosto *et al.,* 1999].

Antioxidant activity

Room temperature [RT] storage negatively affected antioxidant activity, both in mango ginger juice and pulp, showing a downward trend **[Fig. 4.5A and 4.5B]** throughout the storage time. The antioxidant activity of the juice remained unchanged or increased slightly after 80 days at LT. Increasing trend in accumulation of phenolics was exhibited both in juice and methanolic extract of pulp stored at LT. Accumulation of phenolics has been shown to be in response to a range of biotic and abiotic stresses [Dixon and Paiva, 1995; Solecka and Kacperska, 2003].

Fig. 4.5: Changes in DPPH radical scavenging activity of mango ginger juice [3A] and aqueous methanol extract of mango ginger pulp [3B] during storage



Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

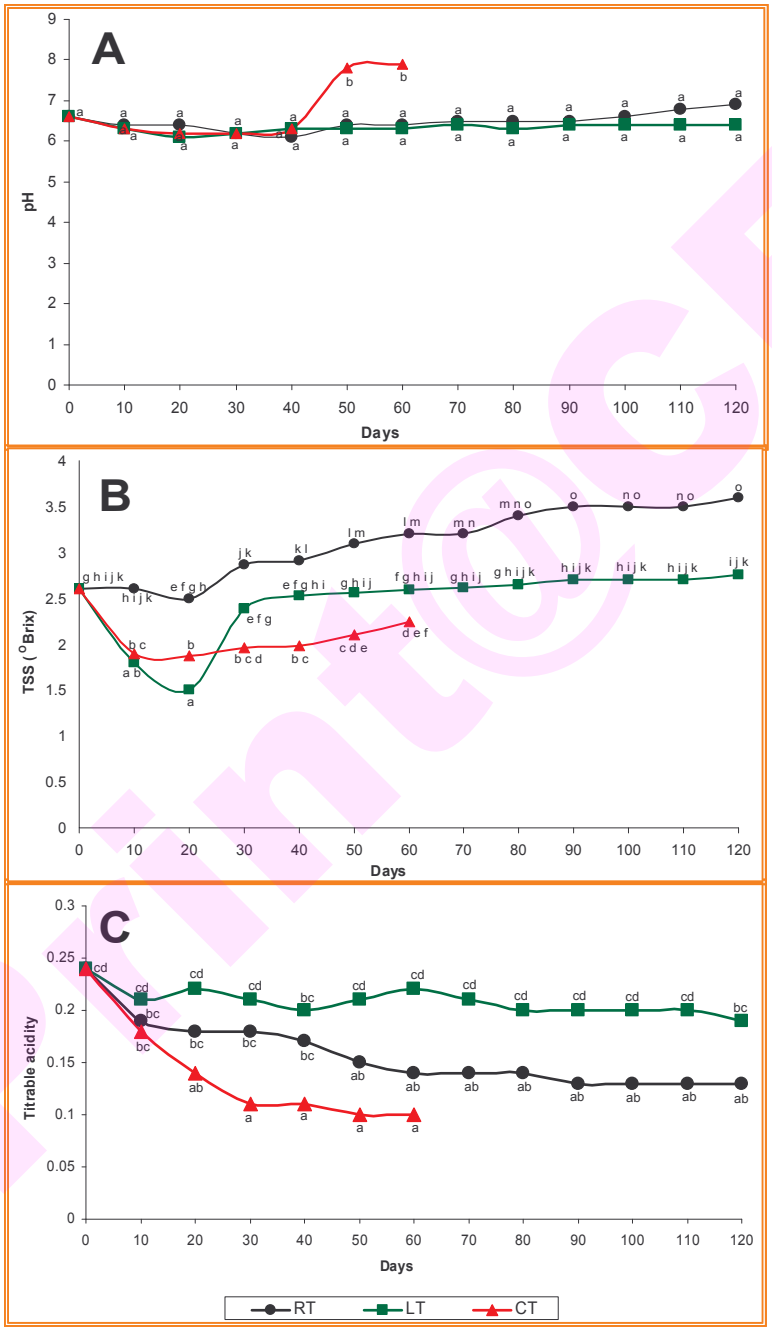
Chilling temperature affected antioxidant activity positively both for juice and pulp for a period of 30 days followed by a decline with the advance of time. The antioxidant activity of many fruits and vegetables are mainly attributed to phenolic compounds [Kalt, *et al.,* 1999]. However, antioxidant activity of mango ginger appears to be independent of total phenolics.

pH, titrable acidity and total soluble solids

The fresh mango ginger juice had a pH of 6.6 immediately after harvest. It remained stable in rhizomes stored at RT and LT. However, storage at CT resulted in an increase from 6.3 to 6.9 from 40 to 60 days **[Fig. 4.6A].** Titrable acidity measured as citric acid showed a downward trend in all the three storage temperatures **[Fig. 4.6B].** The decrease in acidity was highly significant at CT when compared to RT and LT. Loss of acidity was least in LT [0.11 %] when compared to RT [0.05 %].

A steady increase of 1 % in total soluble solids was exhibited in the mango ginger on the 120th day at RT. Low temperature exerted a dual effect on TSS, with an initial decrease of 1.5 % until 30 days followed by a gradual increase [2.7 %] to 120 days. There was no significant change in the TSS of rhizomes stored at CT **[Fig. 4.6C].**

Fig. 4.6: Changes in pH [4A], titrable acidity [4B] and total soluble solids [4C] during storage

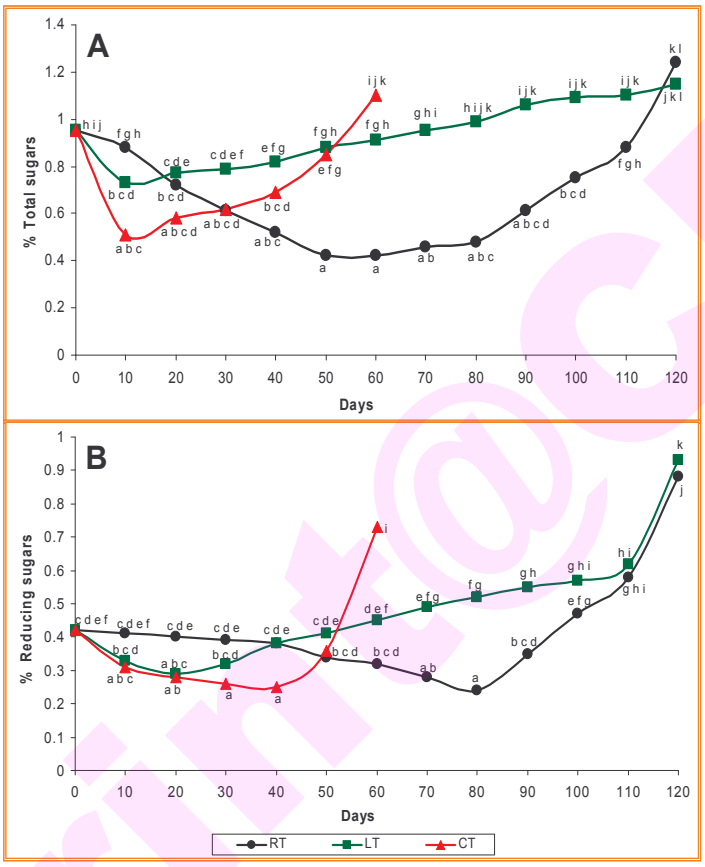


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Total sugars and reducing sugars

Freshly harvested mango ginger had 0.95 % and 0.42 % total sugars and reducing sugars respectively. Rhizomes showed a decrease in total sugars and reducing sugars by 0.53 and 0.10 % respectively up to 60 days of storage at RT. An interesting observation was an increase in total sugars and reducing sugars from the 80th day onwards during storage. A sudden increase in both the sugars by 0.82 and 0.56 % respectively was observed up to 120 days **[Fig. 4.7A and 4.7B].** Shift in nutritional and metabolic sink of rhizomes, due to harvest causes severe physiological stress. This was effectively overcome initially at the expense of total sugars and reducing sugars, acidity and TSS. Thus the increase in sugars which may be attributed to hydrolysis of starch into simple sugars [Biale, 1960]. CT influenced accumulation of both sugars after 30 days of storage. With an increase in time, there was a linear increase in concentration of total sugars and reducing sugars throughout the storage period. Low temperature sweetening by accumulation of total and reducing sugars was exhibited by rhizome. The process of sweetening is accompanied with conversion of starch to sugars. This phenomenon could also be explained by the glucose consumption by the respiration, which was inhibited at the LT [Salunkhe and Desai, 1984; Brecht, 2003]. High temperature negatively influenced the rhizome by attenuating the metabolic activity resulting in decrease in total sugars and reducing sugars.

Fig. 4.7: Changes in total sugar content [5A] and reducing sugar content [5B] of mango ginger juice during storage

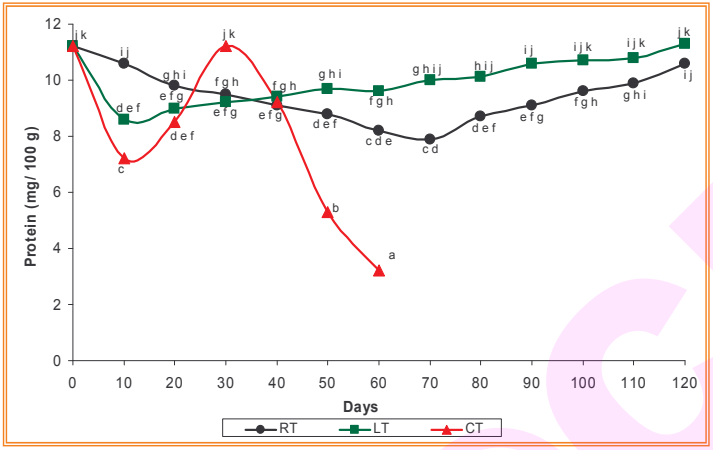


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Total protein content

Total protein content of freshly harvested mango ginger rhizome was 11.2 mg/ 100 g, which was reduced to 7.9 mg on the 70th day in RT storage. Further, storage of rhizomes at RT showed an increase in protein content up to 10.6 mg/100g on the 120th day **[Fig. 4.8].** Low temperature also exerted a similar trend on protein content of rhizomes, i.e. an initial sudden decline in the protein content to 8.6 mg/100 g for 10 days of storage followed by slight increase [11.3 mg] on the 120th day. Chilling temperature negatively affected total protein content of the rhizomes and this reached the lowest concentration of 3.2 mg/100 g on the 60th day. There was a significant increase in protein content prior to sprouting of rhizome at RT, which may be due to *de novo* synthesis of proteins. Increase in proteins and nucleic acids prior to sprouting were observed in many vegetables [Macdonald and Osborne, 1988]. Protein synthesis required for sprouting [Madison and Rappaport, 1968; Alam *et al.,* 1994].

Fig. 4.8: Changes in total protein content of mango ginger juice during storage



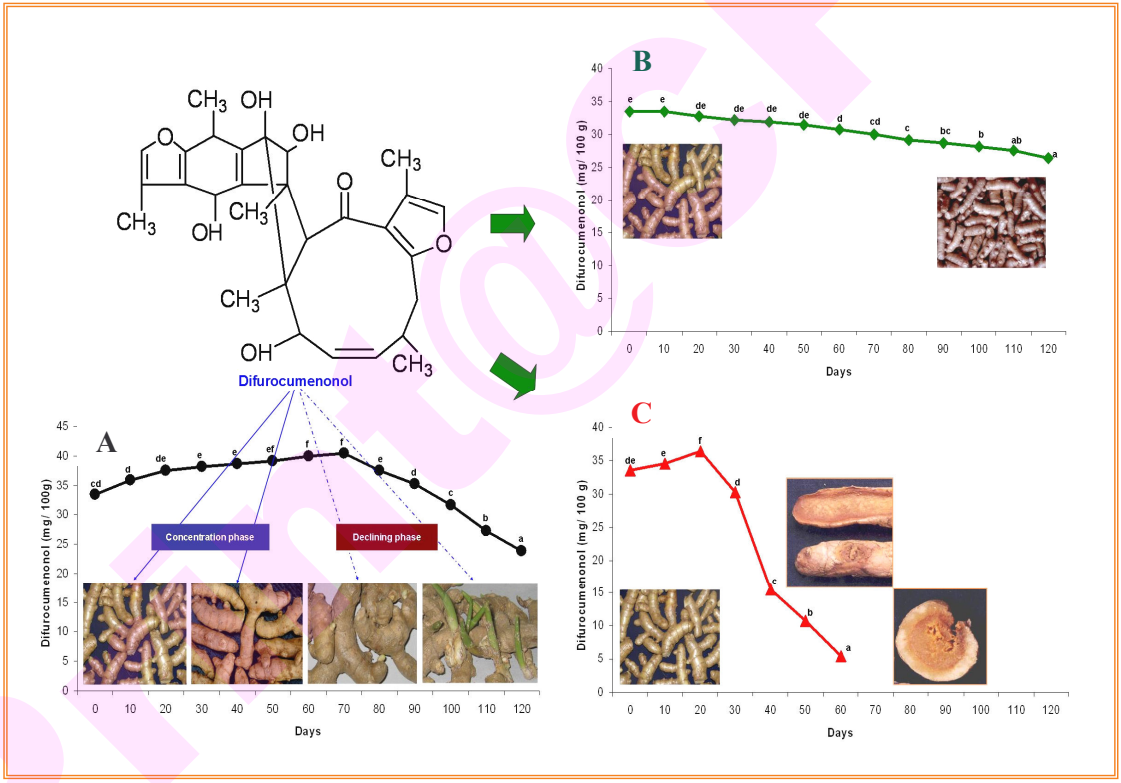
Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Effect of storage temperatures on difurocumenonol

The concentration of difurocumenonol in mango ginger rhizomes during storage up to 120 days was carried out using HPLC. Difurocumenonol **[Fig. 4.9]** - a terpenoid compound, has been characterized and reported to have antimicrobial activity against a wide range of microbes [Policegoudra *et al.,* 2007]. This has been used as marker to evaluate the quality of mango ginger rhizomes during storage at different temperatures. The highest concentration of difurocumenonol [40 mg/100 g] was noticed in 70 days old rhizomes stored at RT and decreases thereafter till 120 days **[Fig. 4.9A].** This may be due to the loss of water rather than de novo synthesis of compound. The peak accumulation of difurocumenonol at RT storage associated with conspicuous display of shriveling, a visual

quality marker of rhizome. Later decrease in its concentration heralds the onset of sprouting a major limiting factor to extend the shelf life of rhizome at RT. At CT difurocumenonol exhibited slight increase in concentration till 30th day followed by a drastic decrease **[Fig. 4.9C].** Changes in difurocumenonol during storage at CT appear to be influenced by chilling injury. At LT gradual decrease in the concentration of difurocumenonol was observed with increase in storage period **[Fig. 4.9B].** . It is interesting to note that difurocumenonol exhibited different pattern of accumulation as a function of temperature during storage period

Fig. 4.9: Effect of different temperatures on difurocumenonol during storage of mango ginger rhizomes



A- Room temperature B- Low temperature C- Chilling temperature

Effect of temperatures on shelf-life

Room temperature aggravates rapid depletion of water, increased TSS, sugars, phenolics and antioxidant activity in mango ginger rhizome. These changes may be responsible for short shelf life of 3 months at RT, beyond which shriveling and sprouting makes rhizomes unsalable. Chilling temperature negatively affected the rhizome and showed a 'bell' shaped curve, which is characterized by, initial accumulation of TSS, total sugars, phenolics that decrease rapidly in later stages. The peak period of accumulation of the above chemical constituents on 30^{th} day may heralds the onset of chilling injury and thus limits the storage period. Low temperature [14±1°C] was found to be optimum for storage of mango ginger rhizome. It alleviates excessive moisture loss, avoids chilling injury, delay sprouting, and ultimately extends the shelf life of rhizome by 4-5 months. Thus, biochemical changes, antioxidant activity and shelf-life of mango ginger are directly governed by temperature and time of storage.

Conclusion

This is the first report on biochemical changes and antioxidant activity of mango ginger at different storage temperatures. Within the range of temperatures, lower end [4°C] was characterized by chilling injury manifested by discoloration, softening, browning of internal tissue, loss of quality and failure to sprout, whereas, upper end [24°C] was characterized by sprouting, shriveling. Moderate low temperature [14°C] is optimum for storage of mango ginger. It retained mango flavour, and minimized the changes in composition, and also biochemical and antioxidant properties. It reduced excessive moisture loss, avoid chilling injury, delay sprouting, maintain freshness, ultimately extended the shelf life for 4-5 months. This suggests that mango ginger rhizome quality is a function of temperature and time during storage and exerts a decisive role in determining its biochemical quality. For the first time difurocumenonol a multi functional bioactive compound has been successfully used as biomarker to asses the quality changes in mango ginger rhizome during storage. These aspects, which I believe have commercial applications to obtain rhizome with high bioactive components a preferred quality for preparation of pharmaceutical of nutraceutical products.

Chapter - 5

Functional Properties of Starch

A Major Storage Component of Mango Ginger Rhizomes

You cannot depend on your eyes
When your imagination is out of focus
– Mark Twain

Introduction

Starches from cereals, tubers and roots are the major dietary source of energy for humans. Starch determines the functionality of many food applications, accordingly they are used in the food industry as stabilizers, thickening agent in snacks, meat products, fruit juices, etc [Betancur and Chel, 1997]. The enthalpy and temperature of gelatinization of starch depend on the microstructure, size, shape and composition of granules, amylose/amylopectin ratio in particular [Hizukuri, 2004]. The granule size in tubers varies from 0.5-85 μm, it is also reported to vary from 1 to 110 μm, depending on the source, while the shape varies from irregular to oval, elliptical, spherical and polygonal [Hoover, 2001; Mara, *et al.,* 2006]. Research on characterization of potato, cassava and sweet potato starch has resulted in their extensive utilization in food industries. On the other hand, structural and biochemical properties of many starchy tubers and spicy roots like mango ginger have not yet been studied. There is a need to explore these starches, to use in food industry.

Mango ginger is composed of 6.39 % of starch by fresh weight basis and 45.64 % of starch by dry weight basis [Shetty, *et al.,* 1963]. It is interesting to investigate the structure and biochemical properties of mango ginger starch for development of product of health benefits. A valuable, but little understood storage polysaccharide from an unconventional source like mango ginger can give a complete picture of the true diversity of natural starches in tuberous crops.

MATERIALS AND METHODS

Plant material

Fresh [2 days old after harvest] and healthy mango ginger [*Curcuma amada* Roxb.] rhizomes were procured from the local market, Mysore, India. Rhizomes were washed, sliced and dried in a hot air oven at 50°C for 72 hrs and powdered to 100-120 meshes in an apex grinder [Apex Constructions, London].

Isolation of starch

Mango ginger powder [100 g] was extracted with 70 % methanol by constant stirring at 40°C for 36 h. The slurry was poured through three layers of muslin cloth and centrifuged at 8,000 rpm for 25 min. The yellowish residue settled was collected and stirred for 10 min with 200 mL of petroleum ether and acetone [1:1, v/v] followed by 500 mL of water. The procedure was repeated till the residue becomes colourless. The starch was allowed to settle. Then starch was washed with distilled water repeatedly till starch becomes completely white and free from debris. The starch was then collected and dried at room temperature [26 ± 2°C] and then stored at ambient temperature.

Moisture and ash content

Moisture and ash content of mango ginger starch were determined according to the AOAC official procedures [AOAC, 1990].

Solubility

The solubility of mango ginger starch was recorded at 25, 35, 45, 55, 65 and 75°C by using the method of Ju and Mittal [1995].

Differential scanning calorimetry [DSC]

Gelatinization of mango ginger starch was measured according to the method of Hoover, [2001] using differential scanning calorimeter [Mettler DSC 30, Switzerland]. Starch was mixed with de-ionized water in 1:3 ratio and transferred to DSC pans, which was then sealed and allowed to stand for 2 h at room temperature before analysis. The sample was heated at 10°C/min over a temperature range of 25-100°C. The thermogram

was recorded with an empty aluminium pan as the reference. The transition temperatures reported are the onset *[T_o]*, peak *[T_p]* and conclusion *[T_c]*.

Water holding capacity [WHC]

Water holding capacity of mango ginger starch at 25, 35, 45, 55, 65 and 75°C was determined by the method of Ju and Mittal [1995].

Amylose content

Amylose content in the isolated mango ginger starch was determined by using the method of Hoover and Ratnayake [2002].

Light transmittance

Light transmittance of mango ginger starch solution [1 %] in DMSO and water was measured by method of Yeh and Yeh [1993] and Craig, *et al.,* [1989]. The transmittance [%] of the solution was recorded at 1, 2, 4, 6, 12 and 24 hrs at 650 nm on a Shimadzu UV-160A instrument [Shimadzu, Singapore] at room temperature.

X-Ray Diffraction

The mango ginger starch was analyzed by powder X-ray diffraction method for quantitative phase identification. The X-ray powder diffraction patterns were obtained using Rigaku X-ray diffractometer [Rigaku Co., Tokyo, Japan], Cu Kα radiation operating at 40 kV and 30 mA. The diffracted intensity was measured from 4°- 50° as a function of 2θ at a scanning speed of 4°/ min. The starch was equilibrated at 100 % relative humidity for 24 h at 25°C prior to analysis.

Fourier-transform infrared [FTIR] spectroscopy

The IR spectrum of mango ginger starch was recorded on Nicolet 5700 [Thermo Electron Corporation, Madison, WI, US] IR Spectrometer at room temperature. The starch powder was blended with KBr powder, and pressed into tablets before measurement. A region from 400-4000 cm^{-1} was used for scanning.

Scanning Electron Microscopy [SEM]

The scanning electron micrographs of mango ginger starch were recorded on LEO-435 VP scanning electron microscope [Cambridge, UK]. Starch granules were sprinkled on cellophane tape attached to a stub and coated with gold. The starch granules were analyzed for surface morphology, shape and size.

Statistical analysis

All the biochemical analysis were carried out in triplicates. Significant differences [$P < 0.05$] were determined by Duncan's Multiple Range Test [DMRT].

Results and Discussion

Isolation of starch

The mango ginger starch granules could not be separated from colored pigments and adhered cell components by adopting the earlier methods [Lilia, *et al.,* 1999; Ana, *et al.,* 2004; Singh, *et al.,* 2005; Jayakody, *et al.,* 2005]. The starch obtained after centrifugation of filtered methanol extract was yellowish in color. The colouration may be attributed to presence of terpenoid pigments. Among different solvent gradients tried, petroleum ether and acetone at ratio of 1:1, v/v, was effective in removal of adhered pigments completely on the starch. Organic layer containing dissolved pigments was separated from starch by repeated wash with distilled water. The starch thus obtained was pure and white in colour. They were dried at room temperature [26±2°C] and stored for further characterization.

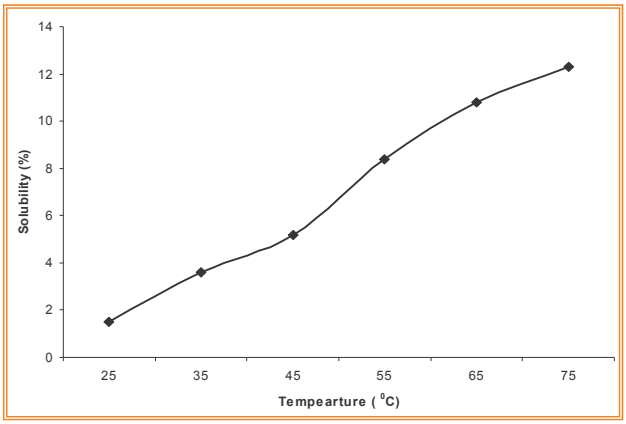
Moisture and ash content

Mango ginger starch contains 9.8 % of moisture and 1.3 % ash content [Table 1]. The moisture content of dry starch varies from 6-16 % among tuber starches, which have been reported by several researchers [Takeda, *et al.,* 1986; Moorthy, 2002].

Solubility

Mango ginger starch [1 g/100 ml] exhibited very low solubility [1.5 %] in water at 25°C, when stirred it formed a suspension. The solubility of mango ginger starch was high [12.3 %] at 75°C. There was a linear increase in solubility of starch in water with increase in temperature **[Fig. 5.1].**

Fig. 5.1: Solubility of mango ginger starch at different temperatures



Differential Scanning Colorimeter [DSC]

Thermal analysis of mango ginger starch by DSC showed that, the gelatinization onset temperature [T_o] be 69.70°C, peak temperature [T_p] was 77.33°C and conclusion temperature [T_c] was 90.52°C. The gelatinization temperature range [T_c-T_o] for *C. amada* starch was 20.82°C, which is lower than that of *C. longa* starch [27°C], *Z. officinale* starch [30°C] and higher than that of *Curcuma malabarica* starch [7.8°C] and *Curcuma zedoaria* starch [17.4°C] [Mara, *et al.,* 2006; Jyothi, *et al.,* 2003]. According to Noda, *et al.,* [1998], T_o, T_p and T_c are influenced by the molecular architecture of the crystalline region, which corresponds to the distribution of amylopectin short chains and not by the proportions of crystalline region. The low T_o, T_p and T_c reflect the presence of abundant short amylopectin chains.

Table 5.1: Composition and morphological features of mango ginger starch

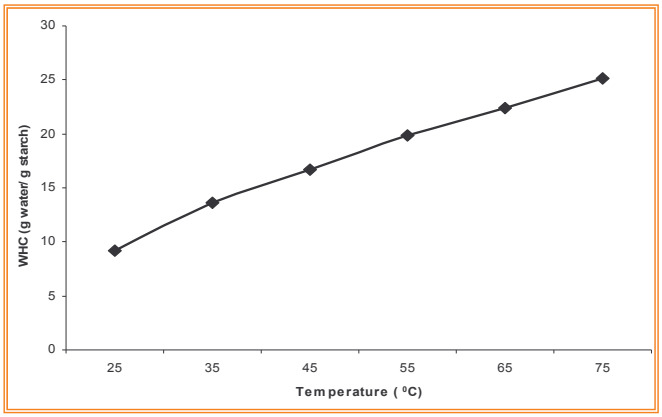
Moisture content	9.8 ± 0.6 %
Total ash content	1.3 ± 0.1 %
Amylose content	43 ± 1.8 %
Granule size	
1) Small granules	3-20 µm
2) Large granules	20-48 µm
Granule shape	round, elliptical, polygonal
Fissures	absent
Concentric rings	absent

* Each value represents mean of three different observations ± S.D.

Water holding capacity [WHC]

Water holding capacity of mango ginger starch increased linearly with increase in temperature **[Fig. 5.2].** The WHC has been reported to depend upon the degree of the engagement of hydroxyl groups to form hydrogen and covalent bonds between starch chains [Hoover and Sosulski, 1986]. Differences in the degree of availability of water binding sites in the starches can have role in the variation of water binding capacity [Wotton and Bamunuarachchi, 1978].

Fig. 5.2: Water holding capacity of mango ginger starch at different temperatures



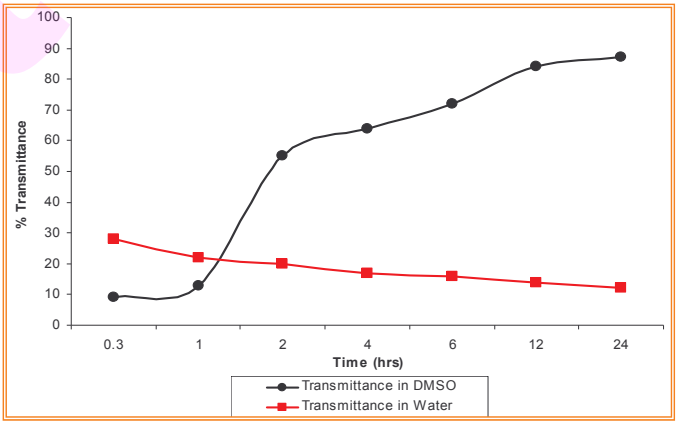
Amylose content

The amylose content in mango ginger starch was 43 % [Table 1]. This is 5 % less than that of *Curcuma longa* starch [Mara, *et al.,* 2006] while, 18 % and 16 % more than *C. malabarica* and *C. zedoaria* starches respectively [Jyothi, *et al.,* 2003]. However the amylose content of *C. amada* was significantly higher than the *Z. officinale,* which has 34 % amylose. Studies indicated that high amylose content was accompanied with lowered blood glucose and insulin responses [Granfeldt, *et al.,* 1995]. This lowers the digestibility of starch due to positive correlation between amylose content and formation of resistant starch [Berry, 1986; Sievert and Pomeranz, 1989]. Such foods, with a low glycaemic index, are considered to produce metabolic advantages [Jarvi, *et al.,* 1999; Bjorck, *et al.,* 2000]. The advantage of high amylose content in mango ginger and its associated functional attributes are yet to be explored.

Light Transmittance

Light transmittance of mango ginger starch dissolved in water decreased progressively as the time progress. On contrary, light transmittance of starch dissolved in DMSO showed significant increase from 1-2 h **[Fig. 5.3]**. Being a hydrogen bond acceptor DMSO breaks ass ociative hydrogen bonding in the starch molecules through surface erosion thus increasing its solubility and light transmittance [French, 1984; Cooreman, *et al.,* 1995]. Light transmittance can be used to indicate the clarity of starch paste which varies considerably with its source, solubility and amylose content [Kittiwut, *et al.,* 2003].

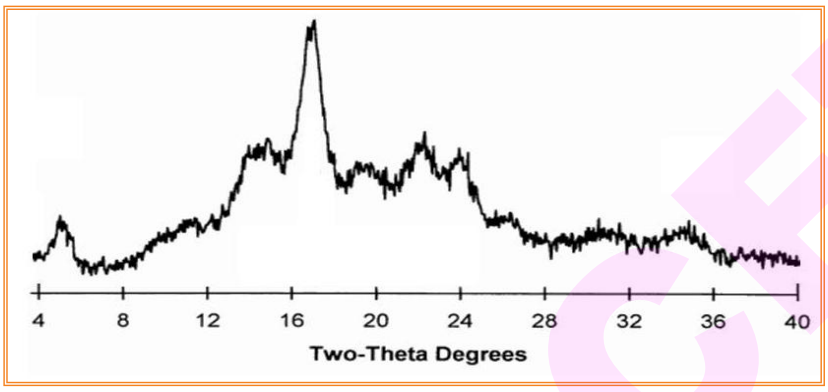
Fig. 5.3: Light transmittance [%] of mango ginger starch dissolved in DMSO and water



X-ray crystallography

X-ray diffraction has been used to reveal the presence and characteristics of the starch crystalline structure [Singh, *et al.,* 2003]. The X-ray diffractogram of mango ginger starch **[Fig. 5.4]** showed five peaks, the strongest diffraction peak at around 17° at 2θ and small peaks around 5°, 15°, 22° and 24° indicated that the mango ginger has a characteristic B-type starch. This pattern is typical to tuber and root starches [Hoover, 2001], which is characterized by a small peak at 5.6°, strong peak at 17° and a doublet at 22° and 24°. The X-ray diffractogram of *C. amada* was found to be similar with that of *C. longa* [turmeric] rather than *Z. officinale* [ginger] and *Mangifera indica* [mango] that have C-type starch [Bello-Perez, *et al.,* 2005; Mara, *et al.,* 2006].

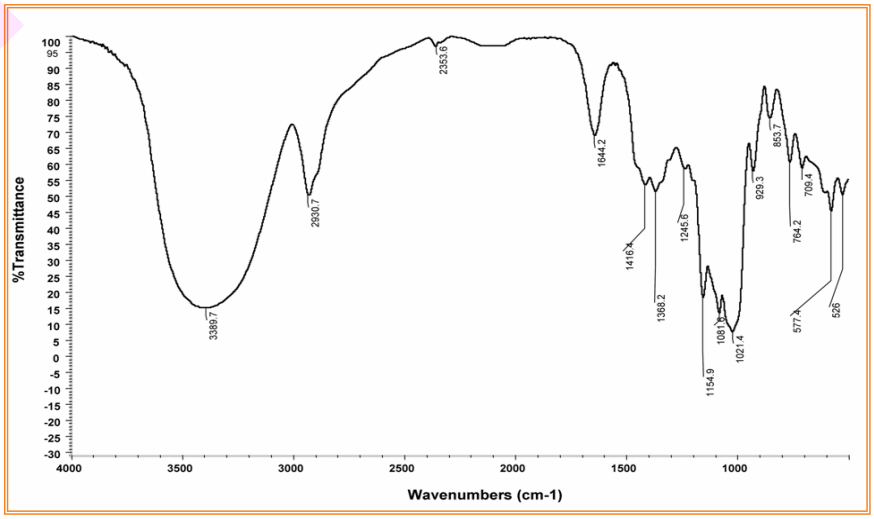
Fig. 5.4: X-ray diffractogram of mango ginger starch



Fourier Transform Infrared [FTIR] spectroscopy

The FTIR spectrum of the mango ginger starch is given in **Fig. 5.5**. The characteristic peak of starch between 1019 and 1156 cm^{-1}, attributed to C-O bond stretching [Fang, *et al.,* 2002]. We observed peaks around 1021 cm^{-1} was ascribed to

Fig. 5.5: FTIR spectrum of mango ginger starch

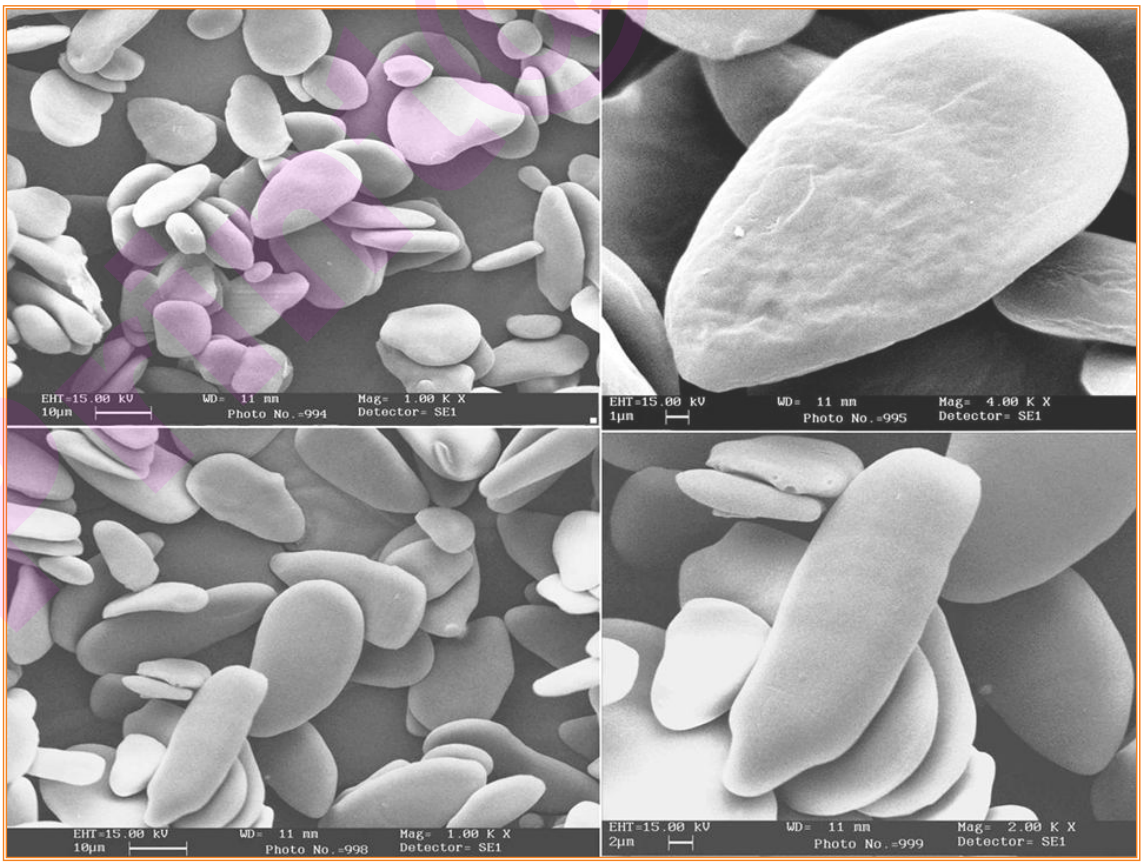


the C-O stretch of C-O-C in starch, and the peaks near 1081 and 1154 cm^{-1} were mainly attributed to C-O stretch of C-O-H in starch. The wide band observed at 3389 cm^{-1} can be attributed to the O-H stretching. The bands at 2930 cm^{-1} may be attributed to the asymmetric stretching of C-H, while the band at 1644 cm^{-1} was ascribed to adsorbed water and the bands at 1416 and at 1368 cm^{-1} to the angular deformation of C-H. Thus, the IR spectra revealed the purity of the starch isolated.

Scanning Electron Microscopy [SEM]

As shown in **Fig. 5.6,** SEM revealed significant variations in shape and size of mango ginger starch. The granule shape appeared to be oval, elliptic, irregular or cuboidal and polygonal. The elliptic-shaped, large-size granules were more in number. The shape of the mango ginger starch appears to be a combination of turmeric [elliptical] and ginger [oval] starch. Absence of fissure on the surface of mango ginger starch revealed that it is similar to ginger, than turmeric starch that showed fissures [Mara, *et al.,* 2006]. The granule diameter ranges between 3-20 µm for small and 20-48 µm for large size. Granule

Fig. 5.6: Scanning electron micrographs of mango ginger starch granules



structure, size and their distribution are important because they can affect the functional properties of starch [Rasper, 1971]. The difference in the granule structure may be attributed to their biological origin [Svegmark and Hermansson, 1993], mainly depends on the biochemistry of the chloroplast or amyloplast, as well as physiology of the plant [Badenhuizen, 1969].

CONCLUSION

Starch from an unconventional source like mango ginger was isolated and characterized for the first time. It exhibited distinct structural and biochemical features of its own. With B type X-ray diffractogram and high amylose content, it showed similarity with turmeric starch. Though morphologically rhizome resembles ginger, starch granules differ by the absence of fissures on the surface and its X-ray diffractogram pattern. Thus it occupies a position between turmeric and ginger starch. High amylose content and low solubility of mango ginger starch, preferred characters that need to be explored for preparation of products of metabolic advantages.

Summary

Summary

The strategies for selection of mango ginger for research was due to its high medicinal value and few reports on bioactive properties and also on chemical constituents and their coordinated changes either at different stages of growth or at storage of rhizome. A work on these aspects, which we believe has some commercial applications. Accordingly the summary of the systematic work that has been carried out on mango ginger is presented below.

Chapter 1: Functional properties of mango ginger extracts

As a first step towards isolation of bioactive molecule, dehydrated powder of mango ginger rhizome was sequentially extracted with hexane followed by chloroform, ethyl acetate, acetone and methanol. All the extracts were screened for antibacterial activity against selected clinical isolates. The extracts were also screened for DPPH radical scavenging activity, superoxide radical scavenging activity, lipid peroxidation-inhibitory activity, metal chelating activity and total reducing power. Platelet-aggregation inhibitory activity and cytotoxicity were also tested for the mango ginger extracts. Chloroform extract showed highest antibacterial and antioxidant activity, when compared to other extracts. Hence, it was selected for the isolation and characterization of antimicrobial and antioxidant compounds.

Chapter 2: Isolation and characterization of bioactive compounds and their functional properties

Bioactivity [antibacterial/antioxidant] guided fractionation of the chloroform extract by repeated silica gel column chromatography yielded three pure compounds. The purified bioactive compounds were subjected to UV, IR, LC-MS and 2D-HMQCT NMR spectra. They were identified as difurocumenonol, amadannulen and

amadaldehyde, which are new compounds and not reported previously from any other source. Potential antibacterial activity of these compounds against wide range of clinical isolates was exhibited. Interestingly multiple antioxidant activities like DPPH radical scavenging activity, superoxide radical scavenging activity, metal chelating activity, lipid peroxidation-inhibitory activity and total reducing activity of these compounds are enumerated. In addition, Difurocumenonol and amadannulen showed platelet-aggregation inhibitory activity and lipoxygenase inhibitory activity. Among these bioactive compounds, difurocumenonol was a major terpenoid compound with highest yield and multiple bioactive properties. Hence, work was undertaken to enumerate the physiological role of difurocumenonol as a biomarker to determine the physiological maturity and quality during development and storage of mango ginger rhizome.

Chapter 3: Physiological role of bioactive compounds during development of mango ginger rhizomes

For the first time, four distinct developmental phases in mango ginger were defined namely; [i] vegetative phase, [ii] initiation and development phase, [iii] maturation phase and [iv] senescence phase, based on bioactive components and biochemical changes. For the first time, difurocumenonol, a bioactive terpenoid compound and phenolics were identified as biomarkers, to determine the optimum physiological maturity for harvesting mango ginger rhizome. Coordinated changes in biochemical components and their accumulation pattern at various developmental stages of rhizome revealed that phenolics accumulate in newly initiated rhizomes. The concentration was higher in mango ginger pulp compared to its juice. The accumulation of difurocumenonol was observed 120 days after planting. Peak accumulation of difurocumenonol and phenolics were noticed in 180 days old rhizome and decline later. Accordingly, the abundance of these components on 180 days was set as an optimum maturity standard for harvest of mango ginger rhizome, compared with conventional harvest period that ranges from 200 to 240 days. Defining the different stages of growth and to evolve chemical index is critical to determine the quality and harvest maturity of rhizome. Existence of link between the bioactive components and constituents of rhizomes

has been exploited to design and develop a physiological maturity index for harvest of mango ginger rhizome. It has direct application for production of quality rhizomes for pharmaceutical or nutraceutical product.

Chapter 4: Functional properties and biochemical changes in mango ginger rhizomes during storage

Concentration of difurocumenonol, antioxidant activity and other biochemical changes in mango ginger rhizomes during storage at different temperatures viz; RT [25°C], LT [14°C] and CT [4°C] were studied as a function of time. Within the range of temperatures, mango ginger rhizomes exhibited chilling injury symptoms as water-soaked lesions with tissue softening, browning, loss of mango flavour and failure to sprout at the lowest temperature [CT], and rapid deterioration of physical, physiological and antioxidant factors at room temperature [RT]. Shriveling and sprouting were the limiting factors for storage at RT. Physiological loss of water of about 32±2 % was identified for apparent commercially objectionable levels of shriveling. Moderate low temperature [LT] minimized the biochemical changes, maintained or increased the antioxidant activity and doubled the shelf life as a function of temperature with storage time. Difurocumenonol was identified as suitable biomarker in defining the quality of the product during storage. It effectively formed an index to predict the chilling injury in rhizome stored at CT. High concentration of difurocumenonol in shriveled rhizome was mainly due to loss of water in tissue rather than de novo synthesis. However, decline in concentration of difurocumenonol indicate the initiation of sprouting of rhizome at RT. A little change in the concentration of difurocumenonol accompanied with other bioactive compounds and storage components in mango ginger rhizome indicate that LT has a positive impact.

Chapter 5: Functional properties of starch - a major storage component of mango ginger rhizomes

For the first time starch from mango ginger was characterized with respect to amylose content, solubility, gelatinization, ash, moisture content, X-ray diffraction pattern and morphological structure of starch granules. Solubility and water holding capacity of mango ginger starch increased linearly with increase in temperature. An increase in light transmittance [%] with increase in time was observed in DMSO, this is in contrary with decrease in water. Scanning electron micrograph revealed the variations in shape of granules that appeared as round, elliptic, irregular and polygonal. The X-ray diffractogram of mango ginger starch revealed B-type of starch, a characteristic feature of *Curcuma* sp. and majority of tuber starch, but in contrast with C-type pattern of ginger starch. Low solubility accompanied with high amylose content of mango ginger starch is a metabolic advantage for preparation of products for diabetics, which need further investigation.

Finale with Futuristic Note

Present investigation encompasses successful attempts in isolation and characterization of three novel, multifunctional bioactive compounds from rhizome of mango ginger, a plant of panacea for an array of ailment in *Ayurvedic* medicine. Application of difurocumenonol other bioactive compounds to determine the harvest maturity and quality marker during development and storage is a showcase of this inimitable investigation. Unique feature of mango ginger starch and its functional properties were established. These aspects, which I believe have commercial applications for design and development of new generation nutraceutical products.

The list of publications and patents follows……

Publications:

1) **Policegoudra R. S.,** Divakar S., and Aradhya S. M. [2007]. Difurocumenonol – a novel antibacterial compound isolated from mango ginger [*Curcuma amada* Roxb.] rhizome. **Journal of Applied Microbiology**, 102, 1596-1602.

2) **Policegoudra R. S.**, Abiraj, K., ChanneGowda D. and Aradhya S. M. [2007]. Isolation and characterization of Amadannulen, a novel antioxidant compound from mango ginger [*Curcuma amada* Roxb.] rhizome. **Journal of Chromatography B,** 852, 40-48.

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Patents:

1. Aradhya, S. M., **Policegoudra R. S.** and S. Divakar. A process for the isolation of difurocumenonol, a novel antibacterial and antioxidant compound from the mango ginger [C*urcuma amada* Roxb.] rhizome. *Indian Patent, IPMD, New Delhi.* **Patent No. 302 DEL /07.**

2. **Policegoudra, R. S.** and S. M. Aradhya. A process for the isolation of Amadannulen, a novel antioxidant compound from the mango ginger [C*urcuma amada* Roxb.] rhizome. *Indian Patent, IPMD, New Delhi.* **Patent No. NF 013/07.**

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