Characterization of Bioactive and Biochemical Constituents in *Spondias mangifera* Fruit

A Thesis Submitted to
The University of Mysore

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

By

Mr. Siva Prasad, M. M.Sc.

Department of Fruit and Vegetable Technology
Central Food Technological Research Institute, Mysore-570 020, INDIA

December 2008

Siva Prasad, M.
Department of Fruit and Vegetable Technology
CFTRI, Mysore-570020

Declaration

I here by declare that, the thesis entitled **"Characterization of Bioactive and Biochemical Constituents in *Spondias mangifera* Fruit"** 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-December 2008.

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

(Siva Prasad, M.)

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 **"Characterization of Bioactive and Biochemical Constituents in *Spondias mangifera* Fruit"** submitted to the University of Mysore, Mysore, for the award of the Degree of **Doctor of Philosophy** in the Faculty of Biotechnology by **Mr. Siva Prasad, M.,** 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 - December 2008.

(S.M. Aradhya)

Date:

Place: Mysore


Dedicated to my beloved...
Parents who laid the foundation...
Teachers who paved the way...
Friends who encouraged me...
Wife who made it possible...

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"Thus far has the Lord helped us." – 1 Samuel 7:12

At times our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us. It gives me immense pleasure to thank everyone, on my way to the success.

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- Siva Prasad, M.

List of Figures

List of Tables

Abbreviations

°C	Degree Celsius
µl	Microliter
µM	Micromolar
2D-HMQCT	Two dimensional heteronuclear multiple quantum coherence transfer spectroscopy
BHA	Butylated hydroxy anisole
BSA	Bovine serum albumin
cfu	Colony forming units
DPPH	1,1-Diphenyl-2-picrylhydrazyl
g	Gram
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
N	Normality
NMR	Nuclear magnetic resonance spectroscopy
pH	Negative logarithm of hydrogen ion concentation
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
UV	Ultraviolet
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		Page No.
General Introduction and Review of Literature		
Scope of present Investigation		
Chapter 1	**BIOACTIVITIES OF SELECTED FRUITS** ❖ Introduction ❖ Materials and methods ❖ Results and discussion ❖ Conclusion	
Chapter 2	**ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUNDS AND THEIR FUNCTIONAL PROPERTIES FROM *SPONDIAS MANGIFERA* FRUIT** ❖ Introduction ❖ Materials and methods ❖ Results and discussion ❖ Conclusion	
Chapter 3	**BIOACTIVE AND BIOCHEMICAL CHANGES DURING GROWTH AND DEVELOPMENT OF *SPONDIAS MANGIFERA* FRUIT** ❖ Introduction ❖ Materials and methods ❖ Results and discussion ❖ Conclusion	
Chapter 4	**BIOACTIVE AND BIOCHEMICAL CHANGES IN *SPONDIAS MANGIFERA* FRUIT DURING STORAGE** ❖ Introduction ❖ Materials and methods ❖ Results and discussion ❖ Conclusion	
Summary		
Bibliography		

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

Ever since 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. The most convincing evidence for protective benefits is attributed to their antioxidant property or multiple activities against free radicals.

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 are well documented [Halliwell, 1994; Maxwell, 1997]. Antioxidants that are rich in fruits, vegetable 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]. Further, conception of 'French Paradox' has evoked interest to explore the polyphenols from selected fruits and vegetable source. 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. Fruits and vegetables are fast

emerged as "Healthy foods of the millennium". They were also described as "Nutraceutical foods of the century," owing to their health benefits. Nutraceutical property of fruits and vegetables are attributed to the presence of phenolics, flavonoids and pigments.

India is the second largest producer of wide variety of fresh fruits and vegetables in the world. In addition, India endowed with many little known indigenous crops that play vital roles in the nutritional, nutraceutical and economy of rural population. *Spondias mangifera, wood apple* and *Momordica cymbalaria* are one such horticultural crop, of nutritional and nutraceutical importance, which has received little research attention. Screening of these fruits revealed that *Spondias mangifera* is more potential and exhibited multiple bioactive activities. Hence, *Spondias mangifera* fruit is the subject of this study. The current knowledge on nutritional and nutraceutical components, their bioactivity and teleological role in *Spondias mangifera* is: (i) scarce and inconclusive and, (ii) fragmented amongst the scientific community working in the field. Hence, a detail investigation on characterization of bioactive and biochemical changes in *Spondias mangifera* fruit was undertaken A comprehensive account of review of literature on the above subject follows.

Review of Literature

Fruits and Vegetable are 'treasure houses' for a repertoire of nutritional compounds and also nutraceutical molecules, which are mostly produced as secondary metabolites. Most of these bioactive phytochemicals are reported to be polyphenols which has multiple functions. Recently the antioxidant activity has much explored due to increasing oxidative related degenerative diseases. In this connection available literature on bioactive and biochemical components *Spondias mangifera*, wood apple and *Momordica cymbalaria* fruits have been reviewed.

The fruits selected for this study are little known albeit their excessive use in Ayurveda and other traditional medicine for wide range of vital disease. Extensive review of literature has indicated scanty literature on wood apple, Momordica cymbalaria and Spondias mangifera. The available literature on wood apple and Momordica cymbalaria has been presented in the beginning of this chapter, followed by Spondias mangifera fruit, both on nutritional components and on nutraceuticals, their preventive or therapeutic properties on various degenerative diseases. The available literature on other species of Spondias is inclusive. This is to provide a comprehensive account of the possibly related bioactive compounds in Spondias species. The present review encompasses a relevant, up to-date published literature from various sources on different yet related topics. However, references from other plant sources have been copiously cited for critical evaluation and meaningful conclusion of results.

1. WOOD APPLE

Feronia limonia (L.) Swingle

Scientific classification

Kingdom/Regno:	Plantae (Plants/Piante)
Subkingdom/Sottoregno	Tracheobionata (Vascular plants/Piante vascolari)
Superdivision/Superdivisione	Spermatophyta (Seed plants/Piante con semi)
Division/Divisione	Magnoliophyta (Flowering plants/Piante con fiori)
Class/Classe	Magnoliopsida (Dicotyledons/Dicotiledoni)
Subclass/Sottoclasse	Rosidae
Order/Ordine	Sapindales
Family/Famiglia	Rutaceae
Genus/Genere	Feronia Correa
Species/Specie:	*(= Feronia limonia (L.) Swingle)* *(=Feronia elephantum Correa)* *(= Limonia acidissima L.)* *(= Schinus limonia L.)*

Limonia acidissima (syns. *Feronia limonia* Swingle; *F. elephantum* Correa; *Hesperethusa crenulata* (Stone and Nicolson, 1978), *Schinus limonia* L.) belonging to the Rutaceae family, native to India, Pakistan, Sri Lanka, and southeast Asia to Java.

Botany

It is a small tree growing to 9 m tall, with rough, spiny bark. The leaves are pinnate, with 5-7 leaflets, each leaflet 25-35 mm long and 10-20 mm broad, with a citrus-scent when crushed. The fruit is a berry 5-9 cm diameter, and may be sweet or sour. It has

a very hard rind which can be difficult to crack open, and contains sticky brown pulp and small white seeds.

Origin and Distribution

Wood apple (Feronia *limonia* (L.) Swing. family Rutaceae) is found in the wild tropical forests of India, Srilanka and Thailand. It is called wood apple because the shell is hard and woody. It has a very rough outer surface. The unripe fruit checks diarrhea where as ripe fruit is used for kidney complaints (Solomon, 1998).

Medicinal Uses

The pulp of the raw fruit is useful in arresting secretion or bleeding. The fruit is much used in India as a liver and cardiac tonic, and, when unripe, as an astringent and carminative. It is an effective treatment for hiccough, sore throat, diseases of the gums, urtecaria (allergic disease marked by painful red round weal's on the skin) and relieving of flatulence. The pulp is poultice onto bites and stings of venomous insects, as is the powdered rind. It is also useful in preventing cancer of the breast and uterus and helps cure sterility due to a deficiency of the hormone progesterone. Ripe fruit is used for kidney complaints (Morton, 1987).

Bioactivities

The consolidated report on bioactivities of wood apple fruit is presented below.

Table R1: Consolidated report on bioactivities of wood apple fruit

Bioactivity	Source	Reference
Vasodilator, Anti-microbial, Laxative, Purgative, Astringent, Anti-hypertensive	Fruit	Kirtikar and Basu, 1935, Tewari and Misra, 1965
Gastrointestinal disorders	Fruit	Gupta et al., 1979, Mehta et al., 1983
Antifungal activity	Unripe wood apple fruit stem-bark and root-bark	Badikaram et al., 1989
Mosquito larvicide	Leaves	Rahuman et al., 2000
Antitumor	Fruit	Saima et al., 2000
Antimicrobial	Stem bark	Rahman and Gray, 2002

2. MOMORDICA CYMBALARIA

Momordica cymbalaria Hook. f.

Scientific classification

Kingdom/Regno:	Plantae (Plants/Piante)
Subkingdom/Sottoregno	Tracheobionata (Vascular plants/Piante vascolari)
Superdivision/Superdivisione	Spermatophyta (Seed plants/Piante con semi)
Division/Divisione	Magnoliophyta (Flowering plants/Piante con fiori)
Class/Classe	Magnoliopsida (Dicotyledons/Dicotiledoni)
Subclass/Sottoclasse	Dilleniidae
Order/Ordine	Violales
Family/Famiglia	Cucurbitaceae
Genus/Genere	Momordica L.
Species/Specie	*Momordica cymbalaria Hook. f.* *(= Luffa tuberosa Roxb.)* *(= Momordica tuberosa (Roxb.) Cogn.)*

Current Status:

The consolidated reports on bioactivities of *Momordica cymbalaria,* presented below.

Table R2: Consolidated report on bioactivities of *Momordica cymbalaria* fruit

Bioactivity	Source	Reference
Cardioprotective	Root	Raju et al., 2008
Antidiarrheal activity	Fruit	Mathad, 2008
Antiovulatory and abortifacient	Root	Koneri et al., 2008
Antihyperglycemic antihyperlipidemic	Fruit	Rao et al., 2003
Antihyperglycemic activity	Fruit	Rao et al., 2001
Antidiabetic and Hypolipidemic activity	Fruit	Rao et. al., 1999

3. *SPONDIAS MANGIFERA* (INDIAN HOG PLUM)

- 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 Indian hogplum are depicted in the following Sankrit shloka;

"आम्रातको गुरुश्चोष्णो तुवरोऽम्ळो रुचिप्रदः ।
सरः कण्ठ्यः पित्तकफरक्तकारी च संस्मृतः ॥
आमवातस्य वातस्य चामस्य च विनाशनः ।
सपक्वस्तुवरश्शीतो गुरुर्वृष्यो बलप्रदः ॥
मधुरस्तृप्तिकफकृत् स्निग्धो धातुविवर्धकः ।
मलस्तम्भकरो वातकफपित्तविनाशनः ॥
रक्तरुक्दाहक्षतरुक् क्षयनाशकरो मतः ।
पर्णं तु कोमळं चास्य रुच्यं ग्राह्यग्निदीपनम् ॥" (नि.र.)
["Āmṛātakō guruścōṣṇō tuvarōऽmḷō rucipṛadaḥ
Saraḥ kaṇṭhyaḥ pittakapharaktakārī ca saṁsmṛtaḥ
Āmavātasya vātasya cāmasya ca vināśanaḥ
Sapakvastuvaraśśītō guruṛvṛṣyō balapṛadaḥ
Madhurastṛptikaphakṛt snigdhō dhātuvivaṛdhakaḥ
Malastaṁbhakarō vātakaphapittavināśanaḥ
Raktarukdāhakṣatarukkṣayanāśakarō mataḥ
Paṛṇaṃ tu kōmaḷaṃ cāsya rucyaṃ grāhyagnidīpanaṃ" (Ni.ra.)]

(Source: Nighaturatnakaram)

Spondias mangifera is effective for curing Rheumatoid arthritis

Strengthening of the body,

Development of seven elements of the body: Rasa (Fluids), Raktha (Blood), Mamsa (Body), Medha (Brain), Asthi (Bone), Madhya (Bonemarrow) and Shukra (Vitality)

Normalization of three Doshas: Vata (air type), Pitta(water type) and kafa (heat type) cures all types of itching and skin diseases.

Helps in improvisation of digestive system.

Table R3: Vernacular names of *Spondias mangifera*

Language	Vernacular Name
Botanical	*Spondias mangifera* Willd., *Mangifera pinnata* J. König ex L. f. , *Spondias pinnata* (J.G. Konig ex L. f.) Kurz.
English	Indian hog plum, Indian mombin, Andaman mombin., Wild mango, Daho, Mango-plum
Hindi	Amra
Kannada	Amatae
Konkani	Ambado
Malayalam	Ambazha
Sanskrit	Amraataka
Tamil	Kaatambalam, Mirrey manga
Telugu	Konda-maamidi, Ambra, Amra jouru, Mamedi
Tulu	Ambadae
Chinese	槟榔青 Bing lang xing
German	Mangopflaume.
Japanese	アムラタマゴノキ Amura tamagonoki
Nepalese	Amaaro
Portuguese	Cajamangueira, Cajá-manga, Imbú manga (Brazil)
Spanish	Ciruela mango, Jobo de la India, Mango jobo (Venezuela)
Thai	มะกอก Makok, มะกอกป่า Má kok pa

The vernacular names of Indian hog plum that prevail in different parts of India and world are given in table R1. *Spondias mangifera* is distributed over the sub-Himalayan tract upto 3000', deciduous to semi-evergreen forests of Western Peninsula (Tandon and Rastogi, 1976), Kerala (Sreedharan, 2004), West Bengal (Hasan and Das, 2005), Andaman and Nicobar Islands (Mukhopadhyay et al. 2002), Andhra Pradesh, Maharashtra, also distributed in southern Florida (Campbell and Sauls 1994) China, Bangladesh, Myanmar, Thailand, Burma, Japan and Australia.

Botanical Hierarchy

The present genus name Spondias was coined by Linnaeus in his Species Plantarum in 1753. *Spondias mangifera* Willd. (syn. *Spondias pinnata* J. König ex L. f.) , commonly known as Indian Hog-Plum, Wild Mango, is a member of Anacardiaceae and originated in tropical Asia particularly around Java and other parts of East India (Campbell and Sauls, 1994). The taxonomical hierarchy of *Spondias mangifera* is as follows;

***Spondias mangifera* Willd.**

Kingdom/Regno:	Plantae (Plants/Piante)
Subkingdom/Sottoregno	Tracheobionataz (Vascular plants/Piante vascolari)
Superdivision/Superdivisione	Spermatophyta (Seed plants/Piante con semi)
Division/Divisione	Magnoliophyta (Flowering plants/Piante con fiori)
Class/Classe	Magnoliopsida (Dicotyledons/Dicotiledoni)
Subclass/Sottoclasse	Rosidae
Order/Ordine	Sapindales
Family/Famiglia	Anacardiaceae
Genus/Genere	Spondias L.
Species/Specie:	*Spondias mangifera* Willd. (= *Mangifera pinnata* J. Koenig ex L. f.) (=*Spondias pinnata* (J. Koenig ex L. f.) Kurz)

The present genus *Spondias* L. belongs to the family Anacardiaceae, which is composed of 8-12 species, which are widely spread in tropical regions of the world (Aristeguieta, 1973, Croat, 1974; Hoyos, 1994, De Souza et al. 1998). S. *dulcis* and S. *mangifera* grow abundantly in India (Basu and Rao, 1981; Ghosal and Thakur, 1981) while S. *purpurea* and S. *mombin* are disseminated in Venezuela (Hoyos, 1989). The plants belong to the *Spondias* are well known for their multiple uses as fruits eaten when fresh and it is often used for making low priced pulp, juice and ice cream jelly, jams,

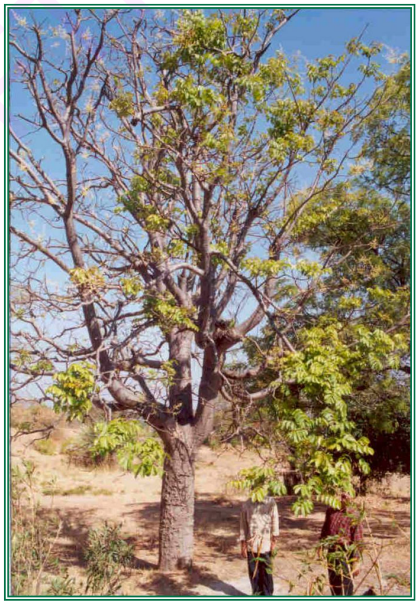
pickles, relishes, soups, stews and juices (Morton, 1987) or may be canned in sucrose (Ramsundar et al. 2002), flavour (Graham et al., 2004; Jirovetz et al., 1999) medicines, ecological, industrial importance, gum exudates (Pinto et al., 2000a; Pinto et al., 1995), additives in the food industry (Koubala et al. 2008), fodder (Jackson, 1987), Mushroom fructification.

The extensive work has been carried out on *Spondias mombin* and of course, *Mangifera indica*. But, *Spondias mangifera* is an untapped medicinal plant among various hog plums in the Mango family.

Habitat

Spondias mangifera is a coarse-grained woody deciduous tree **[Fig. R1]**. The leaves are compound leaves having 3-5 pairs of leaflets. It bears polygamous yellow flowers in dense panicles. *Spondias mangifera* fruit **[Fig. R2]** is an ovoid drupe 4-5 cm (1½-2 in.) in length, bears in terminal clusters of 10-15 fruits, with 1-5 seeds. The fruit is extremely sour, even when completely ripe. The fruit, when largest, is of the size of a goose egg, of a rich olive-green, mottled with yellow and black, with a trifling degree of scent.

Fig. R1: Hog plum tree



Nature of Fruit

S. mangifera is a unique fruit, when largest, is of the size of a goose egg, of a rich olive-green, mottled with yellow, with a trifling degree of scent and none of the quince-like odor of the other species. Fruit belongs to the group called 'drupe' characterized by thin, leathery pericarp, thick, fleshy mesocarp and hard endocarp with a single seed. The fruit is acidic when immature and preferred for pickle industry. After ripening it is astringent acid but is eaten and pickled (Hedrick, 1919).

Fruit Composition

Chemical	Low-High (ppm)
Ascorbic-acid	210 -2165
Ash	5000- 52000
Beta-carotene	2-28
Calcium	360-3710
Carbohydrates	55000-567000
Fat	30000-309000
Fiber	10000-103000
Fructose	18000
Glucose	17000
Iodine	0.04-0.61
Iron	39-400
Kilocalories	218-4810
Niacin	3-31
Phosphorus	110-1135
Protein	7000-72000
Riboflavin	0.2-2.1
Sucrose	29000
Thiamin	0.2-2.1
Water	903000

(Ref.: Duke, 1992)

Uses

Fig. R2: Hog plum fruits

The unripe fruits are widely used to prepare pickles, which are having high commercial value. The fruits are also consumed in fresh form and cooked. The fruit form a rich source of Vitamin-C and its use in indigenous system of medicine as an antiscorbutic and to cure

bilious dyspepsia was recorded in ancient Sanskrit scripts (Watt, 1893; Chopra et al., 1956; Saxena and Singh, 1997). Juice of *Spondias mangifera* leaves is used to cure ear ache and fruits when consumed helps in blood purification. (Sreedharan, 2004) .The fruits are highly perishable in nature resulting in short shelf life (5-6 days) at ambient temperature. The bark of the tree is rubefacient, being used in Indian indigenous medicine for rubbing on the skin over painful joints (Chopra et al. 1958).

Table R4: Therapeutic use of *Spondias* sp.

Spondias sp.	Source	Medicinal Property	Reference
S. mangifera	Bark	Rubefacient	Chopra et al. 1958
	Fruit	Antiscorbutic, bilious dyspepsia	Watt, 1893; Chopra et al., 1956; Saxena and Singh, 1977
	Leaves	Ear ache	Sreedharan, 2004
	Fruit	Blood purifier	
S. mombin	Fruit	diuretic and febrifuge	(Villegas et al., 1997)
	Bark and Leaves	emetic, antidiarrhea, dysentery, hemorrhoids, gonorrhea, leucorrhoea	
	Flowers and leaves	stomachache, inflammations, wound healings	
	Bark	muscular ailment	(Delascio, 1985)
S. cytherea	Bark	Antiseptic solutions	(Villegas et al., 1997)
	Roots	fever, migraine, diarrhea	
	Leaves	gonorrhea, cystitis, urethritis	

Current status

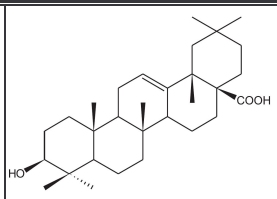
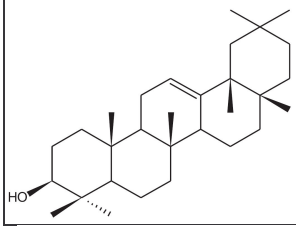
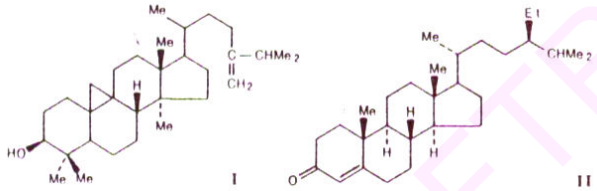
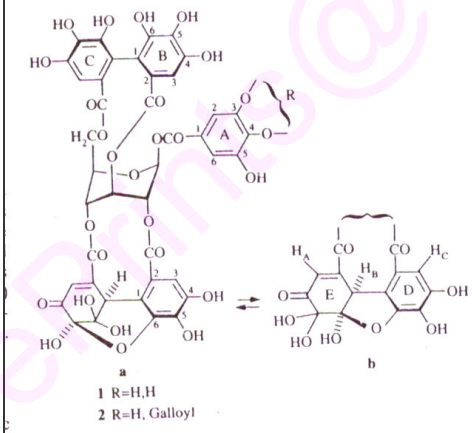
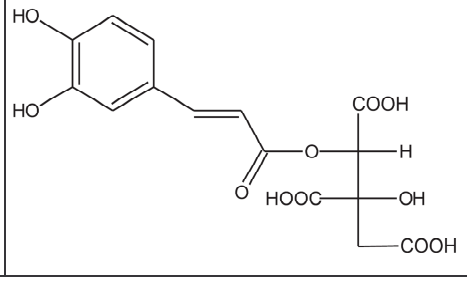
Extensive review of literature on *Spondias mangifera* revealed that, there are no reports on functional properties of bioactive compounds. Very much little work has been carried out on this fruit. Whatever the work carried out was confined to few aspects. Literally there are no reports about this fruit. The fruit is composed of 90.3g moisture, 0.7g protein, 3g fat, 0.5g minerals, 1g fibre, 4.5g carbohydrates, 36mg of calcium, 11mg phosphorous, 3.9mg iron, 270 μg carotene, 0.02mg thiamine, 0.02mg riboflavin, 0.3mg niacin, 21mg vitamin C and it gives 48Kcal of energy per 100g of edible portion (Gopalan et al. 2002). Earlier work carried out revealed that the presence of the polysaccharides which on hydrolysis gave L-arabibnose, D-galactose (Hannan and Haq, 1979). Phytochemical investigation of alcoholic extract of the fruit of *Spondias mangifera* has shown the presence of two terpene compounds, viz., β-amyrin and oleanolic acid (Singh and Saxena, 1976). Presence of Amino acids like glycine, cystine, serine, alanine and leucine were reported by Saxena and Singh (1977). 24-methylene cycloartanone, stigma-4en-3one, lignoceric acid , β-sitosterol and its β-D-glucoside have been isolated from aerial parts of *Spondias mangifera* (Tandon and Rastogi, 1976). In another biological screening report *S. mangifera* found to possess CNS depressant activity (Dhar et al. 1974). New oleanane saponins without trivial names have also been isolated from Spondias mangifera (Saxena and Mukharya, 1997).

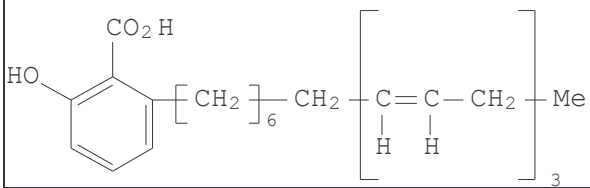
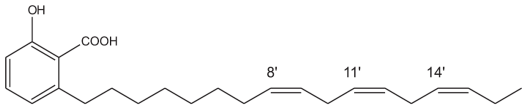
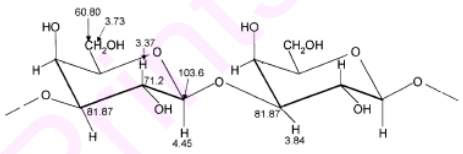
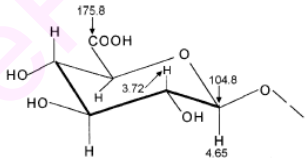
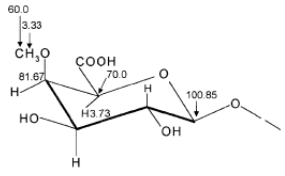
Literature availability on Spondias mangifera, is scanty, Hence related literature on other species of the genus Spondias is inclusive, to provide a comprehensive account of related compounds and their functional properties of Spondias mangifera and presented below.

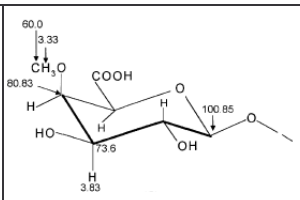
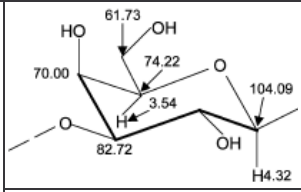
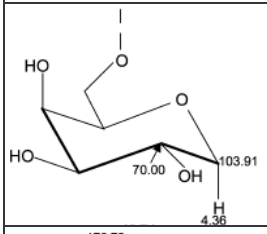
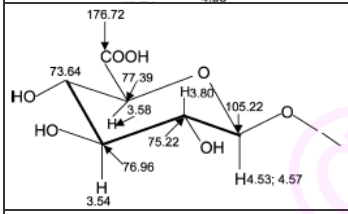
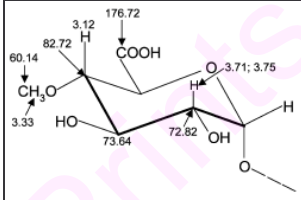
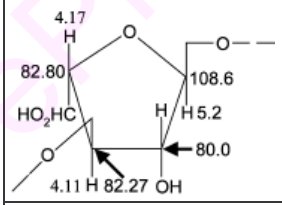
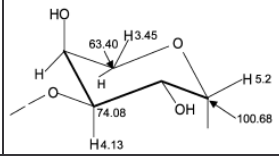
Table R5: Consolidated report on bioactivities of *Spondias sp.*

Spondias sp.	Functional property	Source	Reference
S. *mangifera*	Antioxidant activity	Stem bark	Hazra et al. 2008
	maltase inhibitory		Abesundara et al. 2004
	CNS depressant activity		Dhar et al. 1974
S. *mombin*	Abortifacient activity	aqueous extract of leaves	Offiah and Anyanwu, 1989
	Antimicrobial activity		Ajao and Shonukan, 1985,
	Antibacterial activity		Abo et al., 1999
	Antifungal activity		Corthout et al., 1991
	Antiviral activity		Rodrigues and Hesse, 2000
	Abortifacient activity	aqueous extract	Offiah and Anyanwu, 1989
	Antibacterial and Molluscicidal activity	plant extract (phenolic acid 6-alkenyl-salicylic acid)	Corthout et al.1994
	beta-lactamase inhibitory activity	hexane extract (anacardic acid)	Coates et al. 1994
	Antiherpes and antioxidant activity	plant extract	Corthout et al., 1992, Castner et al., 1998, Shultes and Raffauf, 1990, Mats, 1994
	Sedative, antiepileptic and antipsychotic activity		Ayoka et al. 2006
	Antiviral activity	leaves and stems	Corthout et al. 1991, 1992
S. *lutea*	Antiviral activity		Pereira et al., 1983; Bern and Glass, 1994
S. *cytherea*	Antinematodal activity	Methanolic extracts	Muhammad et al., 1997
S. *purpurea*	Antifungal activity	leaf extracts	Bautista-Baños et al. 2000

Table R6: Consolidated report of list of compounds identified in *Spondias sp.*

Species	Structure	Reference:
S. *mangifera*		Singh and Saxena, 1976
		
	 I = 24-methylene cycloartanone II=Stigma-4en-3one	Tandon and Rastogi, 1976
	Echinocystic acid-3-O-β-D-galactopyranosyl (1→5)-O-β-D-xylofuranoside	Saxena and Mukharya, 1997
S. *mombin*	 a 1 R=H,H 2 R=H, Galloyl b	Corthout et al. 1991
S. *mombin*		Corthout et al. 1992

	Anacardic acid 	Coates et al. 1994
		Rao and Row, 1973
	β-D-galactosyl residues, 6-O substituted D-galactosyl residues, arabinose, furanosyl residues, Arabinopyranosyl residues, rhamnose and mannose, Uronic acids, β-D-glucuronic acid and its 4-O-methyl analogue	Pinto et al. 2000a
	E)-Caryophyllene, Et butyrate, Et hexanoate, myrcene, β-phellandrene , (Z)-caryophyllene, limonene, myrcene, p-cymene	Patricia et al. 2003
S. *cytherea*	cis-β-ocimene, caryophyllene, α-pinene , d-limonene	Regina and Takayushi, 2000
	1,8-cineole, α-pinene, β-pinene, terpinolene, limonene, α-terpineol, Bu acetate, γ-terpinene, and terpinen-4-ol	Jirovetz et al. 1999
	Rhamnogalacturonan, Arabinogalactan	Iacomini et al. 2005
S. *lutea*	Cholesterol, stigmasterol, β-amyrin, lupeol, Palmitic, linoleic, oleic, linolenic, stearic acids, quercetin, quercetrin, rutin, quercetin -7-O-glucoside, glucose, galactose, mannose, rhamnose, arabinose, fructose	El Fiki, 2000
S. *cytherea* & S. *purpurea*	arabinose, mannose, xylose and rhamnose residues, glucuronic acid and its 4-0-methyl derivative, galacturonic acid	Pinto et al. 2000b
S. *purpurea*	 3-O-galactose residues	Gotera et al. 2005
	 β-D-glucuronic acid	
	 two types of 4-O-methyl-α-D-glucuronic acid residues	

			
S. *purpurea*	galactose, arabinose, mannose, xylose, rhamnose and uronic acid residues		Pinto et al. 1996
S. *dulsis*		3-*O*- galactose residues	Maritza et al. 2003
		6-*O*-galactose residues	
		β-D-glucuronic acid	
		4-*O*-methyl-α-D-glucuronic acid	
		3-*O*-α-L-arabinofuranose	
		3-*O*-β-L-arabinopyranose residues	

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 *Spondias mangifera* fruits.

Scope of the Present Investigation

Spondias mangifera (Willd.) also called as Indian Hog plum, is a unique species of *Anacardiaceae* family. The fruits, seeds and bark of the tree are used profusely in *Ayurveda* and *Unani* system of medicines to cure both infectious and degenerative diseases. The fruit is a simple fleshy drupe, which is characterized by single large seed. *Spondias mangifera* – '*a fruit of promise'* is one of the little known indigenous crops that play vital roles in the nutritional, nutraceutical or economy of rural population of India. Further, lack of earlier reports on isolation and characterization of bioactive molecules, their physiological role and biochemical changes during fruit growth and after harvest, offered sufficient scope to undertake a detailed research work on S*pondias mangifera*. These aspects, which I believe have commercial application. Understanding the physiological role of bioactive compounds during fruit growth and maturation is essential. It may provide an opportunity to standardize the stage of physiological maturity, which is a preferred quality for raw material to design and development of products of health benefits. Similar studies on bioactive molecules during storage provide an insight into the storage quality and may depict the problems associated with quality after harvest. This need to know state of scientific affairs was ardently attended, by undertaking detailed investigation on characterization of bioactive and biochemical changes in *Spondias mangifera fruit,* from the time fruit set to attainment of maturity and after harvest during storage at ambient temperature.

The results of scientific pursuit carried out in this regard have been presented in four chapters for the convenience of reading: [1] Bioactivities of selected fruits, [2] Isolation and characterization of bioactive compounds and their functional properties from S*pondias mangifera* fruit, [3] Bioactive and biochemical changes during growth and development of S*pondias mangifera* fruit and [4] Bioactive and biochemical changes of S*pondias mangifera* fruit during storage at ambient temperature.

Chapter 1

Bioactivities Of Selected Fruits

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

INTRODUCTION

Antioxidants nutrients 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. A number of studies have indicated that oxidative stress is reduced in vitro, by a variety of fruits or fruit extracts that contain significant levels of polyphenols, a class of phytochemicals known to have potent antioxidant properties than antioxidant vitamins. (Wilson et al., 1998; Dianne Hyson et al., 2000). Further, conception of 'French Paradox' has evoked interest to explore the polyphenols from selected fruits and vegetable source.

In this contest, natural, multifunctional, stable, non-toxic and natural bioactive compounds from fruits and vegetables may prefer panacea for disease. India is the second largest producer of fruits and vegetables. It produces a variety of fruits and vegetables due its varied agro-climatic conditions. India is endowed with little known fruits and vegetables, which plays a vital role in nutritional, nutraceutical and/or economical role in rural population. The following selected fruits are one belong to such group.

S.No.	Common Name	Scientific Name	Family
1.	Tiny bitter gourd	*Momordica cymbalaria*	Cucurbitaceae
2.	Wood Apple	*Feronia limonia*	Rutaceae
3.	Indian Hog Plum Variety: Kasturi	*Spondias mangifera* Willd.	Anacardiaceae
4.	Indian Hog Plum Variety: Hal	*Spondias mangifera* Willd.	Anacardiaceae

These selected fruit/ vegetables are very important for their nutraceutical value and they are widely consumed in rural India. Further various pharmaceutical properties were attributed in Ayurvedic medicine for these vegetables. Though they were used from time immemorial, the antioxidant properties and associated bioactive molecules in them have not been reported. Hence, the present work has been initiated to screen the above fruit/ vegetables for their antioxidant properties and elucidation of bioactive compounds from the most promising vegetable. The work presented in this chapter describe in detail regarding antioxidant activity of the following vegetables.

MATERIALS AND METHODS

Plant material

Fresh and healthy fruits of *Momordica cymbalaria,* wood apple, *Spondias mangifera* cv. Kasturi and *Spondias mangifera* cv. Hal were procured from the local market, Mysore, India. Hard shell of wood apple was removed before drying, while in case of other fruit species, whole fruits 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 different fruit powder was sequentially extracted using n-hexane, followed by chloroform, ethyl acetate, acetone, methanol 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.

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]. 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.

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 inhibitory 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 different fruit 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 different extracts.

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, Yershenia 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 [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 re-suspended 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.

ANTIFUNGAL ACTIVITY

Poisoned Food Technique or radial growth (RG) test

Poisoned Food Technique was determined as described by Golembiewski et al. (1995) method. Different concentration of fruit extracts in ethanol were prepared and mixed with about 20 ml of molten PDA at about 60° C to give a final concentration of 500 ppm (v/v) poured the mixture of medium into petriplates and allowed the medium to set. Agar disc (8 mm) of five day old culture in which pathogen is cultivated was transferred to agar disc of the plates containing media amended with fruit extracts petriplates were

incubated at 27 °C for 5 days. After 5 days, colony diameter was measured at two positions at right angle to each other and calculated the mean diameter of the colony. The relative growth in different treatments was calculated with the following formula.

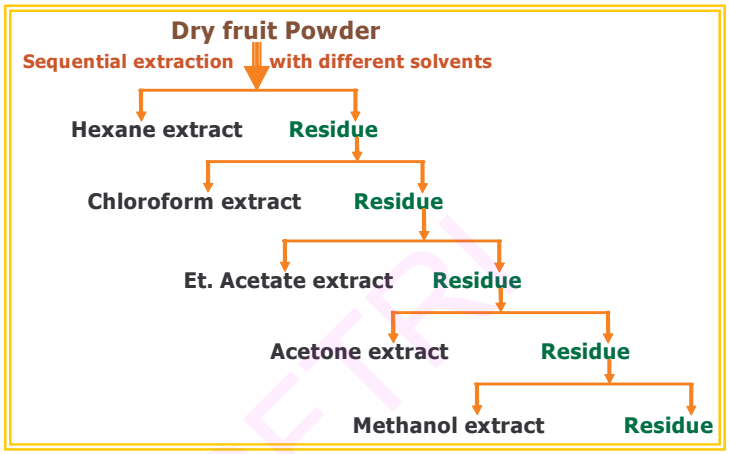
$$\frac{\text{Mean colony diameter in fungicide amended medium}}{\text{Mean colony diameter in un-amended medium (control)}} \times 100$$

RESULTS AND DISCUSSION

Sequential extraction of different fruit powder:

Extraction Method I:

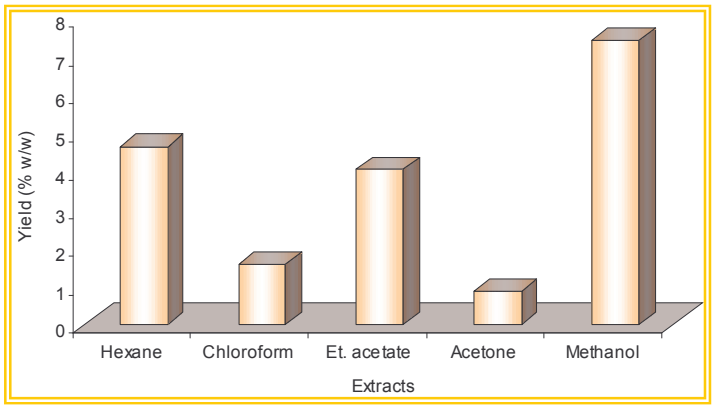
Fig. 1.1: Schematic representation of sequential extraction (method I) of four different fruit powder



Sequential extraction of 100 g of dried fruit powder from *Momordica cymbalaria,* wood apple, *Spondias mangifera* cv. Kasturi fruit and *Spondias mangifera* cv. Hal powder were carried out using hexane, chloroform, ethyl acetate, acetone and methanol **[Fig. 1.1]**.

Yield of the fruit extracts:

Fig. 1.2: Yield of the *Momordica cymbalaria* fruit extracts with different solvents.

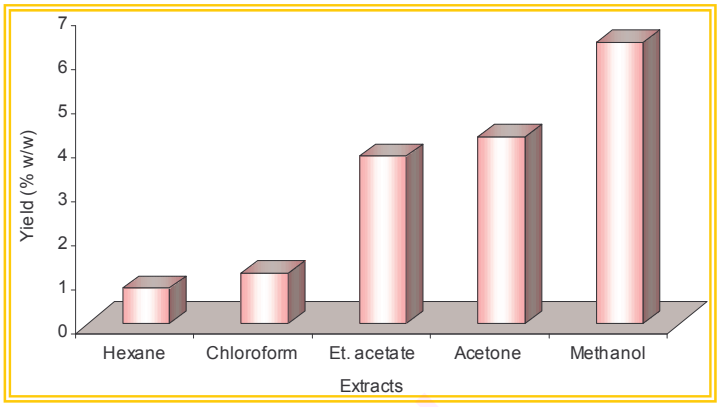


Momordica cymbalaria fruit yielded highest methanol extract (7.40 g) followed by hexane (4.60 g) and chloroform extracts (4.05 g) **[Fig. 1.2]**. Increase in yield of the solvent extracts followed the following order:

Acetone < Chloroform < Ethyl acetate < Hexane < Methanol

Wood apple, methanol extract was observed to have highest yield **[Fig. 1.3]**. Lowest yield was observed in hexane (0.80 g) and it increased gradually up to methanol (6.40 g). Yield increased with increase in the polarity of the solvents. Increase in yield of the solvent extracts followed the following order:

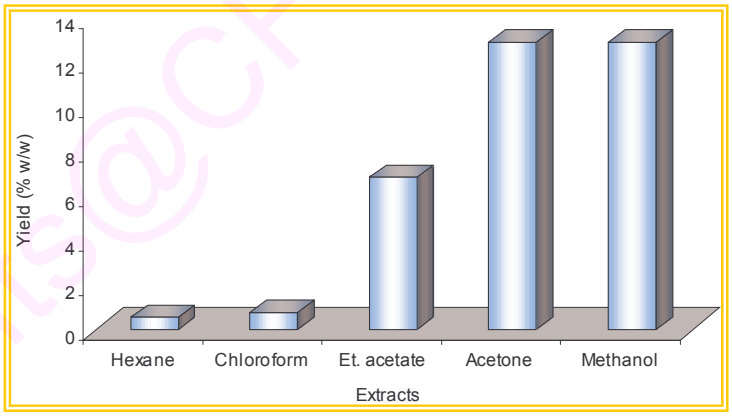
Fig. 1.3: Yield of the wood apple fruit extracts with different solvents.



Hexane < Chloroform < Ethyl acetone < Acetone <Methanol

Fig. 1.4: Yield of the *Spondias mangifera* cv. Kasturi fruit extracts with different solvents.

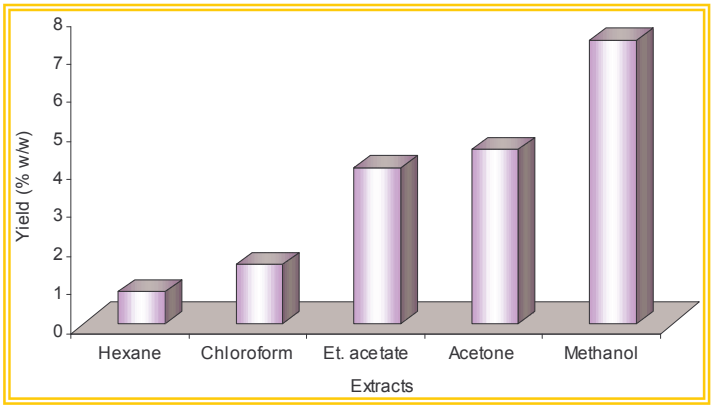
Indian hog plum (cv. Kasturi) fruit powder yielded highest methanol extract (12.95). Lowest yield was observed in hexane (0.56 g) and it increased gradually up to methanol **[Fig. 1.4]**. Yield of the extracts increased as the polarity increased. Increase in yield of the solvent extracts followed the following order:



Hexane < Chloroform < Ethyl acetone < Acetone <Methanol

Fig. 1.5: Yield of *Spondias mangifera* cv. Hal fruit extracts with different solvents.

Indian hog plum (cv. Hal) fruit powder yielded highest methanol extract (7.40 g). Lowest yield was observed in hexane (0.85 g) and it increased gradually up to methanol **[Fig. 1.5]**. Yield of the extracts increased as the polarity increased. Increase in yield of the solvent extracts followed the following order:



Hexane < Chloroform < Ethyl acetone < Acetone <Methanol

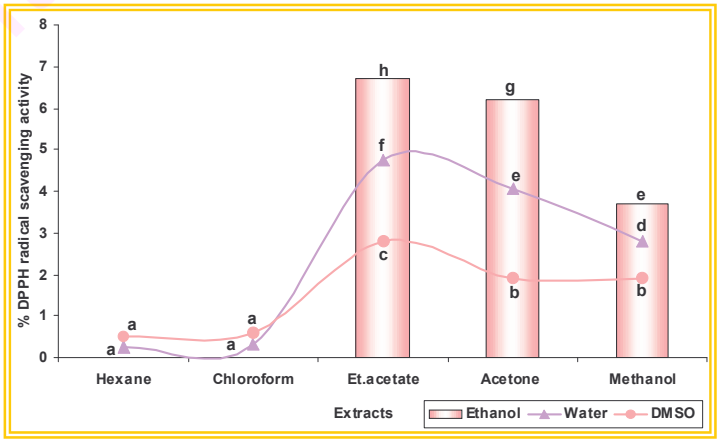
Among different solvents used for extraction from different fruits, methanol extract was found to be having highest yield.

In the present study, sequential extraction of will be helpful to extract wide range of [both non-polar and polar] bioactive molecules in a plant material. Highest yield was observed in methanol extracts in all the fruit extracts. It appears that methanol is the best solvent for extraction of polyphenols, lactones, phenones, quassinoids, flavones, saponons and some terpenoids as observed in earlier studies [Suhaj, 2006]. 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].

ANTIOXIDANT ACTIVITY OF THE DIFFERENT FRUIT EXTRACTS

To find out the potential antioxidant activity rich fruit, five different solvent extracts of the *Momordica cymbalaria*, wood apple, *Spondias mangifera* cv. Kasturi and *Spondias mangifera* cv. Hal were subjected to DPPH radical scavenging assay. BHA was used as a standard for antioxidant assay.

Fig. 1.6: DPPH radical scavenging activity of *Momordica cymbalaria* fruit extracts

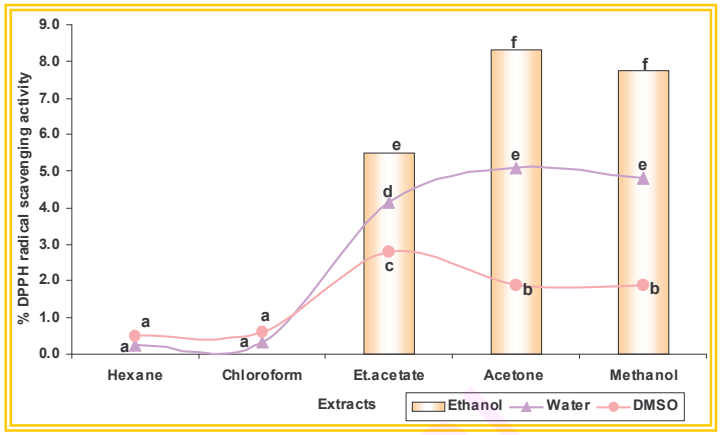


Values with different letters [a, b, c, d, e, f, g and h] differ significantly at P<0.05

Momordica cymbalaria:

Momordica cymbalaria fruit extracts were found to have very less DPPH radical scavenging activity. Highest activity of only 2.8 % of the radicals was scavenged by ethyl acetate extract dissolved in ethanol, while activity shown to be reduced by 50% and 25% when sample was dissolved in DMSO water respectively **[Fig. 1.6]**. This increase may be due to the fact that antioxidant component responsible for the activity is more soluble in ethanol than in DMSO.

Fig. 1.7: DPPH radical scavenging activity of wood apple fruit extracts



Values with different letters [a, b, c, d and f] differ significantly at P<0.05

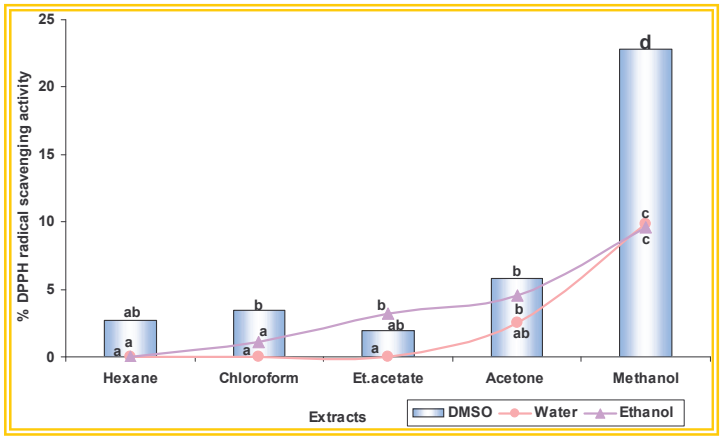
Wood apple:

Wood apple fruit extracts were also found to have less DPPH radical scavenging activity. Acetone and methanol extracts were active. Acetone extract dissolved in ethanol recorded highest antioxidant activity of 8.30%, which is four times higher than DMSO dissolved sample **[Fig. 1.7]**.

***Spondias mangifera* cv. Kasturi:**

Methanol extract of *Spondias mangifera* cv. Kasturi fruit showed highest DPPH radical scavenging activity of 22.8%. Increase in antioxidant activity observed in almost all the extracts when the test sample dissolved in ethanol and it further increased in DMSO solvent **[Fig. 1.8]**. Percentage of antioxidant activity doubled from the ethanol and water dissolved (9.6 and 9.85 %) to DMSO dissolved (22.8 %) methanol extract of *Spondias mangifera* cv. Kasturi. Antioxidant activity of an extract may be depending upon the degree of solubility of antioxidant component of the extracts.

Fig. 1.8: DPPH radical scavenging activity of *Spondias mangifera* cv. Kasturi

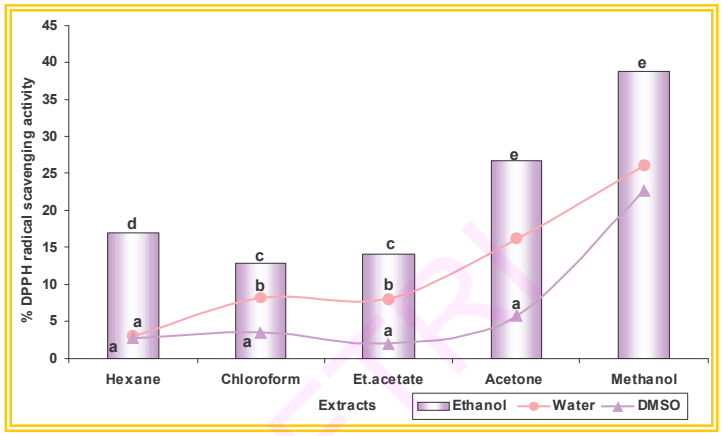


Values with different letters [a, b, c and d] differ significantly at P<0.05

Spondias mangifera cv. Hal:

All *Spondias mangifera* fruit extracts showed considerable DPPH scavenging activity. Methanol extract exhibited highest potential radical scavenging ability of 38.8%. Except, hexane extract, remaining extracts exhibited a steady increase in the antioxidant activity with increase in polarity of the solvent used for extraction **[Fig. 1.9]**.

Fig. 1.9: DPPH radical scavenging activity of *Spondias mangifera* cv. Hal



Values with different letters [a, b, c, d and e] differ significantly at P<0.05

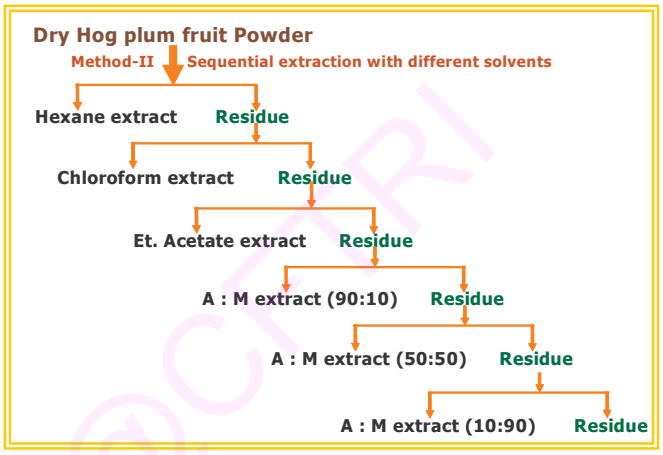
Screening of *Momordica cymbalaria*, wood apple, *Spondias mangifera* cv. Kasturi and *Spondias mangifera* cv. Hal for potential source of antioxidants, lead to the discovery of considerable percentage of antioxidant activity in two different varieties of *Spondias mangifera*. After taking into consideration of the extract yield against antioxidant activity, *Spondias mangifera* cv. Kasturi fruits yielded higher methanol extract (12.95 % w/w) compared to the *Spondias mangifera* cv. Hal variety (7.4 % w/w), but, the antioxidant activity of former was lower (22.8 %), than that of the later (38.8 %).

To exploit the higher yield of *Spondias mangifera* cv. Kasturi and also to find out suitable and efficient sequential solvent extraction system or solvent mixture for effective extraction of available antioxidant components in the fruit, following extraction method carried out apart from the above mentioned extraction system:

Spondias mangifera cv. Kasturi Vs *Spondias mangifera* cv. Hal:

Extraction Method II

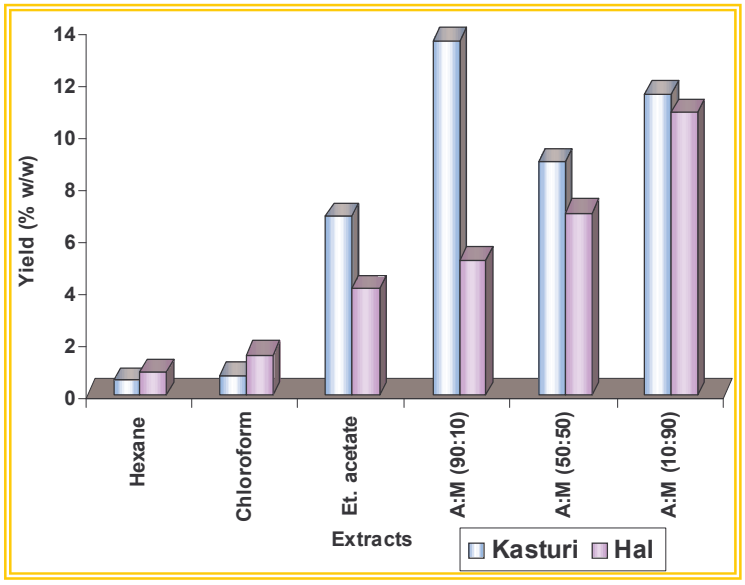
Fig. 1.10: Schematic representation of sequential extraction (method II) of *Spondias mangifera* cv. Kasturi and *Spondias mangifera* cv. Hal. fruit powder



Sequential extraction of 100 g of dried *Spondias mangifera* cv. Kasturi and *Spondias mangifera* cv. Hal fruit powder using hexane, chloroform, ethyl acetate, acetone : methanol (A : M) (90:10), A : M (50:50) and A : M (10:90) was carried out in the manner depicted in **figure 1.10**., and their corresponding yield is shown in **fig. 1.11**.

Yield of the fruit extracts:

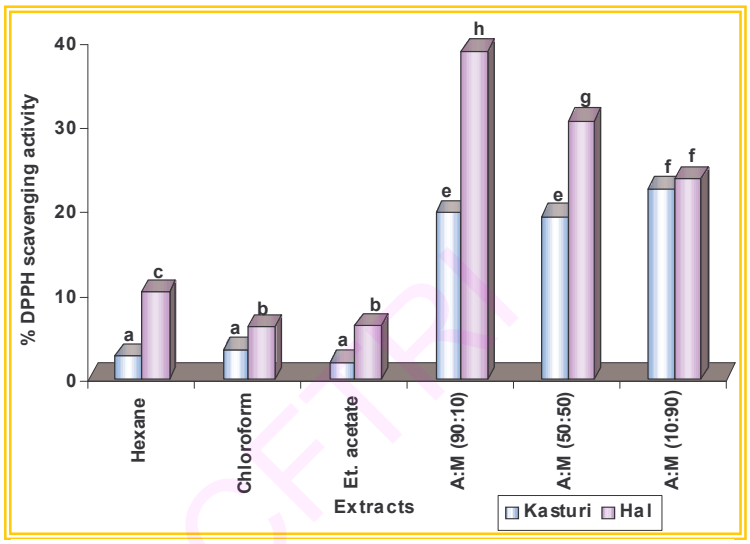
Fig. 1.11: Yield of *Spondias mangifera* cv. Kasturi and *Spondias mangifera* cv. Hal fruit extracts from extraction method II.



Spondias mangifera cv. Kasturi yield was higher than that of the *Spondias mangifera* cv. Hal. There was no significant increase in yield of the any extract in case of the former compared to that of the first method of extraction **[Fig. 1.4 & 1.11]**. But, there was significant increase (30 %) in yield component of the active extract in *Spondias mangifera* cv. Hal **[Fig. 1.5 & 1.11]**.

Antioxidant activity:

Fig. 1.12: DPPH Radical scavenging activity of *Spondias mangifera* var Kasturi and *Spondias mangifera* cv. Hal fruit extracts with different solvents.



Values with different letters [a, b, c, d, e, f, g and h] differ significantly at P<0.05

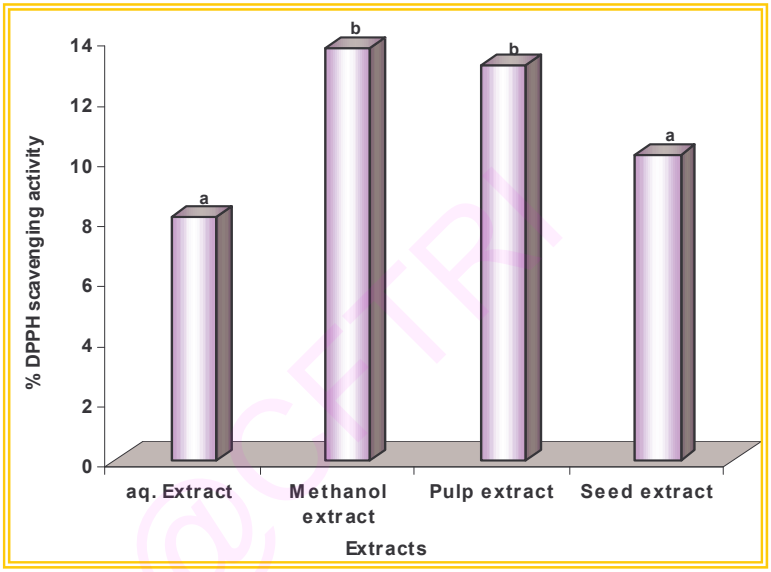
These extracts when tested for their antioxidant activity by DPPH assay, highest antioxidant activity of 38.77 % was observed in fruits of *Spondias mangifera* cv. Hal extracted in A : M (90:10) solvent mixture **[Fig. 1.12]**. This activity was similar to that of the methanol extract in first method **[Fig. 1.9]**. Antioxidant activity decreased with the increase of the methanol concentration in solvent mixture. Similarly, highest antioxidant activity of 22.5 % **[Fig. 1.12]** was observed in fruits of *Spondias mangifera* cv. Kasturi extracted in A : M (10:90) solvent mixture. This activity was similar to that of the methanol extract in first method **[Fig. 1.8]**.

Though there was an increase in the yield of the active extract in second method, but, this increase in extract yield was not expedient in effective extraction of the antioxidant component of the extracts in any of the sequential extraction systems or solvent mixtures. Hence, **the highest antioxidant activity (38.8 %) showed by *Spondias mangifera* cv. Hal extracted from methanol** was found to be a better source of antioxidant screened among the other fruits. This fruit in fresh form was also tested for DPPH radical scavenging activity.

Fresh Extracts:

Fresh fruits of *Spondias mangifera* cv. Hal extracted in methanol showed highest antioxidant activity of 13.7 %, while it was lowest (8.1%) in aqueous extract of the whole fruit **[Fig. 1.13]**. When pulp and seed were separated and extracted in methanol for the radical scavenging activity, pulp extract exhibited higher (13.1 %) activity than seed extract (10.2 %).

Fig. 1.13: DPPH Radical scavenging activity *Spondias mangifera* var. Hal fresh fruit extracts.



The high antioxidant activity of methanol extract may be due to the cumulative effect of its potential antioxidant compounds along with phenolics**.** Phenolics are very important bioactive constituents of *Spondias mangifera* fruit and other fruit crops which are known for their radical scavenging ability due to their hydroxyl groups [Hatano, *et al.,* 1989]. 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].

Antioxidant activities of the methanol extract

Methanol extract of *Spondias mangifera* cv. Hal showed highest antioxidant activity among all the extracts. So, this extract was screened for different antioxidant assays such as DPPH radical scavenging activity, superoxide radical scavenging activity, lipid peroxidation inhibitory [LPI] activity and metal chelating activity.

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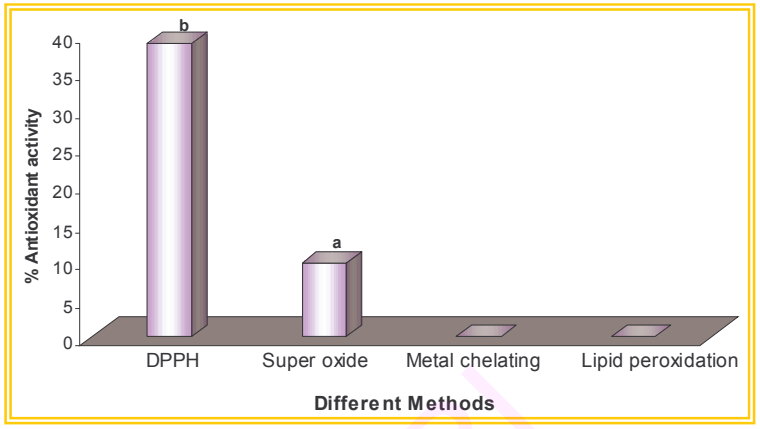
DPPH Radical scavenging activity

Fig. 1.14: Antioxidant activities of *Spondias mangifera* cv. Hal fruit extracts.

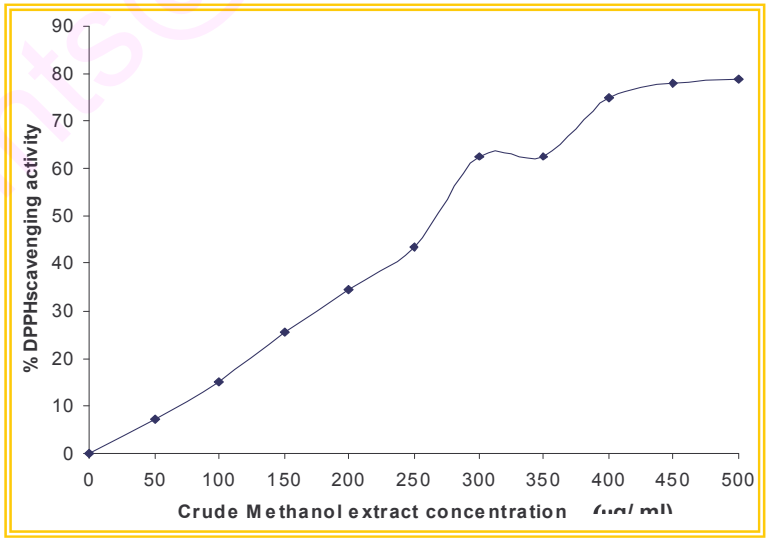


Values with different letters [a and b] differ significantly at P<0.05.

Methanol extract showed highest antioxidant activity compared to that of the other methods of antioxidant assays **[Fig. 1.14]**. So, the concentration required for scavenging of 50% radicals was carried out for DPPH assay. Methanol extract of 290µg of was required for 50% quenching of the DPPH free radicals **[Fig. 1.15].**

Superoxide radical scavenging activity

Fig. 1.15: EC_{50} value for DPPH Radical scavenging activity of methanol extract of *Spondias mangifera* cv. Hal.



Super oxide radicals were successfully quenched by methanol extract **[Fig. 1.14]**. 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].

Methanol extract of *Spondias mangifera* cv. Hal did not show lipid peroxidation inhibitory activity and metal chelating activity.

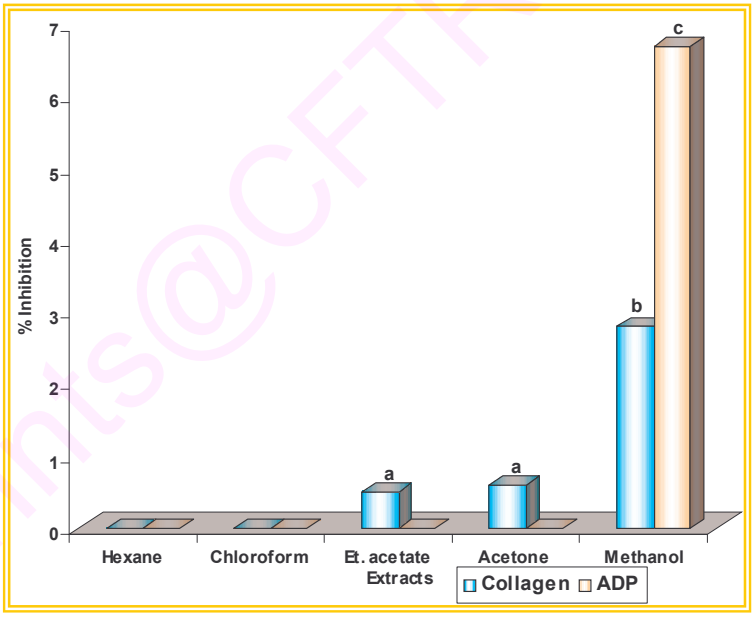
PLATELET-AGGREGATION INHIBITORY ACTIVITY

Among the different fruits viz., *Momordica cymbalaria,* wood apple, *Spondias mangifera* var, Kasturi and *Spondias mangifera* cv. Hal, screened for platelet-aggregation inhibitory activity, except *Momordica cymbalaria,* other fruits showed activity.

Wood apple

Methanol extract (500 µg) of wood apple exhibited platelet aggregation inhibitory activity of 6.7 and 2.8 % against ADP and collagen respectively **[Fig. 1.16]**. Ethyl acetate and acetone extracts showed insignificant activity. Whereas, hexane, chloroform and acetone extracts did not show platelet aggregation inhibitory activity.

Fig. 1.16: Platelet-aggregation inhibitory activity of wood apple fruit extracts.

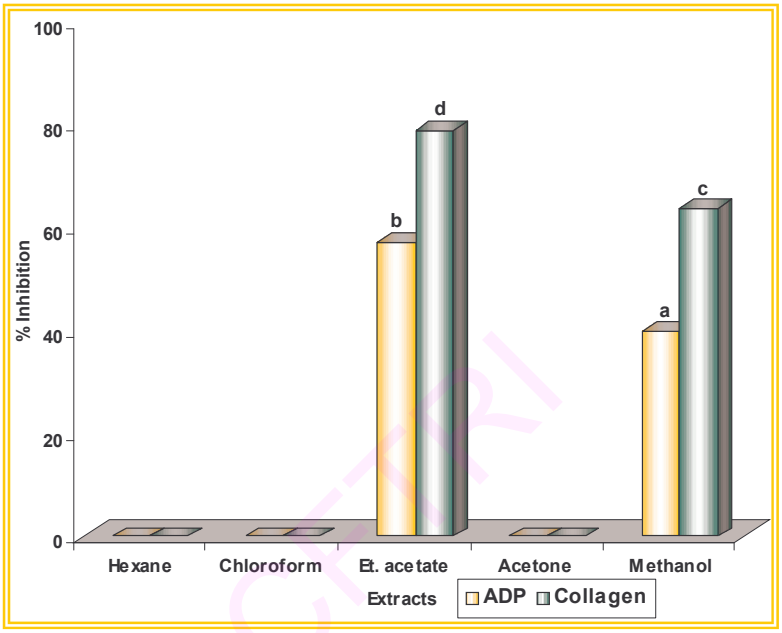


Values with different letters [a, b, and c] differ significantly at P<0.05

Spondias mangifera cv. Kasturi

Ethyl acetate extract (500 µg) of *Spondias mangifera* cv. Kasturi exhibited highest platelet aggregation inhibitory activity of 57.3 and 78.9 % followed by methanol extract inhibiting 40 and 63.9 % against ADP and collagen respectively **[Fig. 1.17]**. Hexane, chloroform and acetone extracts did not show platelet aggregation inhibitory activity.

Fig. 1.17: Platelet-aggregation inhibitory activity of *Spondias mangifera* var. Kasturi fruit extracts.

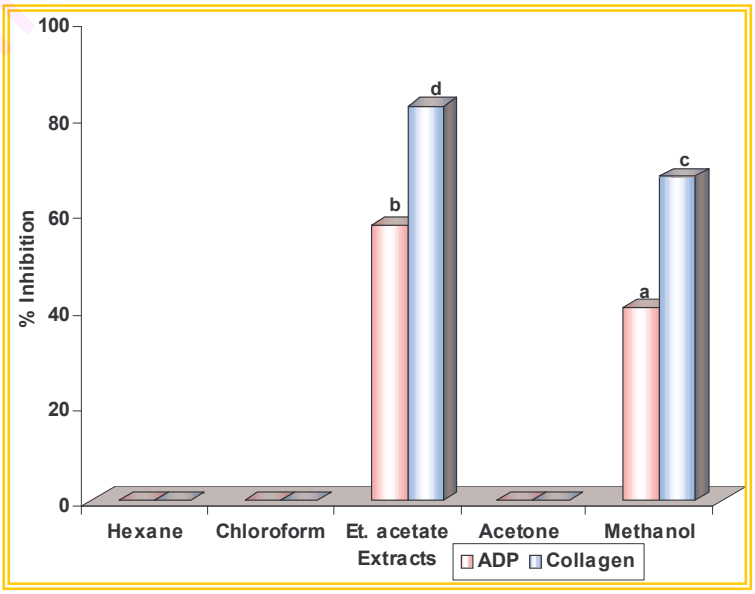


Values with different letters [a, b, c, and d] differ significantly at P<0.05

Spondias mangifera cv. Hal

Ethyl acetate extract (500 µg) of *Spondias mangifera* cv. Hal similar to that of the *Spondias mangifera* cv. Kasturi showed highest platelet aggregation inhibitory activity of 57.3 and 82.3 % followed by methanol extract inhibiting 40 and 67.4 % against ADP and collagen respectively **[Fig. 1.18]**. Hexane, chloroform and

Fig. 1.18: Platelet-aggregation inhibitory activity of *Spondias mangifera* var. Hal fruit extracts.

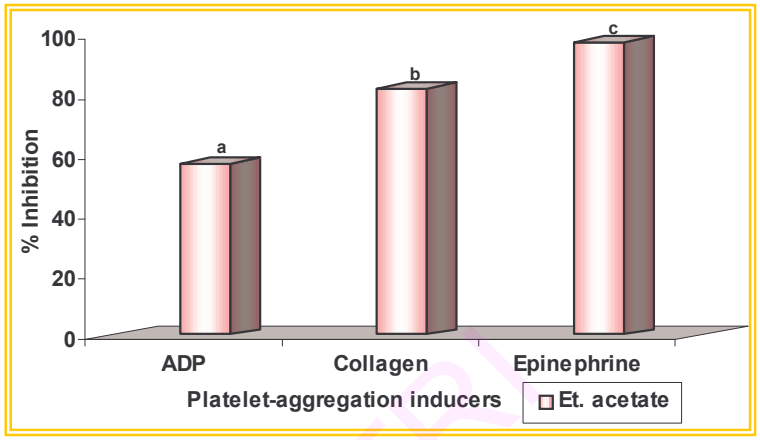


Values with different letters [a, b, c and d] differ significantly at P<0.05

acetone extracts did not show platelet aggregation inhibitory activity.

Ethyl acetate extract of *Spondias mangifera* cv. Hal found to show higher platelet aggregation inhibitory activity than *Spondias mangifera* cv. Kasturi against collagen agonist. To test the potential inhibitory nature of ethyl acetate extract, it was also tested against another agonist 'epinephrine'. It was also promising against epinephrine revealing 98 % activity **[Fig. 1.19]**.

Fig. 1.19: Platelet-aggregation inhibitory activity of *Spondias mangifera* var. Hal fruit extracts against different inducers.



Values with different letters [a, b, and c] differ significantly at P<0.05

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 consists of a series of exquisitely co-coordinated responses. The membrane surface of human platelets is responsible for a variety of reactions to various agonists. Collagen is an important platelet agonist, which enhances tissue factor activity rapidly on the platelet surface bound to monocytes and neutrophils, thus playing a crucial role in physiological haemostasis (Zillmann et al., 2001). Not only that it is associated with a burst in hydrogen peroxide, an oxidant species but also contributes to activation of platelets by acting as secondary messenger (Pignatelli et al., 1999a). It also stimulates arachidonic acid metabolism through the production of thromboxane A_2, a potent platelet aggregator as well as liberation of IP_3 and calcium mobilization (Pignatelli et al., 1999b).

Glycoprotein IV (CD 36) present on platelets plays an important role by acting as a specific receptor for collagen in the early stages of aggregation (Tandon, et al., 1989).

Another receptor Glycoprotein VI has also been identified under flow condition specifically again for collagen induced aggregation (Moroi and Jung, 2004).

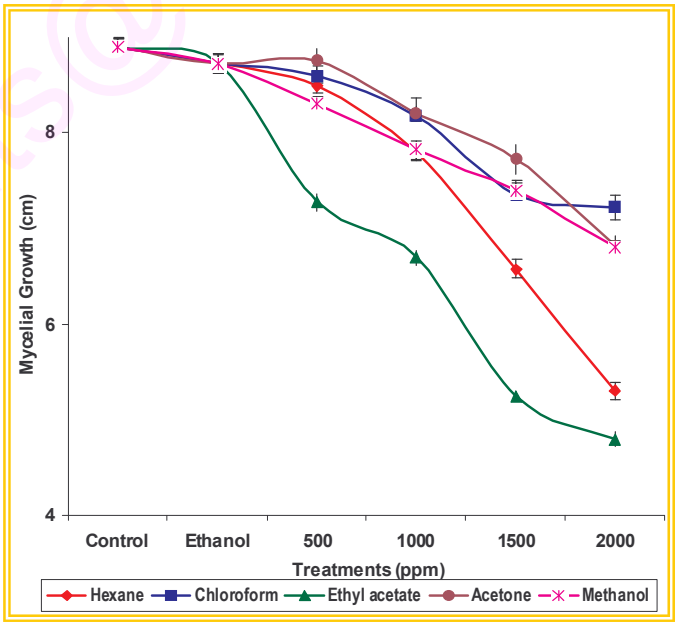
Hence it may be said that wood apple extracts of acetone and methanol and *Spondias mangifera*'s ethyl acetate extracts were acting on the platelet surface resulting in their binding to glycoprotein IV and VI. As a result of this, collagen induced aggregation was being inhibited. Not only that, due to the binding of these components to the glycoproeins, release of hydrogen peroxide, which acts as a secondary messenger was found to be affected.

ANTIFUNGAL ACTIVITY

Among the different fruits viz., *Momordica cymbalaria,* wood apple and *Spondias mangifera* cv. Hal screened for antifungal activity, all fruits showed activity. *Spondias mangifera* cv. Kasturi was not tested for antifungal activity and it was confined to *Spondias mangifera* cv. Hal only.

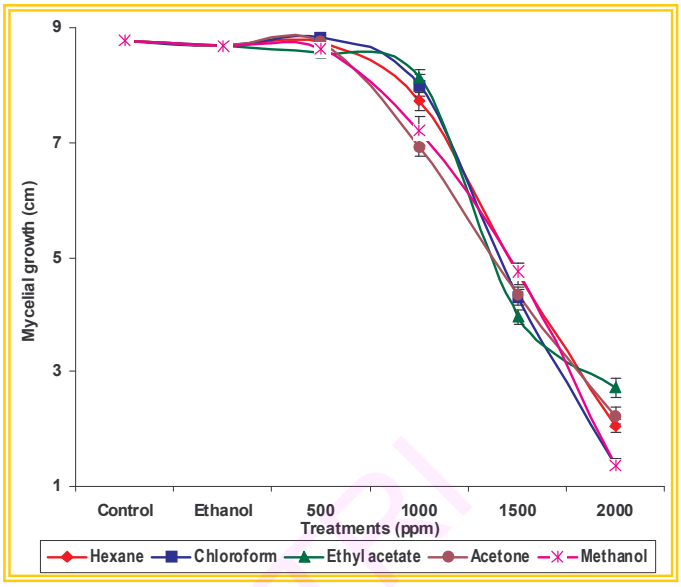
Momordica cymbalaria :

Fig. 1.20: Antifungal activity of *Momordica cymbalaria* fruit extracts .



Ethyl acetate extract of *Momordica cymbalaria* fruit inhibited the mycelia growth better than chloroform, acetone or methanol. Ethyl acetate extract concentration of 1000 ppm restricted the mycelial growth to 7.28 cm while, 1500 ppm of hexane and 2000 ppm of other extracts restricted the same **[Fig. 1.20]**. With the highest concentration of 2000 ppm ethyl acetate extract restricted the mycelial growth to a least of 4.80 cm. So, ethyl acetate extract of *Momordica cymbalaria* may be having component(s) responsible for such antifungal activity.

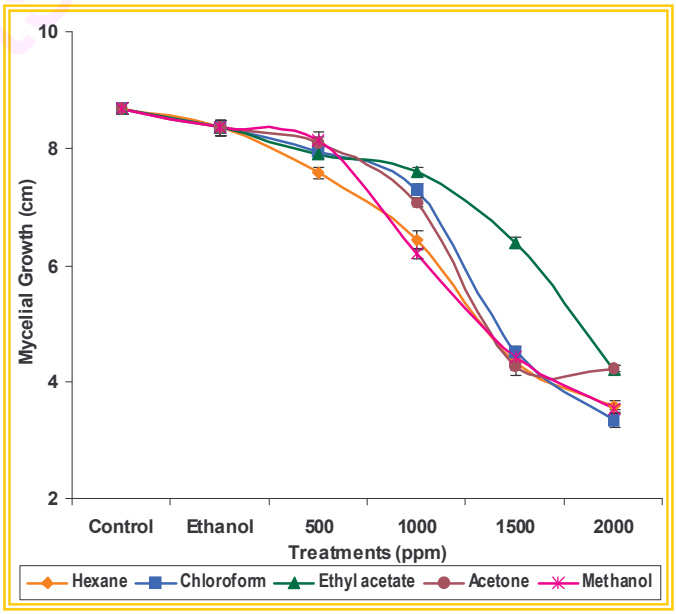
Fig. 1.21: Antifungal activity of wood apple fruit extracts.



Wood apple:

Methanol and chloroform extracts of wood apple at 2000 ppm concentration inhibited and restricted the fungal growth to 1.35 cm which is highest, compared to that of hexane (2.05 cm), acetone (2.23 cm) and ethyl acetate (2.73 cm) extracts **[Fig. 1.21]**. Observing the zone of inhibition, wood apple extracts (1.35 cm) were found to 4-5 times effective and having higher antifungal properties compared to that of the *Momordica cymbalaria* extracts (4.80 cm).

Fig. 1.22: Antifungal activity of fruits of *Spondias mangifera* var. Hal extracts.



***Spondias mangifera* cv. Hal:**

Chloroform, methanol and hexane extracts from the fruits of *Spondias mangifera* cv. Hal at 2000 ppm concentration restricted the mycelial growth of *Colletotrichum capsici* to 3.35 to 3.55 cm, while, ethyl acetate and acetone extracts inhibited 4.2 cm **[Fig. 1.22]**.

Colletotrichum capsici, a widely distributed fungal pathogen in the tropical world, causing anthracnose disease in commercially important fruits and vegetables. Wide array of fungicide sprays on the standing crop as well as on the harvested produce as pretreatments, reported to cause

biological magnification finally hazard to human health. Environmental harmful and health hazardous practice can be substituted by the use of residue free, harmless, non-toxic, environmental friendly natural products. In the wake of this, three different tropical fruits were selected for their potential antifungal activity, as they were reported in the involvement of preformed antifungal compounds for the resistance of subtropical fruits to fungal decay (Prusky and Keen, 1993).

Among five extracts of three fruit species tested for the antifungal activity against this fungal disease revealed that, concentration of 2000 ppm of chloroform and methanol extracts of wood apple (1.35 cm), followed by hexane, chloroform and methanol extracts of *Spondias mangifera* (3.35 to 3.6 cm) and ethyl acetate extract of *Momordica cymbalaria* (4.8 cm) inhibited the mycelial growth to 1.35 cm.

ANTIBACTERIAL ACTIVITY OF HOG PLUM EXTRACTS

Spondias mangifera cv. Hal showed antioxidant activity, platelet-aggregation inhibitory activity and antifungal activity. So, antibacterial activity was restricted to these fruit extracts only. Chloroform extract of *Spondias mangifera* fruits, exhibited effective antibacterial activity against five out of 12 bacteria screened viz. *Bacillus Subtilus*, *Micrococcus luteus*, *Streptococcus faecalis, Staphylococcus aureus*, *Salmonella typhi*, *Enterobacter aerogenes*, *Klebsiella*

Table 1.1: Minimum inhibitory concentrations [MIC] for different extracts of *Spondias mangifera* cv. Hal

Bacteria	MIC [in ppm] * for *Spondias mangifera* extracts				
	Hexane extract	Chloroform extract	Ethyl acetate extract	Acetone extract	Methanol extract
B. Subtilus	140^{c}	60^{a}	140^{c}	180^{d}	--
M. luteus	180^{d}	60^{a}	--	--	220^{e}
S. faecalis	200^{e}	100^{b}	--	--	--
S. aureus	--	--	--	--	200^{e}
S. typhi	--	--	100^{b}	--	--
E. aerogenes	--	--	--	--	--
K. pneumoniae	--	180^{d}	--	--	--
S. dysenteriae	--	100^{b}	--	180^{d}	--
E. coli	--	--	--	--	--
P. mirabilis	--	--	--	--	--
P. aureginosa	--	--	--	--	--
Y. enterocolitica	--	--	--	--	--

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

pneumoniae, *Shigella dysenteriae*, *Escherichia coli*, *Proteus mirabilis*, *Pseudomonos aureginosa*, and *Yershenia enterocolitica*. Chloroform extract showed inhibition similar to hexane extract, in addition to that it also showed inhibition against *K. pneumoniae* and *S. dysenteriae*. Ethyl acetate extract showed inhibitory effect as shown by acetone extract, but, in addition to that, ethyl acetate was showing inhibition against *S. dysenteriae* while acetone extract against *S. dysenteriae.* Methanol extract showed inhibition against *M. luteus,* and *S.aureus*. The chloroform extract of *Spondias mangifera* exhibited highest antibacterial activity against wide range of bacteria, when compared to other solvent extracts **[Table 1.1].** However, *E. aerogenes E. coli, P. mirabilis, P. aureginosa and Y. enterocolitica* were not inhibited by any of the solvent extracts of *Spondias mangifera*.

Different solvent extracts of *Spondias mangifera* 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, M. luteus* with MIC of 60 ppm and also inhibited the growth of *S. faecalis* and *S. dysenteriae* with MIC of 100ppm, while *K. pneumoniae* was completely inhibited at 180 ppm **[Table 1.1].** Lack of outer polysaccharides layer in Gram-positive bacteria may be responsible for more permeable to amphipathic compounds [Cowan 1999]. High resistance of Gram-negative bacteria to *Spondias mangifera* 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 *Spondias mangifera*. 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.

CONCLUSION

In the present investigation, screening of hog plum extracts for different functional properties has revealed that, they are potential inhibitors of platelet aggregation and also scavengers of free radicals and super oxide radicals. Hog plum extracts also showed antibacterial and antifungal properties. The highest platelet aggregation inhibitory activity exhibited by ethyl acetate extract and the highest antioxidant activity shown by methanol extract of hog plum cv. Hal, prompted us to select the *Spondias mangifera* cv. Hal, for the present study. Hence, a detailed studies "Characterization of bioactive and biochemical changes associated during fruit growth maturation and during storage was carried out on *Spondias mangifera* cv. Hal. Accordingly, further, purification and characterization of bioactive compounds from the active extracts of *Spondias mangifera* fruit was carried out. The details are presented in the next chapter.

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. Among the different fruits extracts evaluated for various in-vitro biological activities Different extracts of *Spondias mangifera* cv. Hal fruits exhibited highest, multifunctional bioactive properties compare to other cultivar of *Spondias mangifera* and wood apple, *Momordica cymbalaria*. Earlier review of literature indicated that no reports of bioactive molecules of health benefits. Extensive use of *Spondias mangifera for therapeutic purpose in Ayrveda and other traditional medicine prompted us to undertake isolation and characterization of bioactive molecules*

The current strategy to extract fruit with different solvents of increasing polarity has yielded interesting results. Apparently, the ethyl acetate extract exhibited highest platelet-aggregation inhibitory activity and methanol extract highest antioxidant activity. This provides the deep insight into the use of extracts for therapeutic purpose and totalitarian approach emphasized in traditional medicine. Hence, ethyl acetate and methanol extracts were selected for the isolation, purification and characterization of bioactive molecules. The more inquisitiveness and additional impetus behind selection of ethyl acetate and methanol extracts owe to the fact that, they 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 COMPOUND FROM ETHYL ACETATE EXTRACT OF *Spondias mangifera* CV. HAL

1. ISOLATION OF PLATELET-AGGREGATION INHIBITORY COMPOUND

Fractionation of the ethyl acetate 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 platelet-aggregation inhibitory compound, about 15 g of crude ethyl acetate extract was loaded and eluted stepwise with 500 ml of hexane, 1000 ml of chloroform, 8200 ml of chloroform: ethyl acetate [95:5 to 0: 100, v/v], 3200 ml of ethyl acetate: methanol [90:10 to 70: 30, v/v], 500 ml of acetone and 500 ml of methanol. About 61 fractions measuring 200 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 chloroform: ethyl acetate [90:10], chloroform: ethyl acetate: methanol [50:45:5] and ethyl acetate: methanol [25:75] 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 seven fractions [Fr.1- Fr.7]. All the seven fractions were tested for platelet-aggregation inhibitory activity as described earlier.

Further purification of bioactive fraction four [Fr.4]

Since fraction four [Fr.4] obtained from first step column chromatography showed high platelet-aggregation inhibitory activity, it was selected for further purification. About 4.40 g of bioactive fraction four [Fr.4] was further purified using XAD4 column [450 x 20 mm]. The column was eluted stepwise with 400 ml of chloroform, 300 ml of ethyl acetate, 500 ml of acetone, and 300 ml of methanol. About 17 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.4.1- Fr.4.6]. These six sub-fractions were tested for platelet-aggregation inhibitory activity.

Sub-fraction two [Fr.4.5] obtained from second step chromatography showed high platelet-aggregation inhibitory activity, hence selected for further purification. About 1.1 g of bioactive sub-fraction two [Fr.4.5] was further purified on a silica gel [100-200 mesh] column [600 x 15mm]. The column was eluted stepwise with 100 ml of chloroform: ethyl acetate [90: 10 to 0: 100, v/v], 400 ml of ethyl acetate: acetone [95: 05 to 0: 100 v/v] and 200 ml of acetone: methanol [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.4.5.1-4.5.3]. Among these, sub-fraction two [Fr.4.5.2] 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].

ISOLATION OF BIOACTIVE COMPOUND FROM METHANOL EXTRACT OF *Spondias mangifera* CV. HAL

2. ISOLATION OF ANTIOXIDANT COMPOUND

Fractionation of the methanol 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 antioxidant compound, about 12 g of crude methanol extract was loaded and eluted stepwise with 500 ml of hexane, 500 ml of chloroform, 2000 ml of chloroform: ethyl acetate [90:10 to 0: 100, v/v] and 2500 ml of ethyl acetate: methanol [90: 10 to 0: 100, v/v]. About 22 fractions measuring 250 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 chloroform: ethyl acetate [80:20], chloroform: ethyl acetate [60:40], chloroform: ethyl acetate [50:50], chloroform: ethyl acetate [30:70], chloroform: ethyl acetate [25:75] and chloroform: ethyl acetate: methanol [15:75:10] 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 seven fractions [Fr.1- Fr.7]. All the five fractions were tested for antioxidant activity as described earlier.

Further purification of bioactive fraction seven [Fr.7]

Since fraction four [Fr.7] obtained from first step column chromatography showed high antioxidant activity, it was selected for further purification. About 3.60 g of bioactive fraction four [Fr.7] was further purified using silica gel [100-200 mesh] column [450 x 20 mm]. The column was eluted stepwise with 100 ml of chloroform, 1000 ml of chloroform: ethyl acetate [95:5 to 0: 100, v/v], and 300 ml of ethyl acetate: methanol [90:10 to 0: 100, v/v]. About 15 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 five sub-fractions [Fr.7.1- Fr.7.5]. These five sub-fractions were tested for antioxidant activity **(Fig. 2.3)**

Sub-fraction four [Fr.7.4] obtained from second step chromatography showed high antioxidant activity, hence selected for further purification. About 874 mg of bioactive sub-fraction four [Fr.7.4] was further purified on a silica gel [100-200 mesh] column [600 x 15mm]. The column was eluted stepwise with 100 ml of chloroform: ethyl acetate [90: 10 to 0: 100, v/v], 400 ml of ethyl acetate: acetone [90: 10 to 0: 100 v/v] and 200 ml of acetone: methanol [90: 10 to 0: 100, v/v]. About 20 fractions measuring 25 ml each were collected and concentrated. Fractions having similar R_f values on TLC plate were pooled and numbered [Fr.7.4.1-7.4.4]. Among these, sub-fraction three [Fr.7.4.3] 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].

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 methanol 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 DMSO 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

Platelet-aggregation inhibitory activity

Antibacterial activity

Agar well diffusion assay

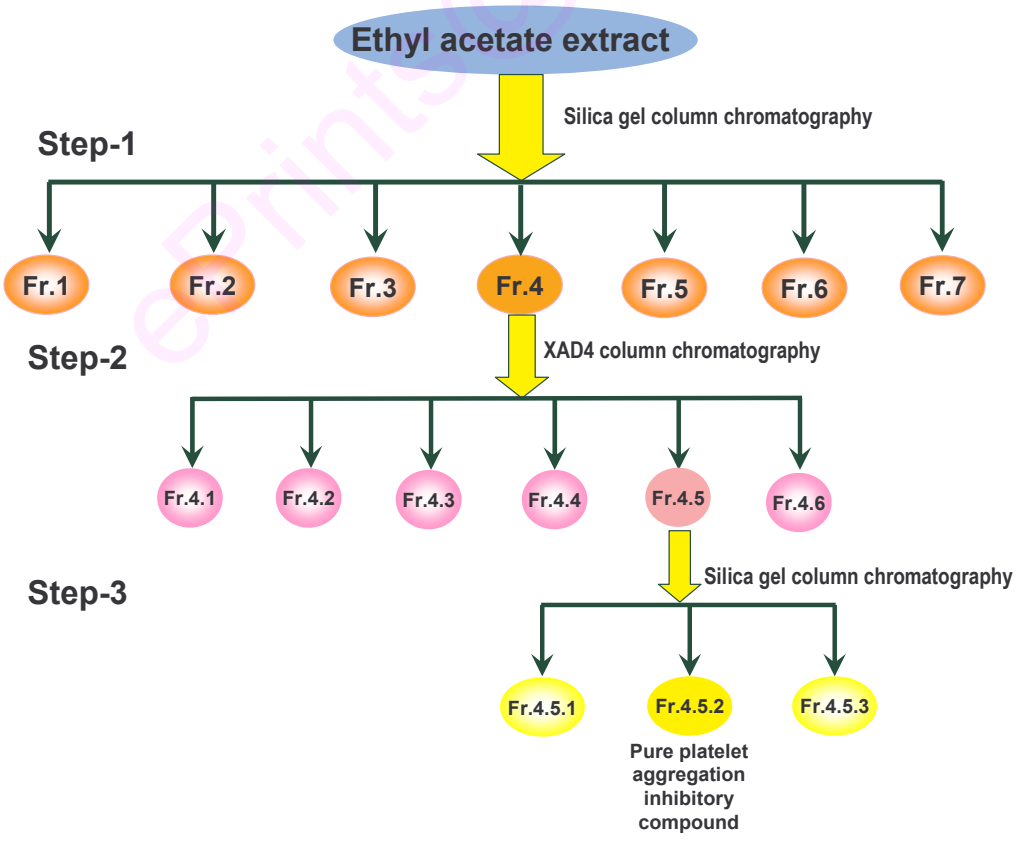
MIC

RESULTS AND DISCUSSION

1. PURIFICATION OF PLATELET-AGGREGATION INHIBITORY COMPOUND FROM ETHYL ACETATE EXTRACT OF *Spondias mangifera*

Platelet-aggregation inhibitory activity guided fractionation of ethyl acetate extract, using silica gel column chromatography has yielded about 61 fractions. The fractions having same number of spots with similar R_f values on TLC plate were pooled into seven fractions [Fr.1- Fr.7]. The schematic representation for the isolation of platelet-aggregation inhibitory compound is given in **figure 2.1**. All the seven-pooled fractions were tested for platelet-aggregation inhibitory activity. Since fraction four [Fr.4] obtained from first step column chromatography showed highest platelet-aggregation inhibitory activity **[Table 2.1]**, it was selected for further purification. Further purification of bioactive fraction four [Fr.4] by using XAD4 column chromatography yielded 17 fractions. Fractions having same number of spots with similar R_f values were pooled into six sub-fractions [Fr.4.1-

Fig 2.1: Schematic representation for isolation of platelet-aggregation inhibitory compound from ethyl acetate extract of *Spondias mangifera* fruit.



Fr.4.6]. These six sub-fractions were tested for platelet-aggregation inhibitory activity. Sub-fraction five [Fr.4.5] obtained from column chromatography showed highest platelet-aggregation inhibitory activity **[Table 2.2].** Further purification of active sub-fraction five [Fr.4.5] by using silica gel column chromatography yielded 28 fractions. Fractions having same number of spots with similar R_f values on TLC plate were pooled into three sub-fractions [Fr.4.5.1-Fr.4.5.3]. Sub-fraction two [Fr.4.5.2] obtained from column chromatography showed highest platelet-aggregation inhibitory activity **[Table 2.3].** Sub-fraction two [Fr.4.5.2] showed a single spot on TLC. The purity of the isolated compound was confirmed by HPLC. This pure compound was subjected to various spectroscopic techniques for the elucidation of the structure.

Table 2.1: Platelet aggregation inhibitory activity and antioxidant activity of different fractions Ψ of ethyl acetate extract

Inducer	Platelet-aggregation inhibitory activity (%) exhibited by different column fractions of ethyl acetate extract						
	Fr. 1	Fr. 2	Fr. 3	Fr. 4	Fr. 5	Fr. 6	Fr. 7
ADP	--	29.8	26.0	33.0	30.7	--	--
Collagen	--	36.7	31.6	78.6	67.8	--	--

Ψ Seven fractions [Fr.1- Fr.7] were obtained from the ethyl acetate extract by silica gel column chromatography.

Table 2.2: Platelet-aggregation inhibitory activity of fractions Ψ obtained from second step column chromatography

Inducer	Platelet-aggregation inhibitory activity (%) exhibited by different column fractions of ethyl acetate extract					
	Fr. 4.1	Fr. 4.2	Fr. 4.3	Fr. 4.4	Fr. 4.5	Fr. 4.6
ADP	--	--	--	--	44.9	39.8
Collagen	--	--	--	--	45.3	38.4

Ψ **Six** fractions [Fr.4.1- Fr.4.6] were obtained by second step chromatography of "active fraction" [Fr.4].

Table 2.3: Platelet aggregation inhibitory activity of fractions Ψ obtained from third step column chromatography

Inducer	Platelet-aggregation inhibitory activity (%) exhibited by different column fractions of ethyl acetate extract		
	Fr. 4.5.1	Fr. 4.5.2	Fr. 4.5.3
ADP	23.4	44.9	29.8
Collagen	27.5	45.3	28.4

Ψ **Three** fractions [Fr.4..5.1- Fr.4.5.3] were obtained by third step chromatography of "active fraction" [Fr.4.5].

ELUCIDATION OF STRUCTURE OF PLATELET-AGGREGATION INHIBITORY 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 216 nm and 260 nm corresponding to π- π* transition of C=C double bonds. IR spectral data showed peaks at 1202 cm^{-1} [C-O stretching], 1427 cm^{-1} [C-H bending], 1048 cm^{-1} [C-O-C stretching] and 3442 cm^{-1} [-OH stretching]. Mass of the purified compound was detected by using LC-MS showed parent molecular ion peak at 579 (M+1) **[Table 2.4].**

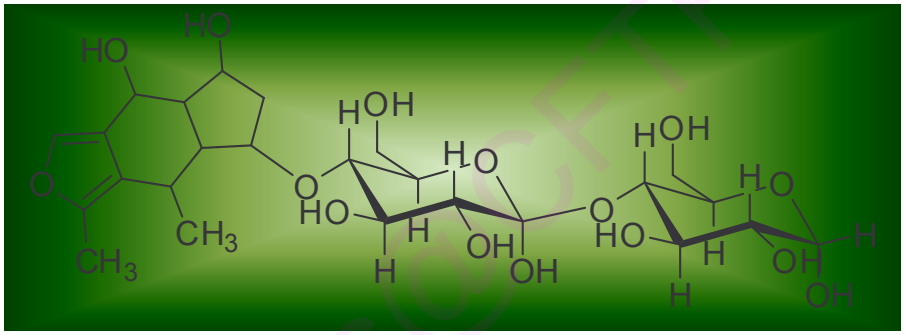
Table 2.4: Spectral data of the Glycospondin.

Spectra	GLYCOSPONDIN		
UV	216 nm and 260 nm		
IR	2964 cm^{-1} [alkyl stretching] 1202 cm^{-1} [C-O stretching] 1427 cm^{-1} [C-H bending] 3442 cm^{-1} [-OH stretching] 1048 cm^{-1} [C-O-C stretching]		
MASS	579 [M^{+1}]		
NMR	Signal	^{13}C [ppm]	^{1}H [ppm]
	1-CH	60.3	4.49
	2-CH	137.3	6.79
	3-C	146.6	-
	4- CH	22.6	2.93
	5-CH	77.8	2.85
	6- CH_2	43.4	1.92; 1.67
	7-CH	69.1	3.23
	8-CH	44.9	2.04
	9-CH	38.6	2.19
	10-C	123.9	-
	11-C	120.9	-
	13- CH_3	11.6	2.30
	14- CH_3	18.1	1.25
	15-OH	-	5.17
	16-OH	-	4.14
	18-CH	82.5	3.02
	19-CHOH	68.3	3.73; 2.08
	20-CHOH	83.0	3.81; 2.10
	21-COH	110.6	2.12
	23-CH	69.6	4.06
	24-CH_2	65.9	3.79; 3.54
	25-OH	-	2.08
	27-CH	65.6	3.02
	28-CHOH	72.5	3.73; 2.05
	29-CHOH	78.3	3.49; 2.08
	30-CHOH	95.6	5.41; 2.08
	32-CH	72.3	4.04
	33-CH_2	73.8	3.79; 3.54
	34-OH	-	2.04

Two-dimensional HMQCT spectrum showed one CH_3 group as singlet and one more CH_3 signal was observed as doublet indicated that they were attached to -CH carbon. The region between 2.0 ppm and 2.93 ppm indicated quite a lot of CH, CH_2 and CH_3 signals with complex multiple splitting. The region between 0.96 ppm and 4.81 ppm in ^{1}H spectrum showed CH signals attached to OH groups along with corresponding ^{13}C signal. Some quaternary carbons and aromatic carbons in the region 120.9 ppm to 146.6 ppm were observed. The glucose signals were observed in the range of 65.6-95.6 ppm. The corresponding proton signals were observed in the range 2.04-5.41 ppm. The details of the spectral data have been given in **Table 2.4.**

Based on all the above spectral data, the structure was deduce (2*R*,3*R*,4*R*,5*S*,6*R*)-5-(7,8-dihydroxy-3,4-dimethyl-4a,5,6,7,7a,8-hexahydro-4*H*-indeno[5,6-c]furan-5-yloxy)-6-(hydroxymethyl)-2-((2*R*,3*R*,4*R*,5*S*,6*S*)-4,5,6-trihydroxy-2-(hydroxymethyl)tetrahydro-2*H*-pyran-3-yloxy)tetrahydro-2*H*-pyran-2,3,4-triol. The structure of the molecule was coined as "Glycospondin" **[Fig 2.2].** Molecular formula of Glycospondin deduced was $C_{26}H_{40}O_{14}$ with a molecular mass of 579. ***The glycospondin was reported for the first time from Spondias mangifera and it is not reported from any other source.***

Fig. 2.2: Structure of Glycospondin.



Molecular Formula: $C_{26}H_{40}O_{14}$
IUPAC Name: (2*R*,3*R*,4*R*,5*S*,6*R*)-5-(7,8-dihydroxy-3,4-dimethyl-4a,5,6,7,7a,8-hexahydro-4*H*-indeno[5,6-c]furan-5-yloxy)-6-(hydroxymethyl)-2-((2*R*,3*R*,4*R*,5*S*,6*S*)-4,5,6-trihydroxy-2-(hydroxymethyl)tetrahydro-2*H*-pyran-3-yloxy)tetrahydro-2*H*-pyran-2,3,4-triol

2. PURIFICATION OF ANTIOXIDANT COMPOUND FROM METHANOL EXTRACT OF *Spondias mangifera*

Methanol extract of *Spondias mangifera* cv. Hal exhibited highest DPPH and super oxide radical scavenging activity **[Fig. 1.11]**. Hence, it was selected for the isolation of antioxidant compound. Antioxidant activity guided fractionation of methanol extract by using silica gel column chromatography yielded about 22 fractions. The fractions having same number of spots with similar R_f values on TLC plate were pooled into seven fractions from Fr.1 to Fr.7. The schematic representation for the isolation of antioxidant compound is given in **figure 2.3.** Among these, seventh fraction [Fr.7] showed 51.8% DPPH scavenging activity, while, Fr.1 and 2 showed no activity while Fr. 3, 4, 5 and Fr. 6 showed 8.2 %, 1.7 %, 4.1 % and 17 % DPPH scavenging activity respectively **[Fig. 2.4].** Further purification of the seventh fraction [Fr.7] by using silica gel chromatography

Fig 2.3: Schematic representation for isolation of antioxidant compound from methanol extract of *Spondias mangifera* fruit.

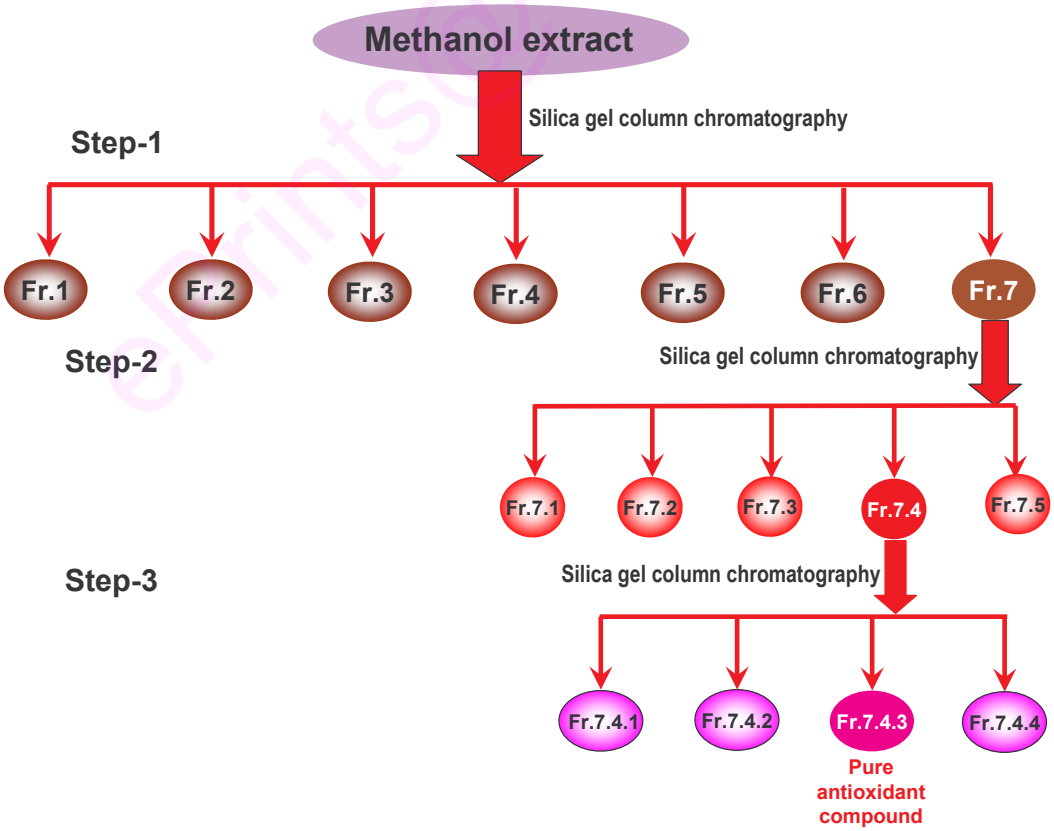
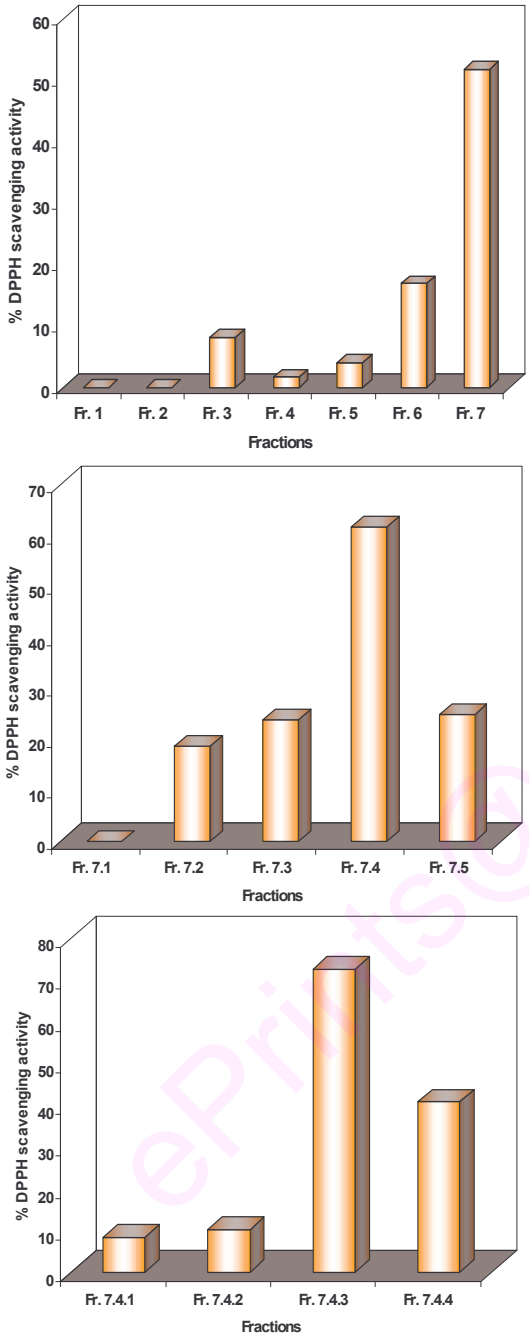


Fig. 2.4: DPPH radical-scavenging activity of different fractions obtained from methanol extract by three steps of column chromatography



Each value is mean of three observations.

yielded about 15 fractions. The fractions having same number of spots with similar R_f values on TLC plate were pooled into five sub-fractions Fr.7.1- Fr.7.5 **[Fig 2.3]**, wherein, fourth sub-fraction [Fr.7.4] exhibited high DPPH scavenging activity [72.5 %], while Fr.7.1, Fr. 7.2, Fr.7.3 and Fr. 7.5 showed 5.6 %, 8.3 %, 10.3 % and 40.8 % DPPH scavenging activity respectively **[Fig. 2.4].** The active sub-fraction four [Fr.7.4] was further purified using silica gel [100-200 mesh] column chromatography and yielded 20 fractions. The fractions having same number of spots with similar R_f values on TLC plate were pooled into four fractions [Fr.7.4.1-Fr.7.4.4]. Among these, Fr. 7.4.1, Fr. 7.4.2 and Fr. 7.4.4 exhibited 38 %, 40% and 36 % DPPH radical scavenging activity respectively **[Fig. 2.4].** The third fraction [Fr.7.4.3] showed highest DPPH scavenging activity [72.5 %] and a single spot on TLC. The purity of the isolated compound was confirmed by HPLC. The isolated purified compound was subjected to various spectroscopic analysis viz. UV, IR, NMR and LC-MS to elucidate the structure.

Elucidation of structure of antioxidant compound

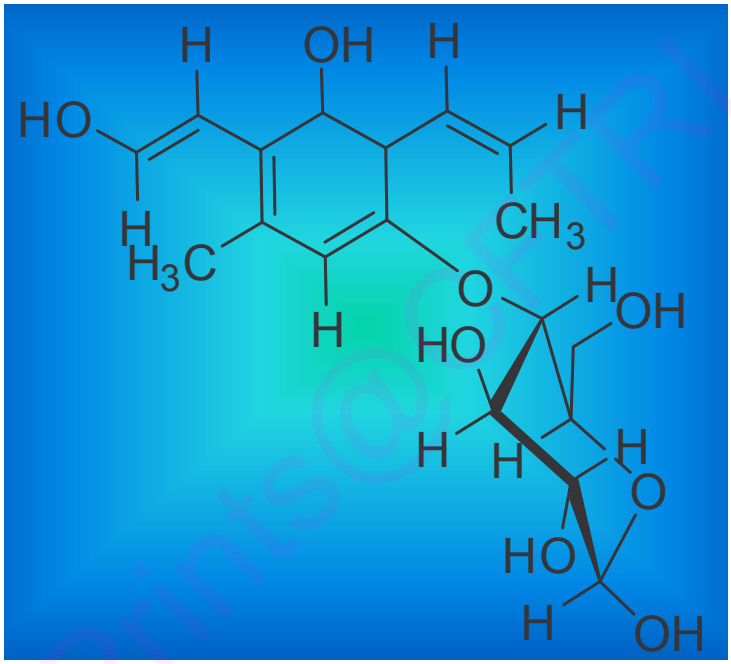
The structure of bioactive compound was elucidated after analyzing the data obtained by various spectroscopic techniques. The molecular weight was determined using LC-MS. Mass spectrum showed the parent molecular ion at 371. The molecular formula of the compound was found to be $C_{18}H_{26}O_8$. The UV maxima were observed at 269 nm, 217 nm and 243 nm. 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 1012 cm^{-1} can be attributed to alkyl and C-O-C stretching vibrations respectively. The alkyl C-H bending vibration has been observed at 1427 cm^{-1}. **[Table 2.5].**

Table 2.5: Spectral data of the Spondiol.

Spectra	SPONDIOL		
UV	269 nm, 217 nm and 243 nm		
IR	3403 cm^{-1} [OH stretching] 2996 cm^{-1} [Alkyl -CH stretching] 1012 cm^{-1} [C-O-C stretching] 1427 cm^{-1} [C-H bending]		
MASS	371 [M^{+1}]		
NMR	Signal	^{13}C [ppm]	^{1}H [ppm]
	1-CH	69.2	3.91
	2-CH	38.0	2.91
	3-C	151.4	-
	4-CH	101.9	4.83
	5-C	133.3	-
	6- C	136.7	-
	7-CH	101.0	6.5
	8-CH	158.7	5.3
	9-CH	129.1	5.43
	10-CH	130.6	5.37
	11- CH_3	12.0	2.05
	14-OH	-	4.14
	16-OH	-	15.0
	18- CH_3	18.5	2.21
	21-CH	70.6	3.67
	22-CHOH	74.2	3.71; 4.81
	23-CHOH	72.6	3.49; 4.81
	24-CHOH	94.8	5.41; 2.0
	26-CH	76.9	3.98
	27- CH_2	62.6	3.54
	28-OH	-	4.78

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 2.5].** The -CH_3 protons showed peaks in the region 2.05-2.21 ppm. There are two -CH_3 groups and the corresponding ^{13}C peaks were detected between 12.0-18.5 ppm. The cyclic -CH_2 and -CH protons exhibited peaks in the range 2.91-5.43 ppm and the corresponding ^{13}C peaks have been observed between 38.0-129.1ppm. The one -CH_2 protons of showed doublet at 3.54

Fig. 2.5: Structure of Spondiol.



Molecular Formula: $C_{18}H_{26}O_8$
IUPAC Name: (2*R*,3*R*,4*R*,5*S*,6*R*)-5-(5-dihydroxy-4-((E)-2-hydroxyvinyl)-3-methyl-6-((*Z*)-prop-1-enyl)cyclohexa-1,3-dienyloxy)-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2,3,4-triol

ppm indicating clearly both the -CH_2 groups are attached to -CH carbon. The three -CH groups attached to -OH moiety showed ^{1}H peak at 3.49- 5.41 ppm and the corresponding ^{13}C peak at 72.6-94.8 ppm. Based on these spectral data, the structure of the compound was deduced as (2*R*,3*R*,4*R*,5*S*,6*R*)-5-(5-dihydroxy-4-((E)-2-hydroxyvinyl)-3-methyl-6-((*Z*)-prop-1-enyl)cyclohexa-1,3-dienyloxy)-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2,3,4-triol**.** The structure of the molecule was coined as a "Spondiol" **[Fig. 2.5].** Molecular formula of Glycospondin deduced was $C_{18}H_{26}O_8$ with a molecular mass of 371. *The compound has been reported for the first time from Spondias mangifera fruit and it is not reported from any other source.*

BIOACTIVE PROPERTIES OF GLYCOSPONDIN

ANTIOXIDANT ACTIVITY

DPPH radical scavenging activity

Glycospondin showed DPPH radical scavenging activity with an IC_{50} of 110 µg/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].

Superoxide radical scavenging activity

Glycospondin showed superoxide radical scavenging activity with an IC_{50} 164 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.

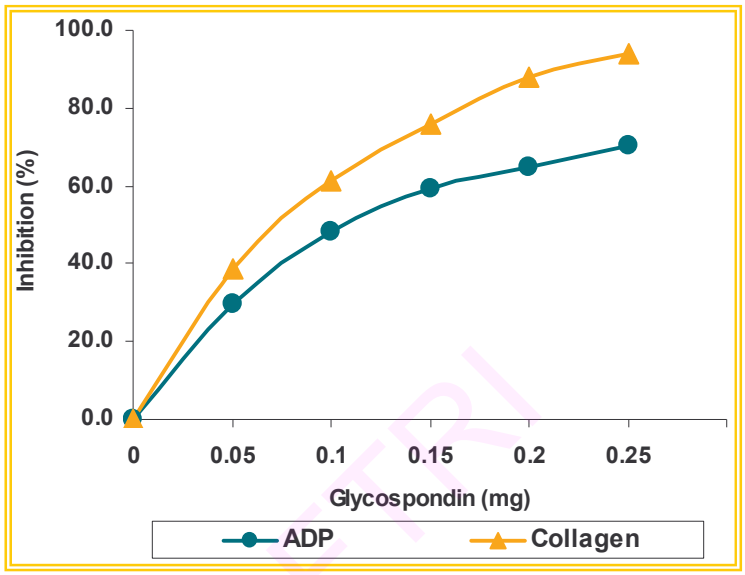
Table 2.6: Antioxidant activity of Glycospondin

Antioxidant activity	EC_{50} value [µg/ml]	
	Glycospondin	BHA
DPPH radical scavenging activity	110 ± 3.5 [b]	5 ± 0 [a]
Superoxide radical scavenging activity	164 ± 3.7 [a]	258 ± 2 [b]
Lipid peroxidation inhibitory activity	N.D.	94 ± 1 [b]
Metal chelating activity	N.D.	N.D.

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

PLATELET-AGGREGATION INHIBITORY ACTIVITY

Fig. 2.6 Platelet-aggregation inhibitory activity of glycospondin.



Glycospondin showed platelet-aggregation inhibitory activity with an IC_{50} of 55 µg and 125 µg against collagen and ADP respectively **[Fig. 2.6].** Glycoprotein IV (CD 36) present on platelets plays an important role by acting as a specific receptor in the early stages of aggregation (Tandon et al., 1989). Another receptor Glycoprotein VI has also been identified under flow condition specifically again for induced aggregation (Moroi and Jung, 2004). Glycospondin concentration of IC_{50} for collagen and ADP were used to investigate the effect of increase at different time of incubation from 1, 2 and 4 minutes [**Table 2.6**]. The platelet-aggregation inhibitory activity was found to be a function of time. Longer the contact with the glycospondin, increased percentage of binding to the surface Glycoproteins.

Table 2.7: Effect of Incubation Time at IC_{50} of Glycospondin on platelet-aggregation inhibitory activity.

Inducer	Amount IC_{50} * (mg)	Per cent Inhibition Incubation Period		
		1 min	2 min	4 min
Collagen	0.055	51.2 ± 2.1[a]	74.8 ± 3.1[c]	83.5 ± 2.4[e]
ADP	0.125	50.6 ± 1.9[a]	63.1 ± 2.4[b]	77.7 ± 2.7[d]

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

Regulation of platelet activity by using specific pharmacological agents has proven to be a successful strategy for the prevention of thrombosis. Platelet-aggregation inhibitory 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 and Steinhubl, 2000; Calverley, 2001]. 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. In the present study, high platelet-aggregation inhibitory activity of glycospondin may be exploited for the pharmacological purpose.

ANTIBACTERIAL ACTIVITY

Glycospondin exhibited an interesting antibacterial activity. It was more effective against wide spectrum of bacteria viz. *B. subtilis, M. luteus, S. fecalis, S. typhi, S. dysenteriae, S. aureus* and *K. pneumoniae.* Glycospondin showed antibacterial activity against *B. Subtilus, M. luteus, S. faecalis* and *S. typhi* with MIC 40, 40, 80 and 80 ppm respectively. In addition, glycospondin also inhibited the growth of other two bacteria viz. *S. aureus* and *K. pneumoniae* with MIC of 130 and 150 ppm respectively **[Table 2.8].** However, these were not inhibited by the chloroform extract. High antibacterial activity of Glycospondin for wide range of bacteria may be attributed to its structural components. It possesses two-hydroxyl and two-methyl along with one furan ring.

Table 2.8: Antibacterial activity of Glycospondin

Bacteria	Glycospondin MIC [ppm] *
B. Subtilus	40
M. luteus	40
S. faecalis	80
S. aureus	130
S. typhi	80
K. pneumoniae	150
S. dysenteriae	90

* Each value represents mean of three different observations.

Glycospondin by virtue of possessing one furan ring, 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 glycospondin compared to its source extract. The bioactivity of glycospondin 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].

BIOACTIVE PROPERTIES OF SPONDIOL

ANTIOXIDANT ACTIVITY

DPPH radical scavenging activity

Spondiol showed DPPH radical scavenging activity with an IC_{50} of 80µg/ml **[Table 2.9].** 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 2.9: Antioxidant activity of Spondiol

Antioxidant activity	EC_{50} value* [µg/ml]	
	Amadaldehyde	BHA
DPPH radical scavenging activity	80 ± 3.5 [b]	5 ± 0.8 [a]
Superoxide radical scavenging activity	104 ± 3.7 [a]	258 ± 2.4 [b]
Lipid peroxidation inhibitory activity	161 ± 2.8 [b]	94 ± 1.6 [a]
Metal chelating activity	196 ± 2.6 [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

Spondiol showed superoxide radical scavenging activity with an IC_{50} 104 µg/ml **[Table 2.9].** The activity was higher than that of BHA [258 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]. Spondiol showed lipid peroxidation inhibitory activity with an IC_{50} of 161 μg/ml **[Table 2.9].** Hydroxyl radical and perferryl ion are highly reactive, and act as the actual initiating species for cellular lipid peroxidation [Fridovich, 1989]

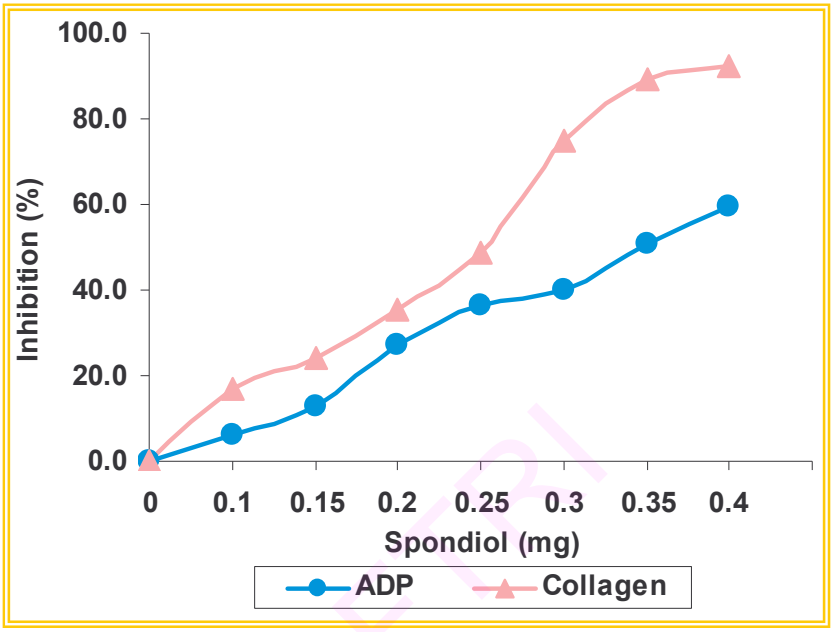
Metal chelating activity

Spondiol showed metal chelating activity with an IC_{50} of 196 μg/ml **[Table 2.9].** 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.

PLATELET-AGGREGATION INHIBITORY ACTIVITY

Spondiol showed platelet-aggregation inhibitory activity with an IC_{50} of 250 μg and 350 μg against collagen and ADP respectively **[Fig. 2.7].** Glycoprotein IV (CD 36) present on platelets plays an important role by acting as a specific receptor in the early stages of aggregation (Tandon et al., 1989). Another receptor Glycoprotein VI has also been identified under flow condition specifically again for induced aggregation (Moroi, 2004). Spondiol concentration of IC_{50} for collagen and ADP were used to investigate the effect of increase in the duration of incubation from 1 to 2 and 4 minutes [**Table 2.10**]. It was observed that as the incubation time doubled the percentage inhibition increased. This showed that the longer the contact with the spondiol, better the binding to the surface glycoproteins.

Fig. 2.7: Platelet-aggregation inhibitory activity of Spondiol.



Regulation of platelet activity by using specific pharmacological agents has proven to be a successful strategy for the prevention of thrombosis. Platelet-aggregation inhibitory 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 and Steinhubl, 2000; Calverley, 2001]. 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.

Table 2.10. Effect of Incubation Time at IC_{50} of Spondiol on platelet-aggregation inhibitory activity.

Inducer	Amount IC_{50} * (mg)	Per cent Inhibition Incubation Period		
		1 min	2 min	4 min
Collagen	0.250	51.2 ± 2.1^{a}	79.2 ± 2.1^{c}	85.5 ± 2.4^{e}
ADP	0.350	49.6 ± 1.9^{a}	64.5 ± 1.4^{b}	76.7 ± 2.7^{d}

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

ANTIBACTERIAL ACTIVITY

Spondiol exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria. It was more effective against wide spectrum of bacteria viz. *M. luteus, S. aureus, B. subtilis, S. typhi, S. dysenteriae, S. fecalis,* and *K. pneumonia*. The most striking increase in activity was observed against *M. luteus, S. aureus, B. subtilis* and *S. typhi* with MIC of 50, 60, 70 and 80 ppm respectively. It also inhibited the growth of five Gram-negative bacteria viz. *S. typhi*, *K. pneumoniae* and *S. dysenteriae* **[Table 2.11].**

However, these were not inhibited by the methanol extract. High antibacterial activity of spondiol for wide range of bacteria may be attributed to its structural components. It possesses two-hydroxyl and two-methyl along with an aromatic ring.

Table 2.11: Antibacterial activity of Spondiol.

Bacteria	Spondiol MIC [ppm] *
B. Subtilus	70
M. luteus	50
S. faecalis	110
S. aureus	60
S. typhi	80
K. pneumoniae	140
S. dysenteriae	100

*Each value is a mean of three different observations.

Spondiol by virtue of possessing an aromatic ring, 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 spondiol compared to its source extract. The bioactivity of spondiol may be similar to several other compounds like capsaicin, caffeic acid, carvacrol, eugenol and menthol [Cichewicz *et al.* 1996; Ali-shtayeh *et al.* 1997; Cowan, 1999].

CONCLUSION

Two multiple bioactive compounds were successfully isolated and characterized from *Spondias mangifera*, viz. 'Glycospondin' from ethyl acetate extract and 'Spondiol' from methanol extract. These compounds were found to be reported first time from the *Spondias mangifera* fruit. They are not reported previously from any other sources. 'Glycospondin' exhibited high platelet-aggregation inhibitory activity while 'Spondiol' showed high antioxidant activity. However, both the bioactive compounds exhibited multiple bioactivities. 'Spondiol' was used as a biomarker for the determination of maturity of *Spondias mangifera* during fruit growth and development. The details are furnished in the next chapter.

Chapter 3

Bioactive and Biochemical Changes During Growth & Development of Spondias mangifera Fruits

All great truths begin as blasphemies

– George Bernard Shaw

INTRODUCTION

Consumption of fruit at its peak accumulation of nutritional and bioactive compounds is governed by vast set of traits, which, in turn a function of fruit species. Thus, identifying the peak accumulation of nutritional and nutraceutical compounds, which associates with fruit growth and development is of vital importance. In post harvest physiology "mature" is defined as "that stage at which a commodity has reached a sufficient stage of fruit growth and development. Biochemical and bioactive changes during this period is critical and dictate harvesting and post harvest quality ultimately, the minimum acceptable characteristic quality to the consumer and/or food industry. Maturity stage differs with individual preferences: appearance and few visual defects are the criteria for growers and shippers, receivers. While, distributors and traders concerned about firmness and shelf life (Parker et al. 1990). Quality of the fruit is perceived by consumers in terms of fruit appearance, firmness, flavor, nutritional and nutraceutical value. Hence, correct maturity stage is essential for harvesting for maximum benefit in terms of catering every individual's need. The problem is multifold, because *Spondias mangifera* cv. Hal fruits tend to harden with the maturity. Consequently, the hog plum fruits have to be harvested after the attainment of maximum size but, prior to the hardening of the endocarp. Such index does not exist for Indian hog plum.

Coordinated biochemical alterations during fruit growth and development of *Spondias mangifera* cv. Hal determine the quality of fruits in terms of maturity, peak accumulation of bioactive compounds. Extensive review of literature showed lack of any maturity index for harvest. This is critical in horticulture to know, when to harvest the fruits. In the present study, successful identification of two bioactive compounds in *Spondias mangifera* cv. Hal gave an impetus to test teleological role of these bioactive compounds to define the maturity of fruits for harvest with preferred nutritional or nutraceutical or pharmaceutical quality. Participation of bioactive compounds in an array of 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 interactions between intrinsic plant factors and external storage factors [Herms and Mattson, 1992; Beckman, 2000; Booij-James *et al.,* 2000]. Lack of such studies in *Spondias mangifera* cv. Hal is apparent despite its pharmaceutical importance and exotic taste.

In the present study, among the isolated compounds, Spondiol exhibited the highest antioxidant, platelet-aggregation inhibitory activity and antimicrobial activity. Since, it is highly stable and easy to estimate, accumulation pattern of Spondiol and other changes in soluble and storage components as a function of physiological maturity in *Spondias mangifera* cv. Hal fruits were studied. A time course study of these changes from the time of fruit set to harvest, which ranged from 1 to 13 week at a time interval of 2 week were carried out. The details were presented in this chapter.

MATERIALS AND METHODS

Sample collection

The fruits of Indian hog plum (*Spondias mangifera* Willd.) were procured from the local orchard of Mysore, Karnataka, India. The first sampling time (one week after fruit set) was conducted after fruit formation. Subsequently, the samples were collected at 1, 3, 5, 7, 9, 11 and 13 weeks after fruit set. Each sample was prepared from fruits obtained from *Spondias mangifera* trees that were harvested randomly from five different trees. All the biochemical analysis and other experiments were carried out in triplicates.

Physical changes

Physical measurement

Three replicates of 10 fruits for each stage of maturity were individually analyzed for physical characteristics. The length and diameter of the fruits were measured with a vernier caliper. The length was measured at the polar axis of fruit, i.e., between the apex and the stem. The maximum width of the fruit, measured in the direction perpendicular to the polar axis, defines the diameter of the fruit.

Firmness measurement

Firmness was evaluated according to the method described by Soliva-Fortuny et al. (2002), with some modification. Force of penetration was measured by using Lloyd texture instrument. Analysis was used to measure the force required for a 5 mm diameter probe to penetrate the *Spondias mangifera* fruits to a depth of 4-5mm at a rate of 50 mm/min using 100 Kg load cell. Samples were placed so that the rod penetrated their geometric centers.

Colour measurement

Skin color and flesh color were determined with a colorimeter (Minolta Spectrophotometer CM-3500d, Osaka, Japan). Color measurements were recorded using the CIE L*a*b* color space. Color values for each fruit were computed as means of 2 measurements taken from opposite sides at the equatorial region of the fruit (Abbott, 1999).

Physiological changes

Respiration

Weighed fruits of each stage were enclosed in 1300 ml hermetic containers for one hour and one ml of the atmosphere of the container was withdrawn and injected into GC model HP 6890 series having poropak Q column with TCD detector using nitrogen as the carrier gas at a flow rate of 30 ml/min. The percentage of CO_2 was calculated by simultaneous running of the standard CO_2 gas. The CO_2 evolution was calculated in mg/ Kg-h by using the formula:

$$CO_2 = \frac{\text{Density of } CO_2 \text{ X } CO_2 \text{ released (\%) X Container Volume X 60}}{\text{Weight of the Sample (Kg) X Enclosure time (min) X 100}}$$

Biochemical analysis

Sample preparation

About 500 g of *Spondias mangifera* fruits were sliced, 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, total soluble solids (TSS), sugar content, protein content, and phenolic content.

Chlorophyll determination

Chlorophyll content was determined by using a UV-VIS spectrophotometer. Sample preparation and spectrophotometric measurements were conducted as described in AOAC method (1990). Chlorophyll content was calculated as follows:

Total chlorophyll $=7.12A_{660.0} + 16.8A_{642.5}$,

Chlorophyll a $= 9.93A_{660.0} + 0.777A_{642.5}$,

Chlorophyll b $= 17.6A_{660.0} + 2.81A_{642.5}$.

Carotenoids determination

Total carotenoid content was determined by spectrophotometric method described by Ranganna (2001). In brief, about 10 g of *Spondias mangifera* fruit pulp was blended with 100 ml cold acetone in a pestle and mortar and filtered over cotton pad. Extraction was repeated until residue was colorless. Acetone extract was placed in the separating

funnel and agitated with 25 ml petroleum ether and 5 ml water. The mixture was left to stand for 30 min. The yellow colored petroleum ether extract was collected and filtered over anhydrous sodium sulphate on a Whatman filter paper No. 1. The extract was made up to 25 ml and the color intensity of carotenoid extract was measured at 450 nm in a UV-Visible spectrophotometer (UV-160A, Shimadzu Co. Japan). The total carotenoid content was calculated on the basis of the calibration curve of β-carotene and expressed as β-carotene equivalents mg/ 100 gm *Spondias mangifera*.

Ascorbic acid, total sugar reducing sugar and total protein content

Ascorbic acid was determined according to the AOAC method (1990). Total Sugar estimations was carried out by the method described by Dubois (1956) and reducing sugar by Miller (1959). 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 pH meter calibrated with standard buffer at pH 4. Titrable acidity was determined by AOAC (1990) method. The total soluble solids (TSS) were determined by an RX-5000 digital refractometer (ATAGO, Japan) calibrated with distilled water. *Spondias mangifera* juice was passed through a filter paper (Whatman No.1) using vacuum before analysis.

Determination of phenolics

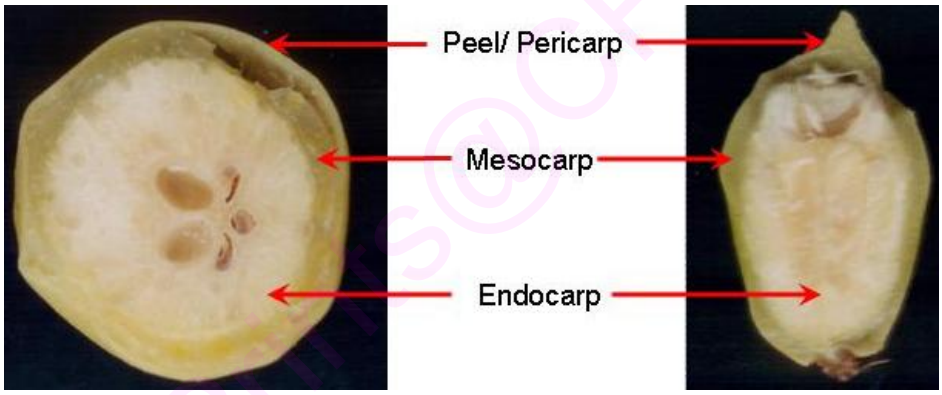
The total phenolic content in *Spondias mangifera* fruit was determined with the modified method of Taga et al. (1984). In brief, 100 μL of sample was mixed with 2 mL of 2% aqueous sodium carbonate solution. After 3 min, 100 μL of 50% Folin-Ciocalteau phenol reagent was added to the mixture. After 30 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.

Statistical analysis

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

Fig. 3.1: Growth and development of *Spondias mangifera* fruit.

Fig. 3.2: Transverse and vertical sections of *Spondias mangifera* fruit.



Depending upon number of weeks from the fruit initiation, three distinct stages were categorized including the following stages for the convenience of explanation of the results carried out:

Table 3.1: Maturity indices of *Spondias mangifera*

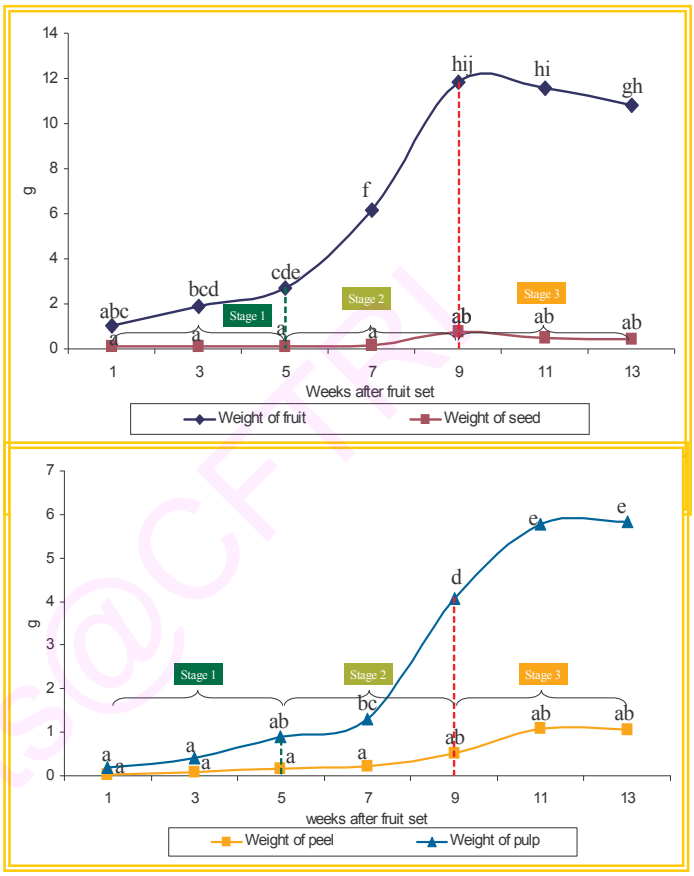
Stage	Weeks after fruit set
Stage 1	1-5
Stage 2	7-9
Stage 3	11-13

PHYSICAL

Length, Diameter, weight, texture, colour

Spondias mangifera fruits showed a simple sigmoid curve with three distinct stages, when all the contingent of fruit growth was plotted against time from fruit set. There is a moderate increase in weight up to 5 weeks after fruit set, followed by two fold increase in weight for every fortnight from fifth to ninth week of development of fruit and finally a static or slight increase in growth for a period of two weeks from 9th week, which coincides with hardening of endocarp. This pattern of growth has been similar to the simple sigmoid growth curve reported in almond, apple and mango (Murkhejee and Dutta, 1967; Rodriguez et al., 1971; Yusof and Suhaila, 1987; Jagtiani et al., 1988). However, this in contrast with development of fleshy drupe fruits of temperate zone like apricot, cherries and plums (Kennard, 1955). The longer growth period of fruits during initial stage of 5 weeks period is probably a response to cooler temperatures. Fruit mass changes showed a trend similar to that observed for diameter **[Fig. 3.3]**. Sigmoid growth curve was exhibited by all the contingecies of other fruit growth parameters such as length, diameter, fresh weight, of exocarp, mesocarp, endocarp and seeds were plotted against time. The ratio between the seed and the fruit increase with increase in time from fruit set. Increase in weight of the fruit from 0.85 to 5.28 g with advance of time was observed till 9th week of fruit set. The exponential increase in weight of the fruit till 9th week was observed.

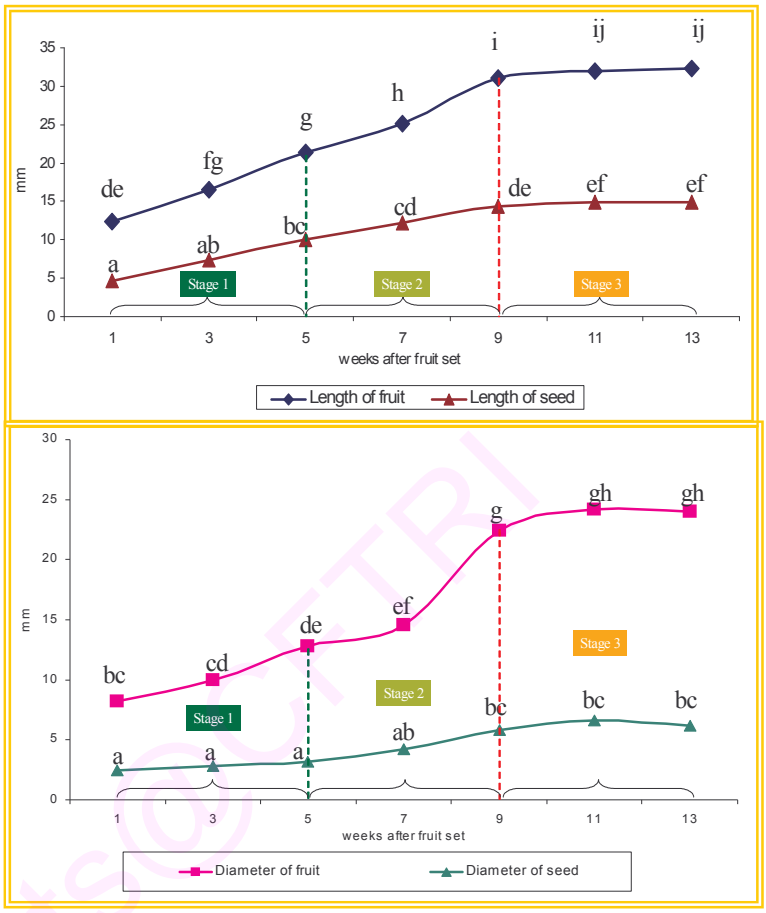
Fig. 3.3: Changes in weight of *Spondias mangifera* during fruit development.



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

Some of structural features of nectarines and peaches resemble to those of *Spondias mangifera* fruit. These are the some of the striking similarities have been observed in *Spondias mangifera* fruit: The fruit is a drupe developed from superior ovary and has no floral residue around the peduncle (Brady, 1993). These fruits have a thin outer skin (epicarp/ exocarp), soft flesh of varying thickness under the skin (mesocarp) and an endocarp that is highly lignified (referred to as the stone or pit) that develops from the inner ovary wall. The skin is composed of cuticle, epidermis and some hypodermal cell layers. Peaches have trichomes or hair ("fuzz") whilst, nectarines and *Spondias mangifera*s have smooth surfaces with no hair (Kader and Mitchell, 1989).

Fig. 3.4: Changes in diameter of *Spondias mangifera* during fruit development.



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

The growth patterns of *Spondias mangifera* fruit unlike other fruits appear to be form of a simple, rather than double sigmoid curve. Coloring and softening of the flesh is from seed outwards; at this stage, the latter has become surrounded by a cartilaginous and finally, strong endocarp. These readily observable changes have been used as a means of assessing the optimal picking date for immediate consumption or for storage. Indian hog plum normally reach maturity in 2 to 3 months from flowering or 9 to 11 week from fruit set. They are harvested at a mature green stage and used as vegetable when unripe and as fruit

The distribution of the various weight components of the fruit is presented in **Table 3.2.** Taking into consideration of all the maturities, it was observed that, on an average, the seed, skin and pulp constituted 4.56 %, 43.00 %, 52.40 % of the weight of the whole fruit respectively. The flesh was found to be white in colour and the skin was green in colour at all the maturity stages. But, the skin turned into yellow colour. When the fruit is still in its immature green, sour and with its seed is not hardened. The total weight of the fruit increased from stage 1 to stage 7.

Table 3.2 Weight of various components of *Spondias mangifera* fruit

	No. of weeks after fruit set						
	Stage 1			Stage 2		Stage 3	
Parameter (mm)	**1**	**3**	**5**	**7**	**9**	**11**	**13**
Weight of fruit	0.85	1.05	2.12	2.56	5.28	11.46	12.68
Weight of peel	0.15	0.16	0.20	0.22	0.36	0.42	0.53
Weight of mesocarp	0.25	0.36	0.56	0.68	0.90	3.96	4.77
Weight of endocarp	0.36	0.44	1.26	1.52	3.84	6.86	7.12
Weight of seed	0.09	0.094	0.096	0.14	0.18	0.22	0.26
Ratio of fruit to Seed weight	9.44	11.17	22.08	18.29	29.33	52.09	48.77
Ratio of pulp to Peel weight	1.67	2.25	2.80	3.09	2.50	9.43	9.00

The mature fruits are ovoid or ellipsoidal in shape, measuring 2.5-3.5 cm in length. The fruits are penta-carpellary with single seed in each carpel **[Table 3.3, Fig. 3.1]**. The endocarp is edible when it is soft. With advance of maturity the endocarp toughened with longitudinal interwoven fibers thus become inedible. The results indicate that hardening of endocarp begins after 9 weeks of fruit set**]**. Thus it reduces 80% of the fruit unavailable for consumption. It is interesting to note that hardening of endocarp associated with various physiological and biochemical changes in the fruit. Hardening of endocarp is the cause or consequence of these changes remains to be solved.

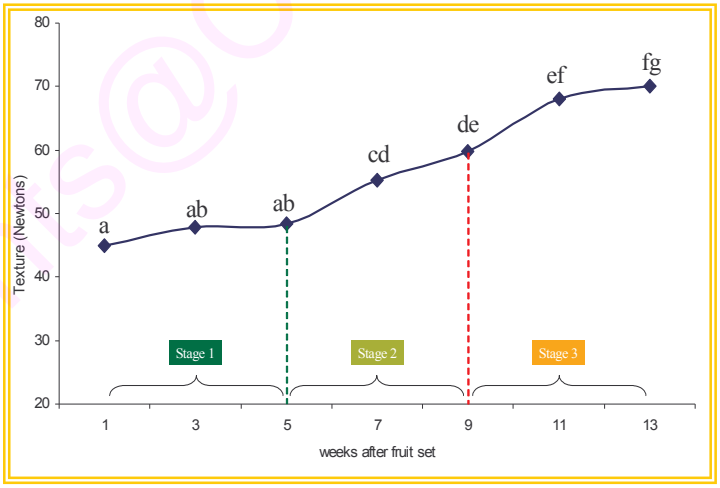
Table 3.3 Physical parameters of the *Spondias mangifera* fruit and its components

	No. of weeks after fruit set						
	Stage 1			Stage 2		Stage 3	
Parameter (mm)	**1**	**3**	**5**	**7**	**9**	**11**	**13**
Length of fruit	12.4	16.6	21.4	25.2	31	38.6	46.6
Diameter of fruit	8.2	10	12.8	14.6	17.4	24.2	31.2
Length of seed	4.6	7.4	10	12.2	13	15.8	20
Diameter of seed	2.5	2.8	3.2	4.2	5	5	5.2
Thickness of endocarp	2.8	2.8	3	3	4	4.8	5.4
Thickness of mesocarp	0.4	0.6	0.8	1.2	1.4	1.6	1.8
Thickness of peel	0	0	0	0.2	0.4	0.6	0.8
Length/ diameter of fruit	1.5	1.7	1.7	1.7	1.8	1.6	1.5
Ratio of fruit to seed diameter	3.3	3.6	4.0	3.5	3.5	4.8	6.0
Ratio of fruit to seed length	2.7	2.2	2.1	2.1	2.4	2.4	2.3
Thickness (endocarp+mesocarp+peel)	3.2	3.4	3.8	4.4	5.8	7	8

For the first time different stages in fruit growth in *Spondias mangifera* is defined based on physical parameters. Maturity indices depend upon the nature of the fruits. The various maturity indices that are followed for different stone fruits were Size and shape (Lill et al., 1989). Flesh firmness (Kader and Mitchell, 1989a; Kader and Mitchell, 1989b), Color (Rood, 1957; Romani and Jennings, 1971; Ryall and Pentzer 1982), Soluble Solids Concentration (SSC) (Mitchell et al., 1990; Mitchell et al., 1991), Titratable acidity (Reid, 1992), TSS : TA ratio Batten (1989) sugar, acid, sugar : acid ratio, Underhill and Wong (1990). However there is no report on use of endocarp development as maturity index for any stone fruits including mango. The present study, development and differentiation of endocarp in the Indian Hog plum, was found to be a function of maturity. It can be used as maturity index in India hog plum.

Texture

Fig. 3.5: Changes in texture in *Spondias mangifera* during fruits development



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

Fruit firmness usually follows a declining trend during maturation and ripening. Unlike the maturation of the fruit, firmness of the *Spondias mangifera* increases during development as the endocarp hardens. The force required for penetration into the fruit gradually increased from fruits harvested after one week to thirteen weeks. During first stage of fruit development increase in percentage of firmness was 7 %. While at the end of stage 2 and stage 3 development, force required for penetration increased to 19 % and 15 % respectively **[Fig. 3.5]**.

Significant increase in pressure during stage 2 & 3 compared to the stage 1 may be due to the hardening of the endocarp during the maturation of the fruit. During Stage 2 fruit developed fully preparing for the hardening of the endocarp during stage 3 of the development of the fruit, wherein, recorded highest firmness of 70N. The rise may also be

attributed to the lignification of secondary cell wall with the advancement of the fruit maturity.

Fruit firmness is one of the practical and an excellent indicator for judging maturity. Though this is a destructive method, development of advanced techniques such as near infrared (NIR) spectroscopy has been used to evaluate internal quality of fresh fruits such as peach (Kawano et al., 1992, 1995), satsuma mandarin (Kawano et al., 1993) and mango (Guthrie and Walsh, 1997; Peiris et al., 1999; Schmilovitch et al., 2000). This can be quantified by hand or mechanical devices (Thompson, 1996). Similar trend in firmness at commercial maturity of early, mid and late season nectarines increased gradually unlike other fruits, where there was decline due to ripening and other associated changes (Kader and Mitchell, 1989a).

ENDOCARP

Endocarp formation and hardening

The fruit of the *Spondias mangifera* attains maturity on 9th week from fruit set. With no or little change in weight of the fruit accompanied with hardening of endocarp was observed till 11th week after fruit set. These visible observable changes can be considered to determine the harvest maturity in Indian hog plum fruits **[Fig. 3.1]**. There is an increase in thickness of the fruit with increase in duration after fruit set. Endocarp contributes more to the fruit thickness than the mesocarp. Thin, papery pericarp contributes to the least either to the thickness or to the weight of the fruit. Dimensions of the *Spondias mangifera* fruit and its components are shown in Table 3.1. The fruit is ovoid to ellipsoidal in shape, being the ratio of length to the diameter of the fruit is 1.7 for all maturities. The thickness of the pulp increased from 0.4 cm in stage 1 to 1.8 cm in stage 7 fruits.

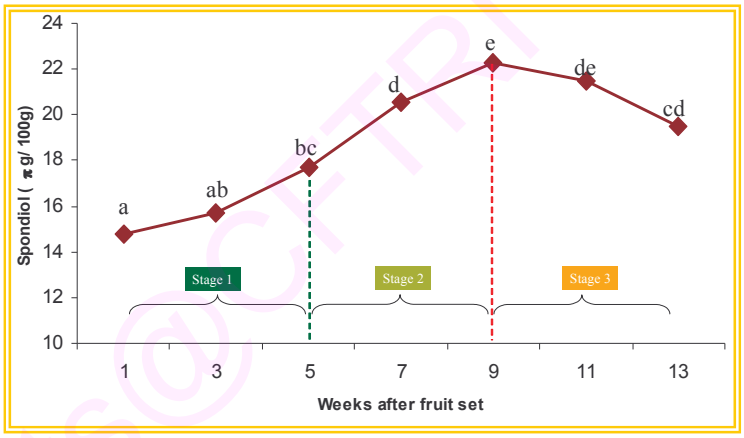
The endocarp is white and cartilaginous and restricted itself to the respective carpels. With advance of development and maturity of the fruits they finally fuse together. This process of carpellary fusion and demarcation of endocarp from surrounding mesocarp completes on 9th week after fruit set **[Fig. 3.1]**. Later the physiological activities may concentrate more on hardening of endocarp, which was achieved by longitudinal

interwoven of fibers for a period of two to three weeks as in mango and drupes such as nectarines and peaches (Kader and Mitchell, 1989b).Thus extending these readily observable changes have been used as a means of assessing the optimal picking date for immediate consumption or for storage.

BIOACTIVE

Spondiol

Fig. 3.6: Changes in Spondiol during fruit development of *Spondias mangifera* fruit.



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

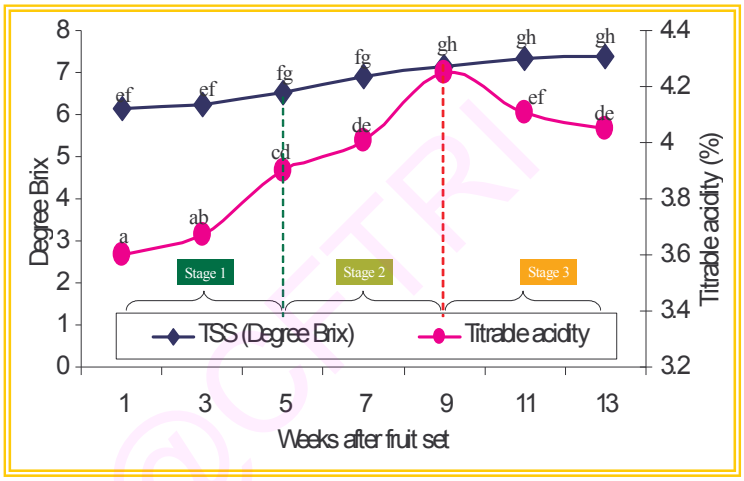
Concentration of the bioactive compound 'Spondiol' increased with the development of the *Spondias mangifera* fruit. There was significant increase in the accumulation of Spondiol after five weeks after fruit set, which reached highest (22.25 mg/ 100g) after nine weeks **[Fig. 3.6]**. This followed by a decrease during third stage of development.

BIOCHEMICAL

pH, titrable acidity and total soluble solids

pH, acidity and ascorbic acid content were highest in the early maturity stages compared to the late maturity stages. Similarly in mango (Rao et al.,1998). *Spondias mangifera* fruit, like stone fruits, lose acidity during maturation and ripening. This maturity feature is also affected by cultivar and seasonal variability (Boggess et al., 1974; Rood, 1957; Salunkhe et al., 1968).

Fig. 3.7: Changes in total soluble solids and titrable acidity in *Spondias mangifera* during fruit development



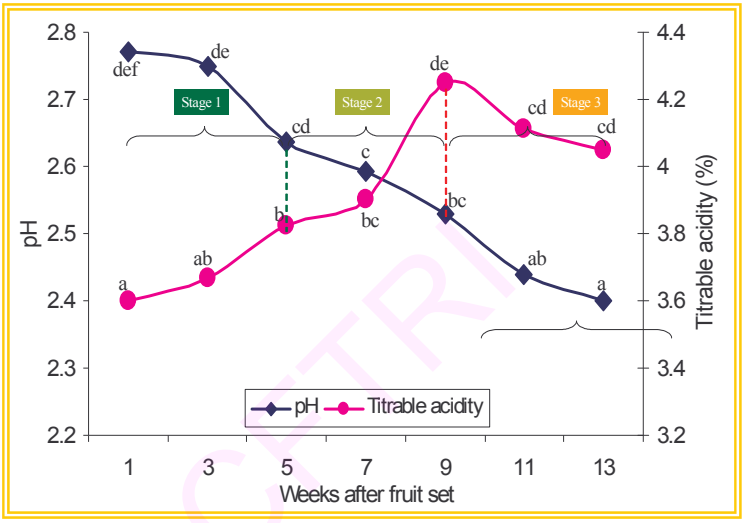
Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Maximum amount of brix (7.35), total sugars (0.88 mg/100g), reducing sugars (0.07 mg/ 100g) and non-reducing sugars (0.82 mg/ 100g) were observed in stage 7 **[Fig. 3.7]**. Similar trend in increase of total soluble solids observed in stone fruit, peach (Kakiuchi et al., 1981). Generally, sugars constitute major quantity in total soluble solids of the fruit juice. Measuring soluble solids in fruit juice can give a reliable measurement of sugar content and serve as a guide to maturation making it one of the indicators of eating quality. But, this may vary widely with varieties, production area and season (Dann and Jerie, 1988; Kader and Mitchell, 1989a) even fruit the orientation in the canopy (Mitchell et al., 1990; Mitchell et al., 1991) rendering its limitations as maturity index.

Conversion of polysaccharides into sugars may be the reason for the increase in sugar content. Gradual decrease in pH, acidity and ascorbic acid may be correlated to the

ripening changes associated with the maturity of the fruit. As the fruit developing from early maturity stage to the fully matured state, there are number of ripening changes associated, involving conversion of polysaccharides into reducing and non-reducing sugars, which may be the reason for the increase in the amount of total sugar content.

Fig. 3.8: Changes in pH and titrable acidity in *Spondias mangifera* during fruit development

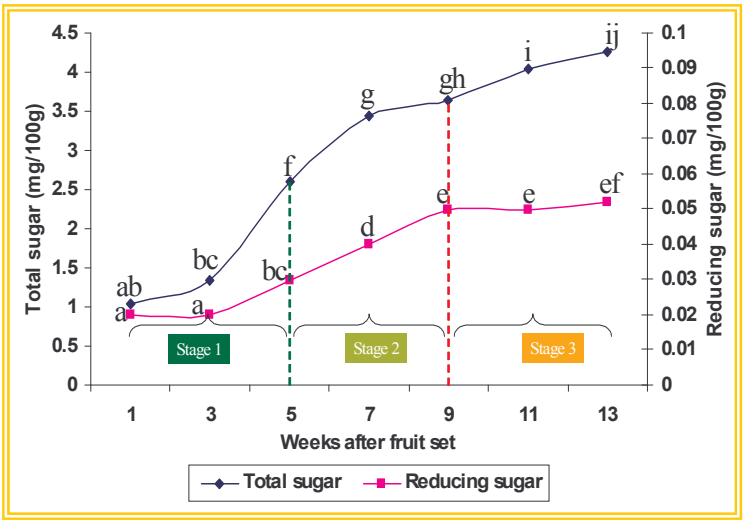


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

The fruit being highly acidic in nature, it tends to show the gradual rise in the acidity and concomitant decline in pH indicating the nature of the fruit. In the developing Indian hog plum fruits, acidity increased at the early growth phase, reached a peak on the 9th week after fruit set and then declined gradually until harvest **[Fig. 3.8]**. In fruit the acidity reached maximum in about 9 weeks and declined slowly at the time of harvest.

Total sugars and reducing sugars

Fig. 3.9: Changes in total sugar and reducing sugar content in *Spondias mangifera* during fruit development



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

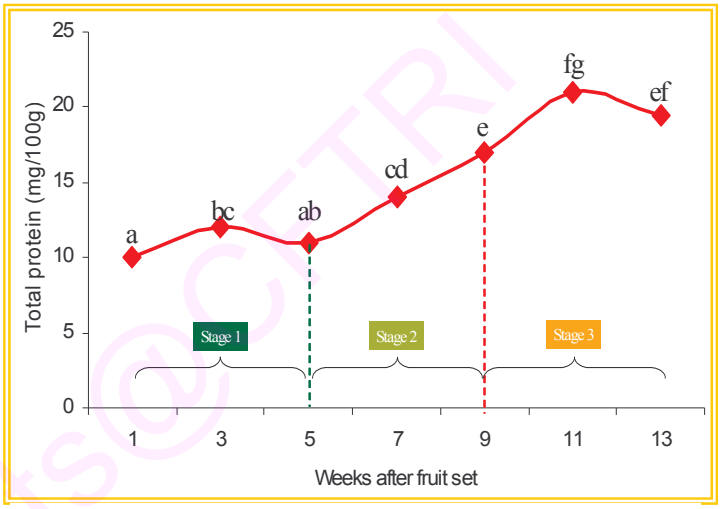
At initial stages of fruit development, no systematic trend was observed in the sugar content, but after 3rd week both reducing and non-reducing sugars were found to be increasing. The total and reducing sugar content in fresh fruit varied between

1.0 and 4.5 mg/100g and 0.02 to 0.05 mg/100g respectively. There was a steep increase in the concentration of total sugars till 7th week, but slow down towards the end of maturity **[Fig. 3.9]**. While reducing sugar after 9th week, showed no change or slight decrease towards the end of maturity. Interestingly accumulation pattern of reducing sugar showed similarity with sigmoid growth curve. The soluble sugars of the fruit pulp consisted mainly of glucose, fructose, and sucrose.

Total protein

Protein content of the fruit was increasing as the fruit was developing from immature juvenile phase to matured fruit. Maximum protein content of 21mg/ 100g was observed in the fruits harvested after eleven weeks **[Fig. 3.10]**. Chaturvedi (1974) reported that alcohol-insoluble solids (AIS) and protein contents of guava fruit, at different stages of maturity, decreased with fruit development.

Fig. 3.10: Changes in total protein content in *Spondias mangifera* during fruit development

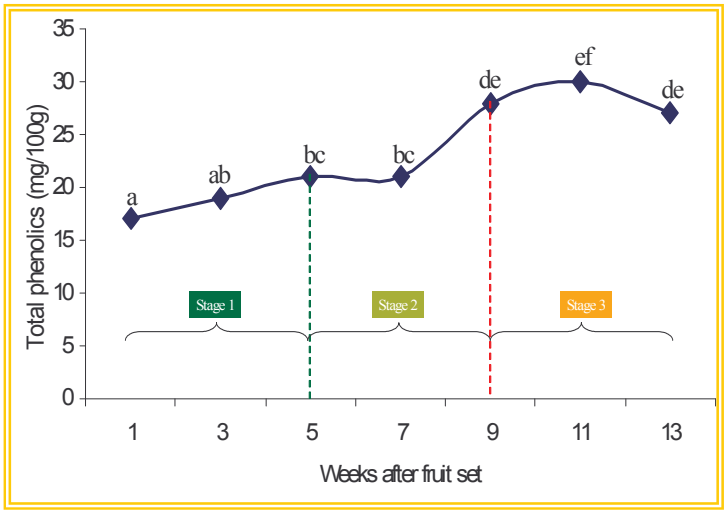


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Total phenolics

Total phenolic content was observed to be an index of fruit development and maturation. The rate of phenols accumulation is slow at the beginning of fruit growth but during the exponential growth phase

Fig. 3.11: Changes in total phenolic content in *Spondias mangifera* during fruit development

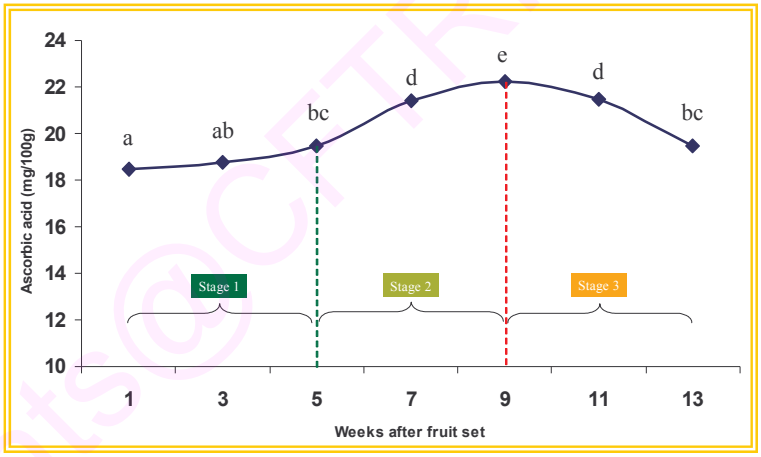


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

of the fruit, total phenolic content increased significantly (28 mg/ 100g) till 9th week after fruit set **[Fig. 3.11]**. During development accumulation of total phenols appear to follow the similar pattern of simple single sigmoid growth the fruit. Decline in total phenolics after eleven weeks after fruit set may be due to the oxidation of phenolic content by polyphenol oxidase (Amiot et al., 1995). Phenolic content is influenced by fruit maturity and similar trends were observed in grapes and pears (Fernandez de Simon et al., 1993; Amiot et al., 1995; Mayr et al., 1995).

Ascorbic acid

Fig. 3.12: Changes in ascorbic acid content in *Spondias mangifera* during fruit development.

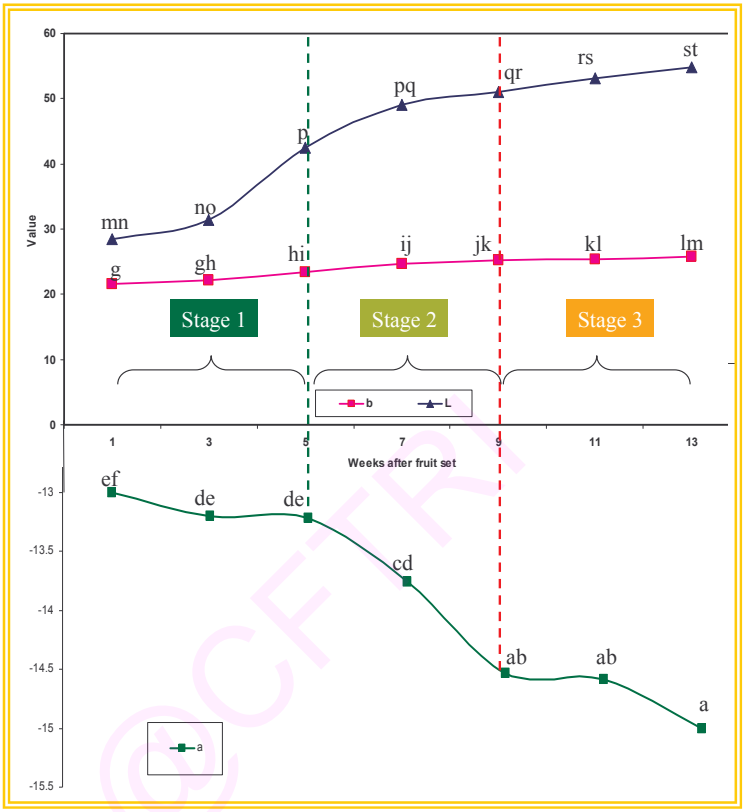


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Ascorbic acid content was observed to be one of the indexes for the optimum stage of harvest of the *Spondias mangifera* fruits. Maximum ascorbic acid content of 22.63 mg/ 100g is accumulated till the fruit reached to the full maturation phase and then it started declining as the maturation advanced **[Fig. 3.12]**. A similar trend was reported in mango and guava fruits (Bashir & Abu-Goukh, 2003). Similar studies on ascorbic acid in guava (El-Bulk et al. 1995, Rodriguez et al., 1971) showed that the ascorbic acid content for developing guava increased slowly during the initial growing period, followed by rapid increase during maturation and ripening. These cumulative changes leading to the increase in total soluble solids, sugars, carotenoids and ascorbic acid, while decrease in titrable acidity, total chlorophyll and total phenolics with fruit maturity rendering optimum eating quality attainment at the climacteric peak is in agreement with the Al-Niami et al. 1992.

Pigments and Colour

Fig. 3.13: Changes in colour readings of *Spondias mangifera* during fruit development.



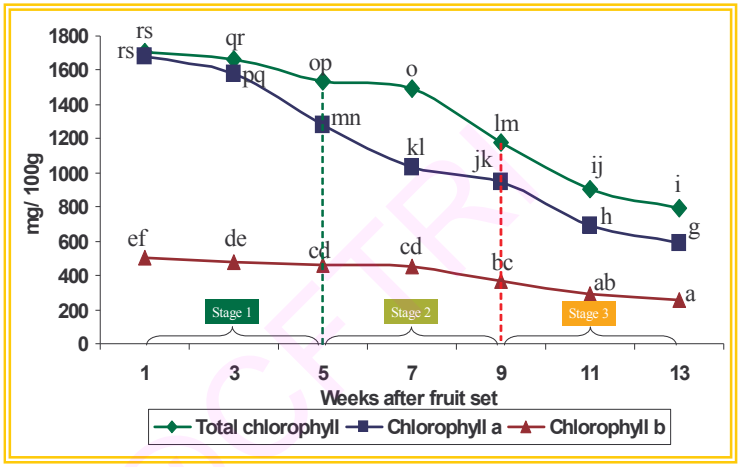
Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

The *L** and Hue values at harvest were higher with increasing maturity stage; Lightness 'L' values were associated with the accumulation of carotenoids and/or deterioration of the chlorophyll pigments. It was highest in late harvested fruits (which appeared more yellowish), consistent with the colour alteration, total chlorophyll content was highest in early harvested fruits (1704.25 µg/100 g) and decreased gradually and reached lowest chlorophyll content in fruits harvested after thirteen weeks (789.36 µg/100 g) **[Fig. 3.14]**. The dark nature of the fruit may be due to the high chlorophyll. Arias et al. (2000) and Shewfelt et al. (1988) reported the decrease of *L** with maturity reflecting the darkening of the tomatoes while in case of *Spondias mangifera*s it is the converse, showing increase of *L** may be due to the absence of dark pigmentation unlike tomatoes.

Individual colour values such as 'a', which is associated with green colour, decreased with fruit maturation. Colour differences during various maturity stages showed a decrease from –13 value to 'a' in stage 1 to –15 in stage 7 (Fig. 3.5). The *a** values were different for each stage of maturity and increased after fruit set. These values correspond to yellowish green fruit. The 'b' values also increased with the gradual change from green to yellow in the skin colour. An increase in yellow colour from 21.56 value of 'b' in stage 1 to 25.75 in stage 5, may be due to the accumulation of carotenoids. These changes are caused by the degradation of chlorophyll and the accumulation of carotenoid

pigments (Song et al., 1997). Increased lightness accompanied with accumulation of carotenoids with concomitant decrease in total chlorophyll converges on ninth week period after fruit set **[Fig. 3.13]** may indicate maturation of the fruit. This is the first report of nondestructive objective determination of external color as a reliable harvest index in Indian hog plum.

Fig. 3.14: Changes in chlorophyll pigments in *Spondias mangifera* during fruit development



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

Pigments such as chlorophyll a, chlorophyll b and total chlorophyll also followed a decreasing trend with the advancement of maturity of the fruit **[Fig. 3.14]**. Maximum and less content of chlorophyll pigments in early and late developmental stages respectively may be due to the degradation of chlorophyll as the fruit develops into fully matured fruit as in other drupaceous fruits such as mango (Va´squez-Caicedo et al., 2004) and *Spondias cytherea* Sonn (Ishak et al., 2005). Contrarily peak accumulation of carotene featured in the fully matured fruits. Total phenolics were found to be highest in case of fully matured fruits.

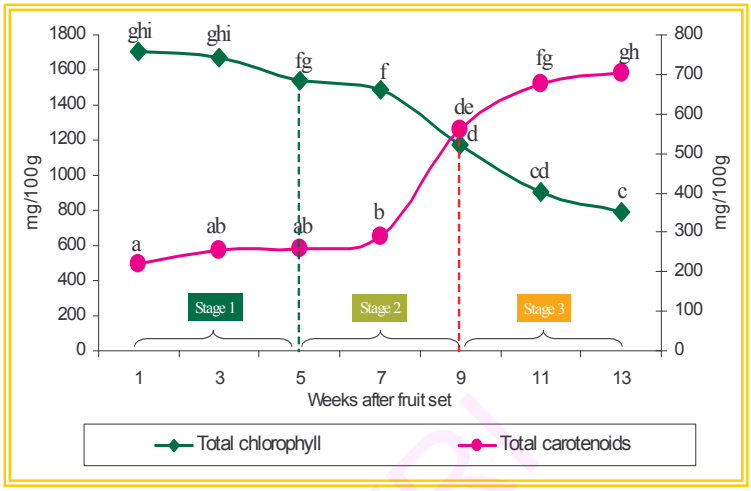
Visual observation of the skin pigmentation is the most evident change during ripening (Kader and Mitchell, 1989a; Lill et al., 1989; Robertson et al., 1990; Romani and Jennings, 1971; Ryall and Pentzer, 1982). Quick, reliable, convenient and ease of using color measurements correlated the long, tedious, and costly chemical methods of quantifying pigment, several studies have correlated the color with the pigment in different food stuffs. Apart from wide variety of leafy vegetables and other food stuffs, tomato (Arias et al. 2000), blueberries (Francis, 1985), grapes (Watada and Abbott, 1975), peaches (Morrison, 1990), are some of the food materials in which the color has been correlated with the pigment content.

Colour of the skin is quantified by determining various pigments in the skin and underlying fruit tissue. As the maturation of the fruit progresses, apparent changes are

brought about by the degradation of chlorophyll and concomitant biosynthesis of either anthocyanins or carotenoids (Tucker, 1993).

Therefore, as *Spondias mangifera* fruit mature and ripen, like nectarines and peaches, show a change in ground colour from green to white or yellow (Kader and Mitchell, 1989b) and this change is a major criterion for determining fruit maturity.

Fig. 3.15: Changes in total chlorophyll and total carotenoids in *Spondias mangifera* during fruits development



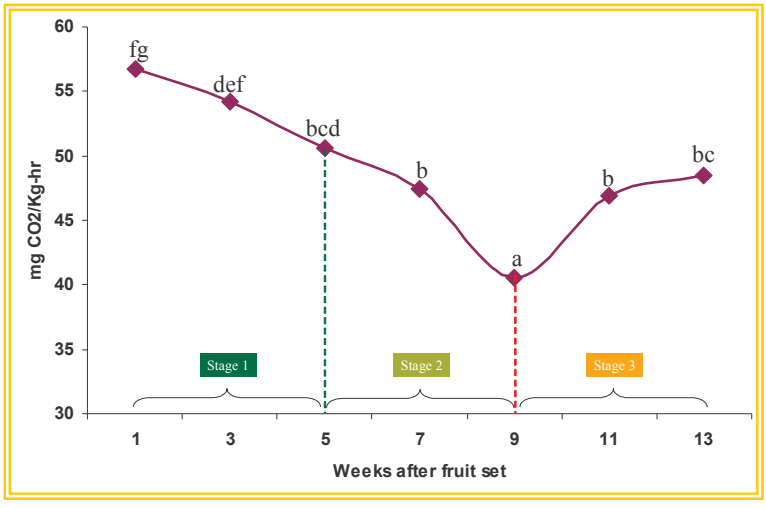
Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

PHYSIOLOGICAL

Rate of Respiration

Rate of respiration in *Spondias mangifera* fruits showed a decreasing trend as a function of development till nine weeks during development leading to a pre-climacteric minimum of 40.57 mg CO_2/ kg-hr during 9 weeks after fruit set at the end of stage 2 followed by a rise in the rate of respiration **[Fig. 3.16]**. The high respiration rate (56.75 mg CO_2/ kg-hr) during initial harvest stage may be matched to the

Fig. 3.16: Changes in rate of respiration in *Spondias mangifera* during fruit development



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

higher surface area and the phase of maximum cell division, rapid growth phase marked by cell expansion phase, diminished surface area may result in decrease in rate of respiration, a phenemonenon commonly recorded in climacteric fruits (Biale and Young, 1981, Kader, 2000). Wills et al. (1989) also reported that respiration rate per unit weight was observed to be highest for immature tissues and then steadily decreased with age. The upsurge of respiration prior to harvest may indicate the completion of maturity or herald the onset of ripening of fruit.

In the present study, the following multiple maturity indices has been evolved in *Spondias mangifera*, depending upon changes in various physical, bioactive and biochemical attributes during fruit growth and development.

Maturity Indices

At present, best time for harvest of fruits has been based on experience. This may vary with the individual's preferences and tastes. For the first time a set of development related physical, physiological and biochemical quality attributes have been identified for determining harvest maturity in Indian hog plum fruit. All the contingencies of physical growth like length, diameter, fresh weight of the fruit, when plotted over the development period of fruit showed simple sigmoid growth curve. Accordingly the bioactive and biochemical changes associated with fruit growth and maturation are discussed. Notable, chemical indices evolved during the present study being; the equilibrium concentration of chlorophyll and carotenoid on the 9^{th} week may mark the attainment of optimum maturity and onset of ripening. The pre-climacteric dip followed by an upsurge in the rate of respiration heralds the optimum maturity and/or onset of ripening.

Field Chart

Maturity indices of *Spondias mangifera*

MATURITY ATTRIBUTES	Stage 1	Stage 2	Stage 3
Weeks after fruit set	1-5 *Immature*	7-9 *Optimally matured*	11-13 *Over matured*
Endocarp differentiation	*Indistinct endocarp*	*Distinct demarcation of endocarp*	*Dominant endocarp*
Physical and chemical attributes (texture, carotene, TSS, sugars)	Increasing/ accumulative	Peak increase and Stabilization	Declining

Thus, in the present study, multiple maturity indices have been evolved and presented in tabular form, which can be used as a field chart along with colour illustrations presented in this chapter. Use of endocarp differentiation as a maturity index was carried out for the first time. Based on this facet, the fruit development was classified in to three phase viz. Indistinct, Distinct and dominant phases. This can be adopted as one of the method for determining the maturity index in other stone fruits. Popularization of nutraceutical food supplements posed new challenges and provided new dimension in identifying the maturity indices in fruits and vegetables. In the present study, use of bioactive molecule isolated from *Spondias mangifera* fruit has been effectively used as a biomarker for maturity. This may facilitate harvesting of fruits with high nutraceutical compounds, which is preferred for pharmaceutical industry. Thus the present study enabled to evolve multiple indexes to cater the needs of producers, distribution market vendors, consumers and also to pharmaceutical and/or nutraceutical industry.

CONCLUSION

For the first time a set of development related physical, physiological and biochemical quality attributes have been identified for determining harvest maturity in Indian hog plum fruit. Endocarp hardening was contingent and function of time during development and post maturity process in Indian hog plum fruit. The inevitable process of endocarp hardening turned out as a hallmark of maturity indices in *Spondias mangifera* fruit. Interestingly, contingents of fruit growth, pre-climacteric plunge in rate of respiration, coordinated changes in pigments with peak accumulation pattern of ascorbic acid, carotene and phenols were defined and featured as maturity indices for *Spondias mangifera* fruit. Based on bioactive and biochemical changes, the physiological/horticultural harvest maturity in *Spondias mangifera* plum fruits was fixed as 9^{th} week after fruit set. Since hardening of endocarp is a post maturity process, commercial harvest time of fruit can be stretched till 11^{th} week or little later.

Table 3.4. Maturity Standards for Harvest of *Spondias mangifera* fruit maturity markers index

S. No.	Maturity Standard	Index
	Physiological	
1	Pre-climacteric dip in respiration	7^{th} - 9^{th} week
	Biochemical	
2	Ascorbic acid (mg/100 g)	20-23
3	Titrable acidity (%)	4-4.5
4	Total soluble solids (%)	6-7
5	Phenolic content(mg/ 100 g)	21-28
6	Total chlorophyll (µg/ 100 g)	1400-1200
7	Carotenoid (µg/ 100 g)	300-600
	Morphological	
8	Fusion of the endocarp (weeks)	7-9
	Fruit *Characteristics*	
9	size of fruit : (a) length (mm)	25–35
10	: (b) diameter (mm)	15–20
11	weight of fruit (g)	3–6

Thus, measuring optimum stage of maturity is of paramount importance in dictating the terms of nutraceutical value, market value as well as consumer acceptance. Immature fruits characterized by low quality parameters viz., shriveling, internal breakdown, mechanical damage and inferior quality, when ripe, including low accumulation of bioactive compounds. While, the over-mature ones either may become soft and mealy and attain insipid flavour, may become hard stony and significant decrease in edible portion or total loss of palatability. Optimum maturation is the end point of final fruit growth with preferred texture, taste, characteristic aroma, nutritional and nutraceutical components. It also heralds the beginning of ripening subsequently senescence in *Spondias mangifera* fruits. Thus the present study enabled to evolve multiple indexes to cater the needs of producers, distribution market vendors, consumers and also to pharmaceutical and/or nutraceutical industry.

Chapter 4

Bioactive & Biochemical Changes in *Spondias mangifera* Fruit During Storage

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

INTRODUCTION

Fruits generally are subject to a number of physiological disorders and spoilages that limits the ability of postharvest technology to maintain freshness and quality. They are also susceptible to high water loss, a major limitation for postharvest storage [Burton, 1982, Brecht, 2003]. Postharvest losses of fruits 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 *Spondia mangifera* includes physiological loss of water, shriveling followed by spoilage. The antioxidant components of fruits are highly susceptible to postharvest temperature and duration of storage. Utility and Shelf life are governed mostly by stage of harvest and storage temperature. Fruits harvested at various stages of maturity show effect on the quality of the fruit, regulation of the shelf life and eating quality of the fruit. Though fruits harvested in early stages have longer shelf life, better tendency to bear transport stress and may ripen off the tree, but it will be of poor quality (Delwiche and Baumgardner, 1985; Culpepper et al., 1955; Deshpande and Salunkhe, 1964 Josan and Chohan, 1982; Kader and Mitchell, 1989a; Shewfelt et al., 1987; Sims and Comin, 1963). Though a mature fruit will reach good quality, when ripe liable to lose its shelf life soon, unable to withstand packing and likely to be spoiled during transport.

Temperature is the most critical factor that alleviates or aggravates the physiological and bioactivity in *Spondias mangifera* after harvest. *Spondias mangifera* fruit being climacteric in nature expected to be highly perishable in terms of rapid deterioration quality and spoilage. However, such information is lacking. Because of high medicinal properties and its importance in the food industry for its nutraceutical properties, there is a need to understand quality after harvest. Hence, storage studies of freshly harvested fruits were planned to evaluate the bioactive and biochemical quality changes as a function of storage temperature and time. This investigation also aimed to find out influence of different maturity during extended period of storage of *Spondias mangifera* fruits. The details of the work carried out presented in this chapter.

MATERIALS AND METHODS

Sample collection

The fruits of Indian hog plum (*Spondias mangifera* Willd.) were procured from the local orchard of Mysore, Karnataka, India. The first sampling time (one week after fruit set) was conducted after fruit formation. Subsequently, the samples were collected at 1, 3, 5, 7, 9, 11 and 13 weeks after fruit set. Each sample was prepared from fruits obtained from hog plum trees that were harvested randomly from five different trees. All the biochemical analysis and other experiments were carried out in triplicates.

Physiological changes

Respiration

Weighed fruits of each stage were enclosed in 1300 ml hermetic containers for one hour and one ml of the atmosphere of the container was withdrawn and injected into GC model HP 6890 series having poropak Q column with TCD detector using nitrogen as the carrier gas at a flow rate of 30 ml/min. The percentage of CO_2 was calculated by simultaneous running of the standard CO_2 gas. The CO_2 evolution was calculated in mg/Kg-h by using the formula:

$$CO_2 = \frac{\text{Density of } CO_2 \text{ X } CO_2 \text{ released (\%) X Container Volume X 60}}{\text{Weight of the Sample (Kg) X Enclosure time (min) X 100}}$$

Physical changes

Firmness measurement

Firmness was evaluated according to the method described by Soliva-Fortuny et al. (2002), with some modification. Force of penetration was measured by using Lloyd texture instrument. Analysis was used to measure the force required for a 5 mm diameter probe to penetrate the hog plum fruits to a depth of 4-5mm at a rate of 50 mm/min using 100 Kg load cell. Samples were placed so that the rod penetrated their geometric centers.

Biochemical analysis

Sample preparation

About 500 g of hog plum fruits were sliced, 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, total soluble solids (TSS), sugar content, protein content, and phenolic content.

Determination of phenolics

The total phenolic content in hog plum fruit 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.

Chlorophyll determination

Chlorophyll content was determined by using a UV-VIS spectrophotometer. Sample preparation and spectrophotometric measurements were conducted as described in AOAC method (1990). Chlorophyll content was calculated as follows:

Total chlorophyll $=7.12A_{660.0} + 16.8A_{642.5}$,

Chlorophyll a $= 9.93A_{660.0} + 0.777A_{642.5}$,

Chlorophyll b $= 17.6A_{660.0} + 2.81A_{642.5}$.

Carotenoids determination

Total carotenoid content was determined by spectrophotometric method described by Ranganna (2001). In brief, about 10 g of hog plum fruit pulp was blended with 100 ml cold acetone in a pestle and mortar and filtered over cotton pad. Extraction was repeated until residue was colorless. Acetone extract was placed in the separating funnel and agitated with 25 ml petroleum ether and 5 ml water. The mixture was left to stand for 30 min. The yellow colored petroleum ether extract was collected and filtered over anhydrous sodium sulphate on a Whatman filter paper No. 1. The extract was made up to 25 ml and the color intensity of carotenoid extract was measured at 450 nm in a UV-Visible

spectrophotometer (UV-160A, Shimadzu Co. Japan). The total carotenoid content was calculated on the basis of the calibration curve of β-carotene and expressed as β-carotene equivalents mg/ 100 gm hog plum.

Ascorbic acid, total sugar reducing sugar and total protein content

Ascorbic acid was determined according to the AOAC method (1990). Total Sugar estimations was carried out by the method described by Dubois (1956) and reducing sugar by Miller (1959). 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 pH meter calibrated with standard buffer at pH 4. Titrable acidity was determined by AOAC (1990) method. The total soluble solids (TSS) were determined by an RX-5000 digital refractometer (ATAGO, Japan) calibrated with distilled water. Hog plum juice was passed through a filter paper (Whatman No.1) using vacuum before analysis.

Extraction and quantification of glycospondin by HPLC

To study the accumulation and quantification of glycospondin, the fresh rhizomes [10 g] were homogenized with ethyl acetate till they became colorless. The extract was filtered and concentrated using a rotary evaporator and freeze dried before using the sample for HPLC analysis. Glycospondin [the isolated compound] and ethyl acetate 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 216 nm

Pathogenic isolates (*Aspergillus niger* and *Penicillium sp.*)

Aspergillus niger and *Penicillium sp***.** were isolated from the fruit of a naturally infected hog plum showing characteristic symptoms of disease. Infected fruit is split longitudinally by a sterile knife. Growing margin of the lesion or water soaked region or leading edge region is carefully cut into small pieces of 3-4 mm blocks using sterile scalpel and surface sterilized it by dipping in 70% ethanol for 1 min. The blocks were washed with sterile water and plated on PDA media. The plates were incubated at 27±1^{o}C for 4-5 days. The major fungal colony developed was re-isolated from the growing margin of the colony and pure cultured on fresh PDA plates. Pure cultures of the isolate were maintained on potato dextrose agar (PDA) at 4^{o}C. Pathogenicity of the culture was maintained by inoculation and re-isolation of the pathogen at regular intervals on the hog plum (El-Neshawy and Wilson, 1997).

Pathogenesity test of the isolates

Aspergillus niger and *Penicillium sp* isolated pure cultures were subjected to Koch's postulate studies respectively to establish host-pathogen relation in the current scenario. Spores are harvested from 7 day old pure fungal culture obtained from infected *Spondias mangifera* fruit showing characteristic symptom. Using sterile water containing 0.1% Tween -20, spore suspension of 10^5 spores/ml was prepared and spread on the freshly cut *Spondias mangifera*. Ten matured fruits are used for the Pathogenicity test (Piano *et al.,* 1997). The fruits are incubated at room temperature (27°C) for 6 days. The fruits are split open to investigate the incidence and severity of the infection. In all the cases the fruit was found to be infected and showing characteristic symptom of disease.

Identification of fungal species

Identification of fungal species was carried out based on their colony characteristics under light microscopy.

Statistical analysis

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

RESULTS AND DISCUSSION

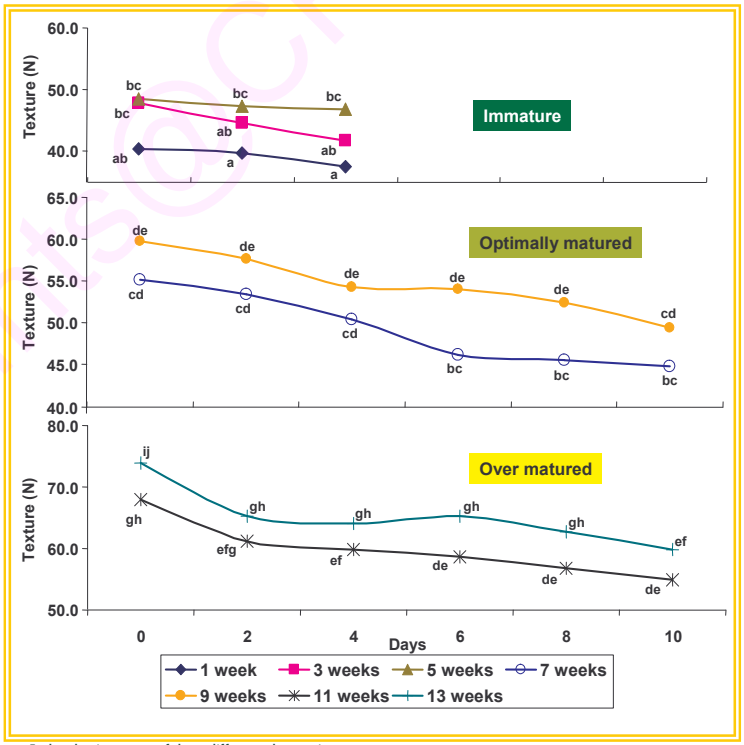
Detachment of a stored food material bearing organ such as fruit or vegetable from parent, suffers sudden and serious stoppage of water and nutrients. An independent harvested produce tries to behave as it had been attached to the plant. These behavioral aspects will determine how long the fruit will survive. The factors such as rate of respiration, water loss and various other biochemical changes override the life of fruit. Appearance of hard endocarp and deterioration of qualitative physiological, bioactive and biochemical changes are studied in fruits harvested periodically at 1, 3, 5, 7, 9, 11 and 13 weeks after fruit set. The various physical, pathological and biochemical changes were recorded till the end of storage period.

PHYSICAL

Texture

Spondias mangifera fruit showed an increase in firmness with advance of maturity. The lowest was observed in immature fruit, while the highest in over matured fruits. The change in firmness was least in immature fruits. But gradual decrease in firmness with advance of storage days was observed in all the maturity fruits **[Fig. 4.1].** Firmness of stored *Spondias mangifera* fruits decreased continuously till 10 days of storage.

Fig. 4.1: Changes in texture of *Spondias mangifera fruits* of different maturities during storage at ambient temperature.



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 change in firmness is associated with the stage of maturity. Decrease in firmness found to be function of storage time. Physiological changes alter the biochemical constituents and affect mechanical properties of the cell wall. The fully

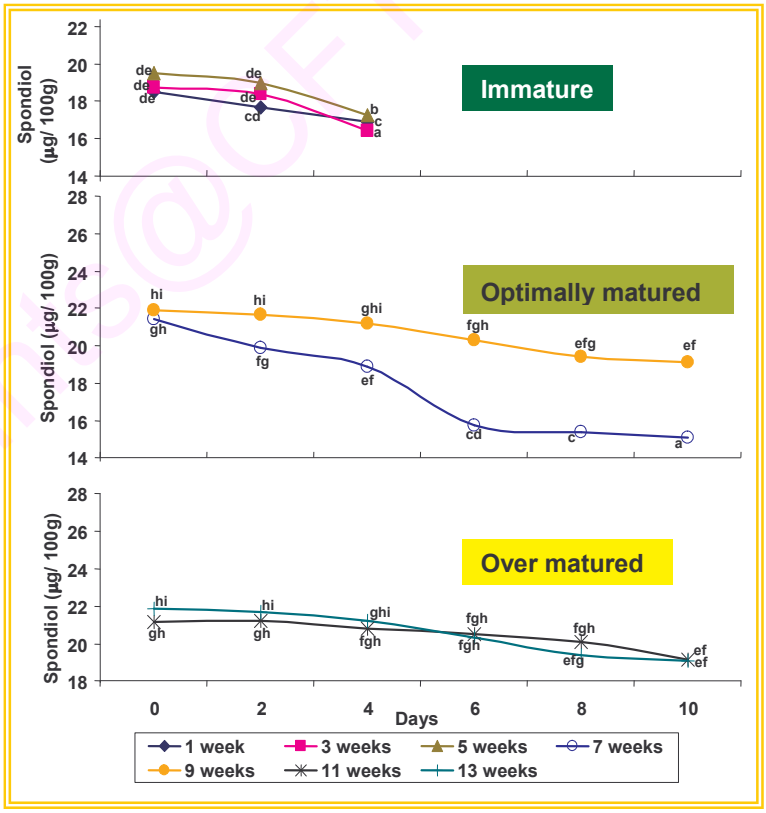
developed fruit allowed changes in structure of the pectin polymers during ripening in the cell wall (Kalra et al., 1995). Further, change in firmness was also associated with ripening and/or storage time. It appears that *Spondias mangifera* fruits had short shelf life period during storage at room temperature. Mature green fruit had the highest penetration values and the yellow ripe fruit had the lowest values at all the stages of maturity, as observed in other fruits [Mercado-Silva et al., 1998]. Rapid increase in alcohol insoluble solids may contribute to the softening of fruits, during growth and development, as indicated by a rapid decrease in shear-resistance of the fruit [El-Buluk, 1995]. Change in texture from raw, green, firm to ripe, yellow, soft fruit during storage, is mainly attributed loosening of cells, due to cellulose and pectic enzymes. Major increases in the activity exhibited by these enzymes immediately preceded the loss of firmness in the fruit (Wilson, 1980).

BIOACTIVE

Effect of storage on Spondiol

The concentration spondiol in spondias mangifera during storage up to 10 days was carried out using HPLC. Spondiol **[Fig. 4.2]** - a terpenoid bioactive compound characterized from *Spondias mangifera* fruits was used as a biomarker to evaluate the quality of *Spondias mangifera* fruits during storage. The fruits are harvested at the interval of 2 weeks, were used for various physico-chemical studies. The highest concentration of Spondiol (22.25 mg/100 g) was noticed in 9 week fruits stored for 8 days. There was loss in the concentration of Spondiol with increase in storage period. Decrease in concentration

Fig. 4.2: Changes in Spondiol of *Spondias mangifera fruits* of different maturities during storage at ambient temperature.



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

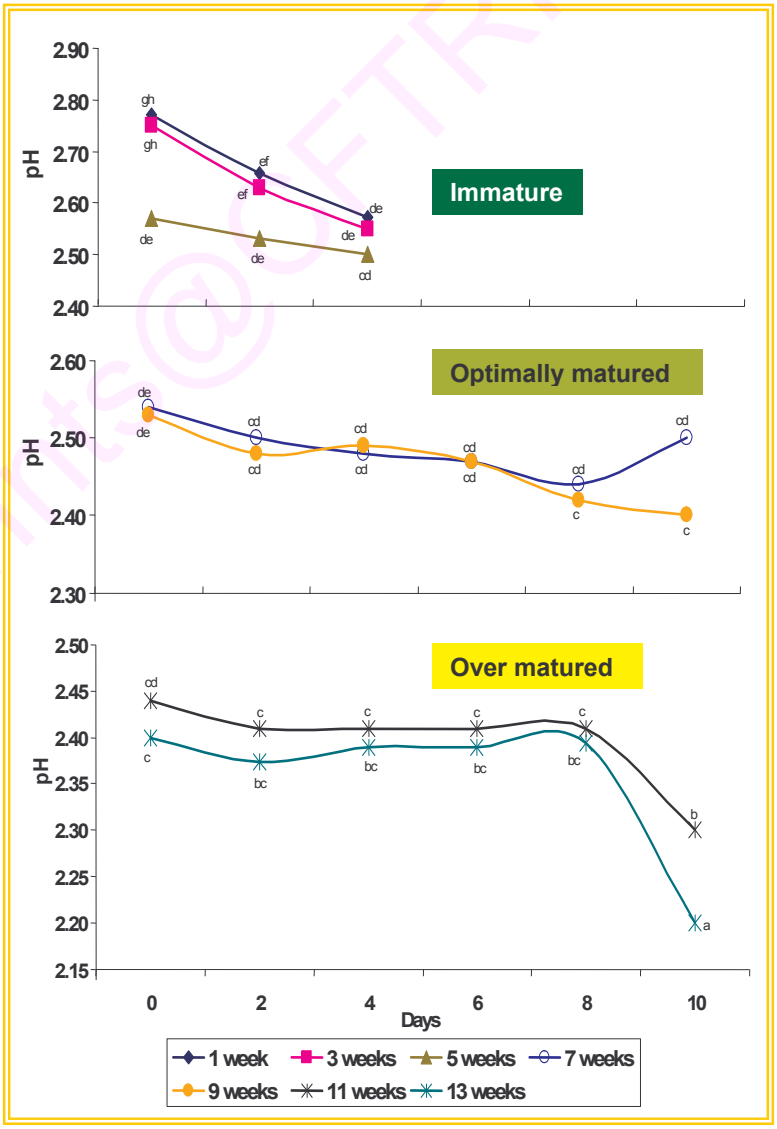
ranged between 8 – 12 % in immature and over matured fruits. While it ranged between 18 - 30 % in optimally matured fruits. It appears that optimum maturity fruits exhibited more retention of *Spondiol* concentration even at the end of storage period, which is a preferred quality criterion for nutraceutical industry and for therapeutic uses. Steep decrease in the Spondiol concentration from 18.89 to 15.73 mg and from 21.45 to 18.32 mg in the *Spondias mangifera* fruits harvested on 7 & 9 week respectively. Interestingly, this sudden decrease in spondiol coincides with their climacteric peak. The reason can not be explained.

BIOCHEMICAL

pH, titrable acidity and total soluble solids

The pH of fresh *Spondias mangifera* fruits harvested at mature and over mature stages did not show significant changes during the storage. However, highest pH of 2.7 was observed in immature, indistinct phase fruits. It decreased to 2.5 on fourth day of storage **[Fig. 4.3].** Decrease in pH was observed with advance of maturity. It varies from 2.55 to 2.6 and 2.4 to 2.5 in distinct and dominant stage of maturity respectively. pH

Fig. 4.3: Changes in pH of *Spondias mangifera fruits* of different maturities during storage at ambient temperature.

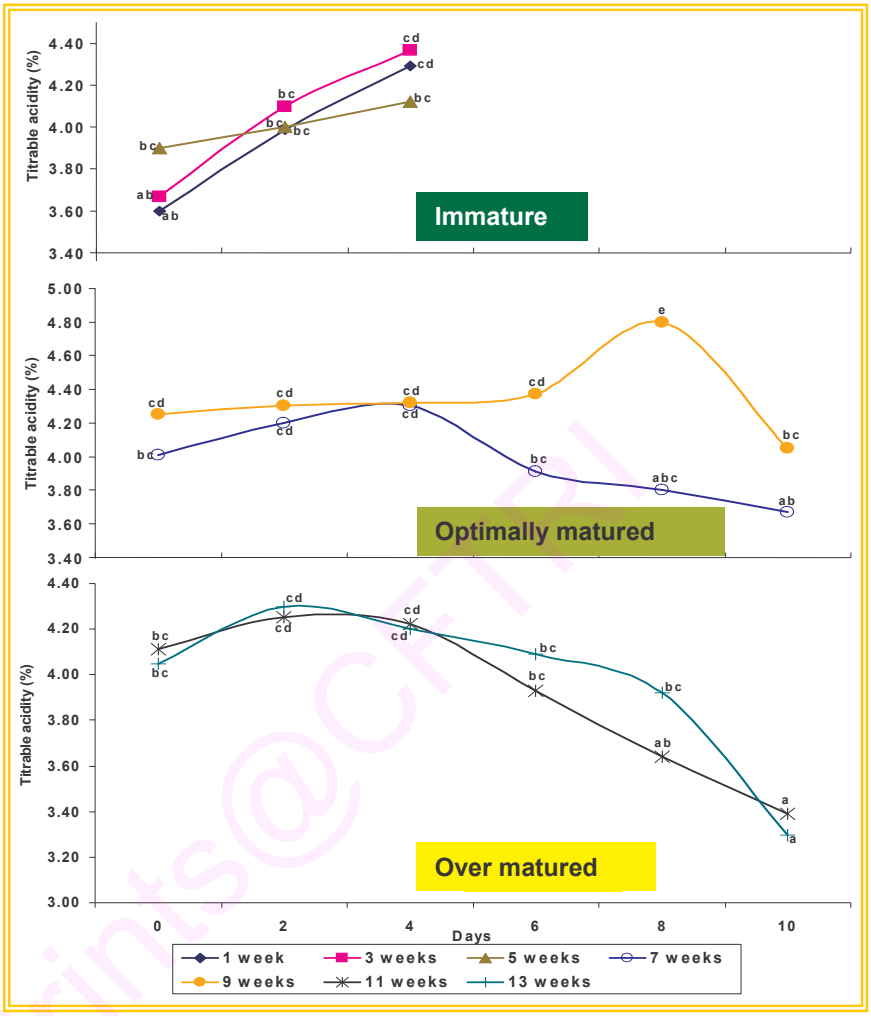


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

was found to be stable through out the storage period except at the end of storage period between 8 to 10 days in overmatured or dominant phase fruits [**Fig. 4.3].**

Titrable acidity calculated as citric acid showed a downward trend in matured and overmatured *Spondias mangifera* fruits encountered a slight climacteric rise in the former. However,

Fig. 4.4: Changes in titrable acidity of *Spondias mangifera fruits* of different maturities during storage at ambient temperature.

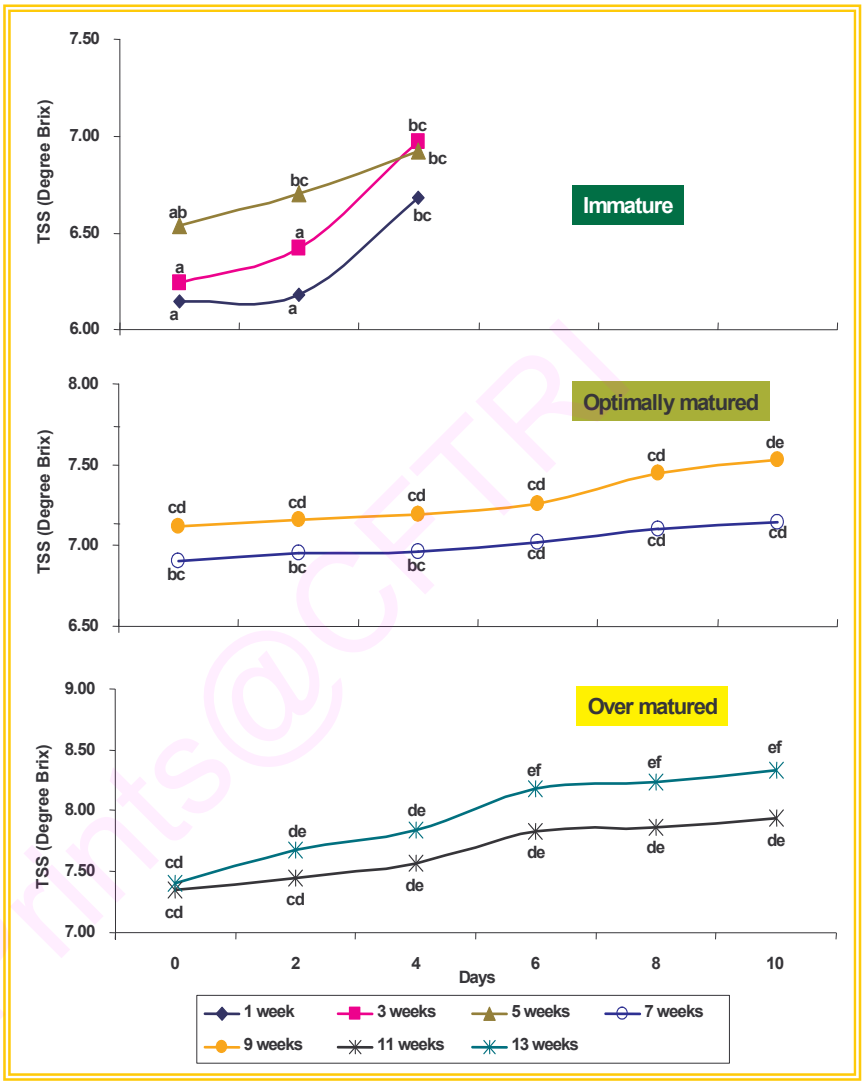


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

decrease in the titrable acidity at the end of storage period was observed in all the fruits, immaterial of maturity stages. Contrarily, immature *Spondias mangifera* fruits showed an increase in titrable acidity from 3.6 to 4.3 **[Fig. 4.4].** Loss of acidity was least in 9 weeks fruits [4.7 %] when compared to 13 week fruits [18.6 %]. The increase in acidity was highest [19 %] in fruits harvested after 3rd week.

A steady and slight increase in total soluble solids was exhibited in the *Spondias mangifera* fruit during storage period. At the end of ten days of storage, optimally matured and over matured fruits accumulated 7-8 degree brix. But, the fruits harvested at 13th week showed highest increase (11.2%) in TSS at the end of storage period. It was observed that increase in TSS with the advance of maturity **[Fig. 4.5].** The increase was mainly attributed to loss of water.

Fig. 4.5: Changes in total soluble solids *Spondias mangifera fruits* of different maturities during storage at ambient temperature.

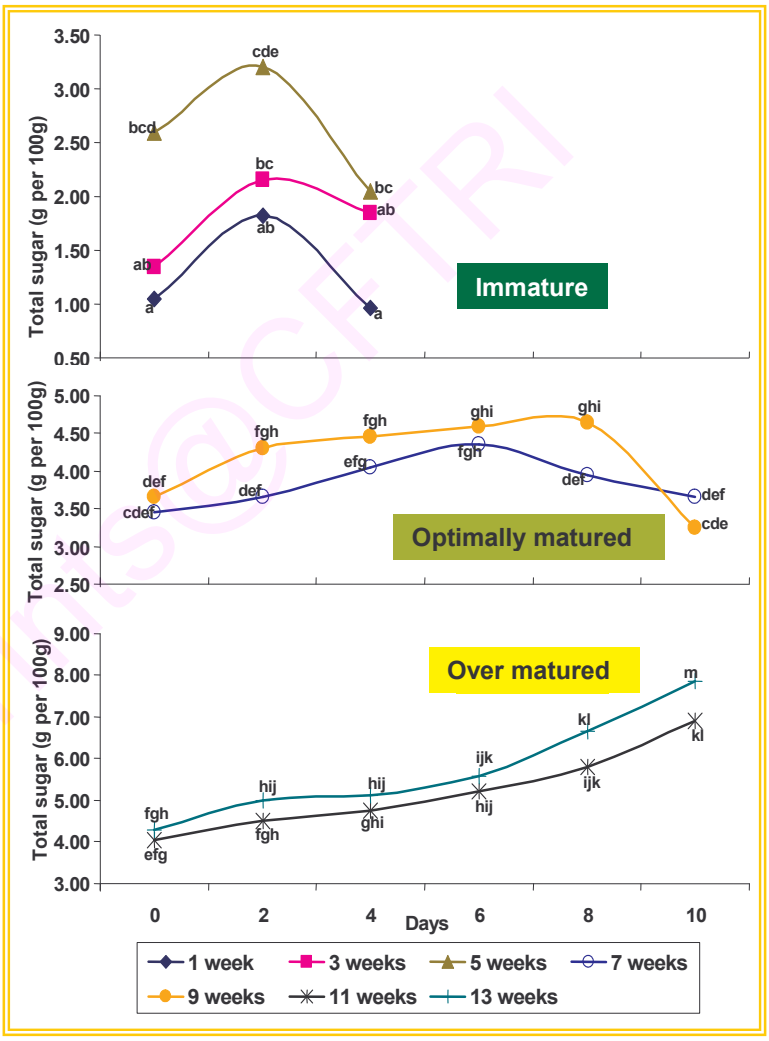


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Total sugars and reducing sugars

Increase in total sugar conternt in *Spondias mangifera* fruits was observed till second day in immature fruits, it was upto sixth and eighth day for optimally matured fruits respectively. Further, observations recorded lower concentration. While, in over matured fruits, total sugar content increased continuously till the end of storage. The increase in percentage of total sugar with advance of fruit growth was recorded in all fruits **[Fig. 4.6]**. Optimally matured and over matured fruits of *Spondias mangifera* showed 20 % and 40-45 % gain in total sugars during the storage period respectively. In contrast, the immature fruits exhibited a typical bell shaped trend in accumulation of sugar during storage.

Fig. 4.6: Changes in total sugar of *Spondias mangifera fruits* of different maturities during storage at ambient temperature.

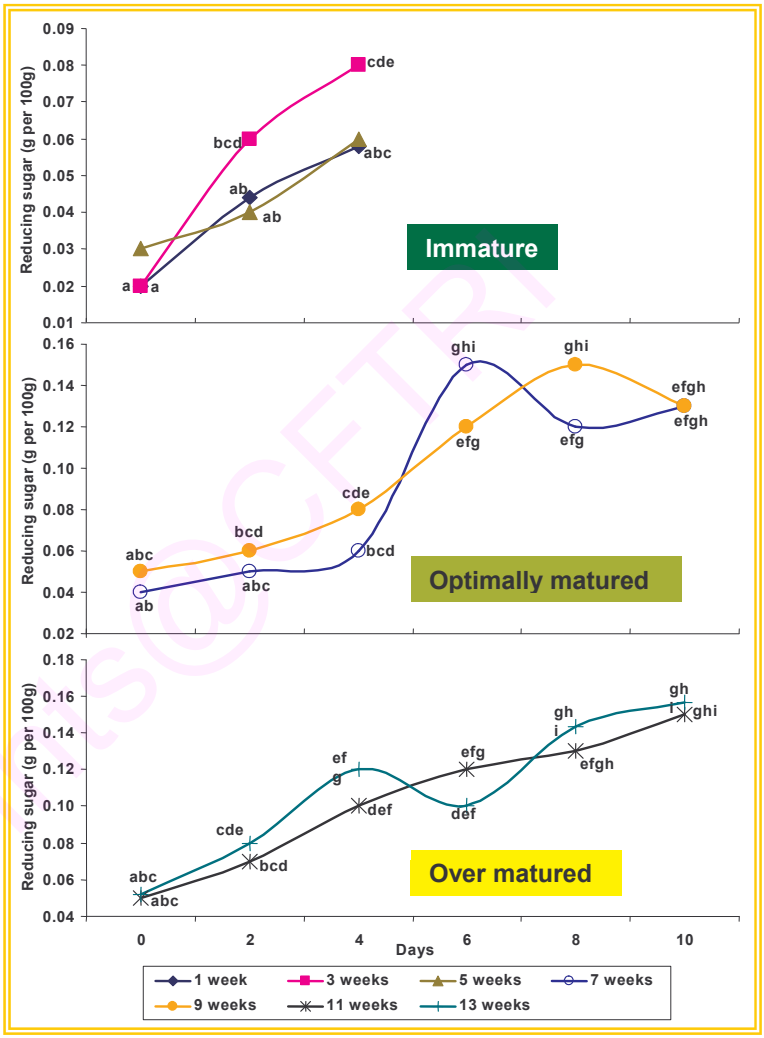


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

There was an increase of 50-75% **[Fig. 4.7]** in the reducing sugar level in case of the immature *Spondias mangifera* fruits at the end of their storage life on fourth day. Fruits

of optimally matured and over matured showed an increase of 60-70 % at the end of 10th day. But, optimally matured fruits recorded slight decrease in reducing sugars after attaining a peak accumulation on sixth and eighth day, which, in turn coinciding with the climacteric peak.

Fig. 4.7: Changes in reducing sugar of *Spondias mangifera* fruits of different maturities during storage at ambient temperature.



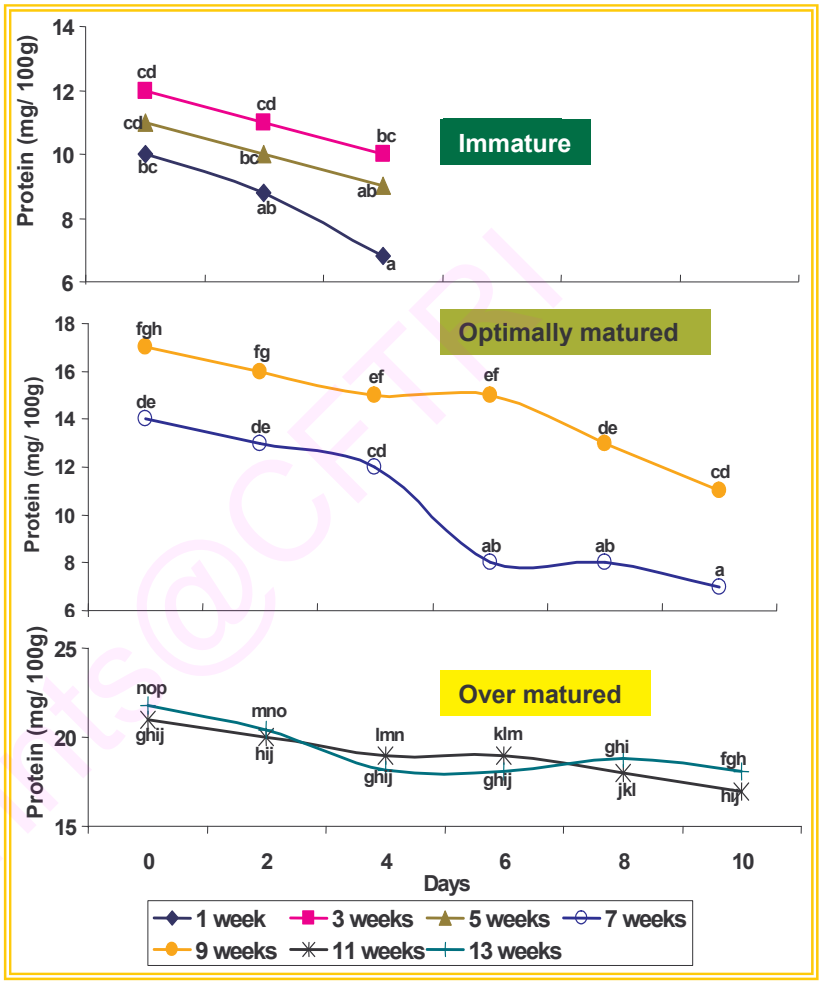
Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Thus the increase in sugars which may be attributed to hydrolysis of starch into simple sugars [Biale, 1960]. 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, [Salunkhe and Desai, 1984; Brecht, 2003]. ambient temperature negatively influenced the rhizome by attenuating the metabolic activity resulting in decrease in total sugars and reducing sugars [Brecht, 2003]. Thus differential trend in accumulation and decline in trend of sugars in different maturity fruits may be the consequence of difference in respiration and associated biochemical solubles rearrangements.

Total protein

Total protein content of immature fruits of *Spondias mangifera* is lower (10-12 mg) when compared to matured fruits (21 & 21.8 mg) **[Fig. 4.8]**. Low protein content in immature fruits, while high protein in optimally and over matured fruits was reported in *Spondias cytherea* by Ishak et al. [2005]. Steady decrease in the protein content was observed in all the maturity stages of *Spondias mangifera* fruits. In seven week fruits the total protein content decreased from 14 to 7 mg/100g while in nine week fruits it was from 17 to 11 mg/ 100mg. These results reveal that during the period of storage, due to the solubulization of the pectin by the pectic enzymes protein level decreased in different fruits such as guava [Chaturvedi, 1974], peach [Shewfelt et al., 1986] and papaya [Jagtiani et al., 1988].

Fig. 4.8: Changes in total protein content of *Spondias mangifera* fruits of different maturities during storage at ambient temperature.

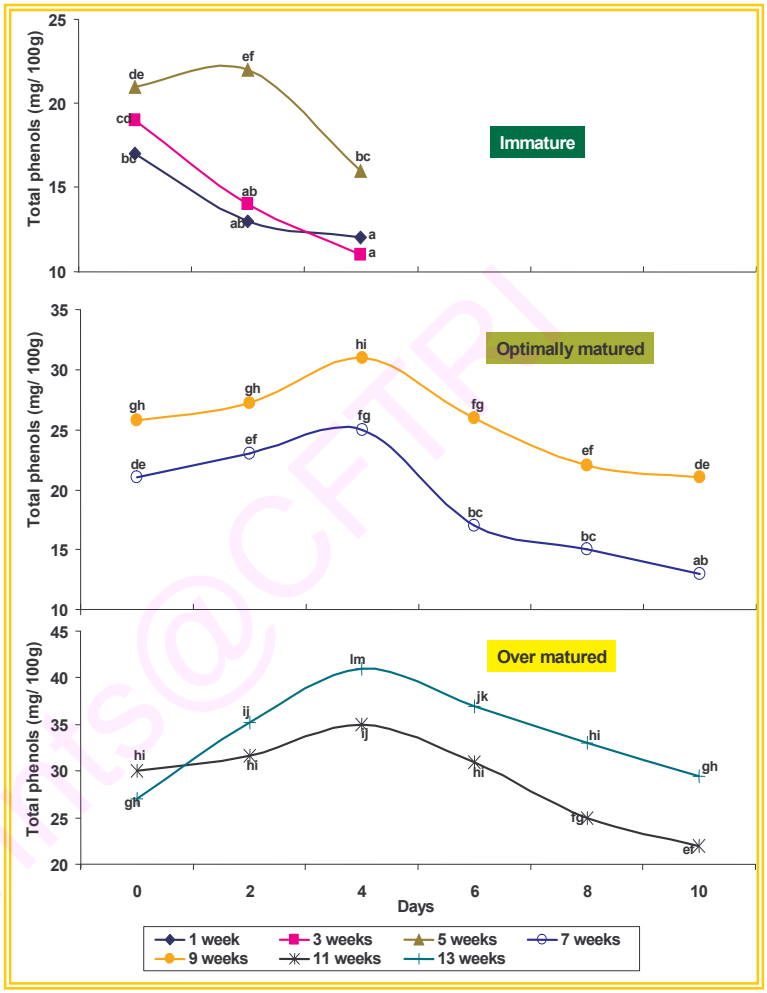


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Total phenolics

The total phenolic content increased with delay in harvest period. After harvest the total phenolic content in immature fruits showed a declining trend through out the storage periodl. While a slight increase with peak accumulation of total phenols on 4th day of harvest was observed in both optimially and over matured fruits. The peak accumulation ranged around 3-4 mg/100 g in optimum matured fruits, when compared to 15mg mg/100g, in over matured fruits harvested on 13th week. This is in contrast with 5mg/100g in 11 week fruits (Fig 4.6). later, the concentration of total phenols declined gradually over the period of storage **[Fig. 4.9**]. Immature fruits showed continuous decline in phenols unlike optimally matured and over matured fruits. It appear that peak accumulation of total phenols in both optimum and over matured fruit precede the climacteric peak of respiration. Increase in concentration of total phenol may not be due to de novo synthesis of phenol but, due to concentration of phenols, as a consequence of rapid loss of water and shriveling **[Fig. 4.14].** The post climacteric reduction in the concentration of phenols may be due to hardening of endocarp.

Fig. 4.9: Changes in total phenolics *Spondias mangifera fruits* of different maturities during storage at ambient temperature.

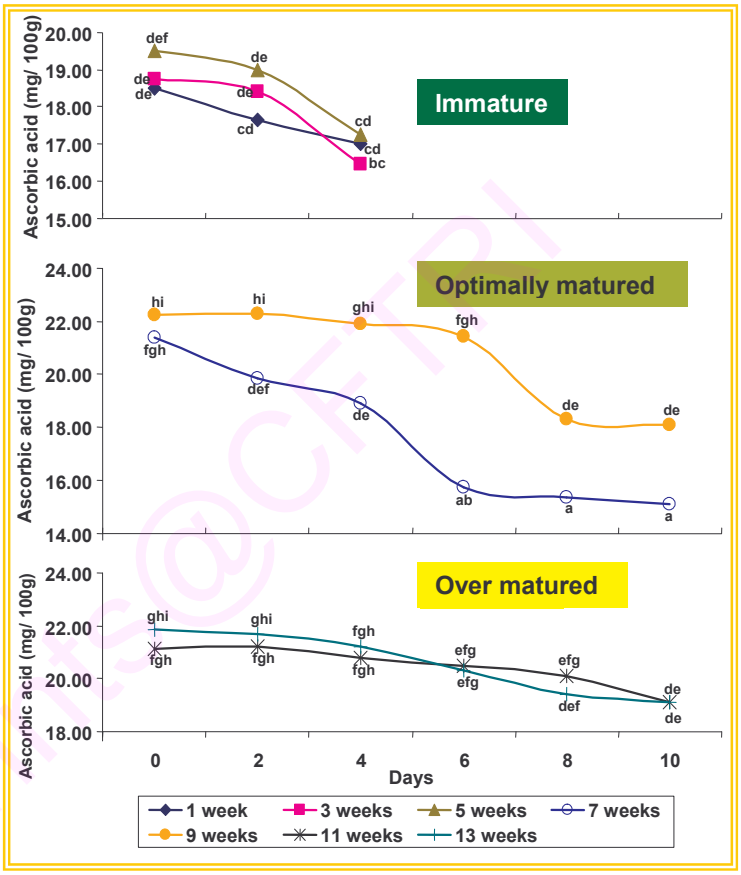


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Ascorbic acid

Fruits harvested after nine weeks accumulated and retained ascorbic acid content of 21.45 mg/ 100g until six days, and then it suddenly declined on eighth day coinciding with the climacteric peak. Similar sudden decline on sixth day observed in seven week fruits. So, ascorbic acid content of optimally matured fruits decline is coinciding with the rise of climacteric peak. These cumulative changes leading to the increase in total soluble solids, sugars, carotenoids and ascorbic acid, while decrease in titrable acidity, total chlorophyll and total phenolics with fruit maturity rendering optimum eating quality attainment at the climacteric peak is in agreement with the Al-Niami et al. (1992). Immature and over matured fruits showed steady decline in ascorbic content over the period of storage **[Fig 4.10]**. The synthesis and loss of ascorbic acid is a function of stage of harvest and time. Early harvested fruits recorded lowest concentration of 18-19 mg/ 100g which slightly reduced on second day, but deterioration of ascorbic acid started after second day recording quick loss on fourth day. This decrease in ascorbic acid is observed in many fruits during storage (Herna´ndez et al. 2007; Manolopoulou and Papadopoulou, 1998; Casas-Alencaster, 1977; Pruthi, 1963; Al-Kahtani, 1992).

Fig. 4.10: Changes in ascorbic acid content of *Spondias mangifera* fruits of different maturities during storage at ambient temperature.

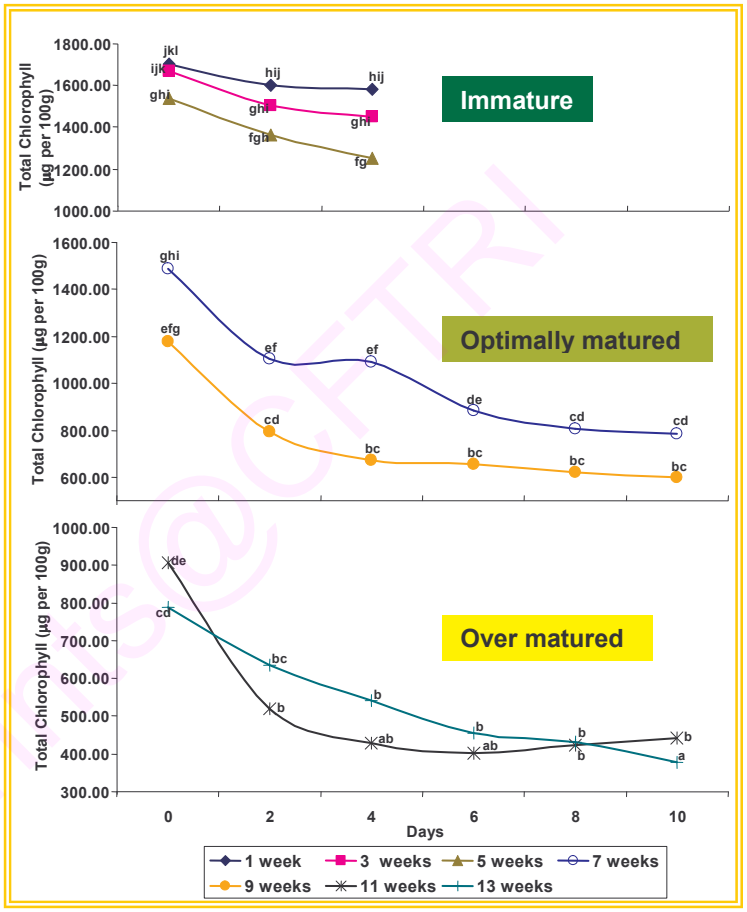


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Pigments

During storage of *Spondias mangifera* fruits total chlorophyll, in contrast to the carotenoids, followed a decreasing trend for all the stages of harvest along the entire period of storage **[Fig. 4. 11]**. Among the immature fruits (harvested after 1, 3 and 5 weeks), only 7-19% chlorophyll degradation was observed leading to 1581.65, 1450.07 and 1249.40 µg/ 100g retention, because of shriveling after four days of storage they lost the market acceptability. Whereas, in stage optimally matured and over matured fruits, 50% of chlorophyll was degraded at the end of storage period of ten days. In optimally matured fruits, total chlorophyll content reduced from 1489.02, 1176.83 to 784.50, 600.20 µg/ 100g and it was from 906.84, 789.36 to 442.17, 377.56 µg/ 100g. Total chlorophyll content reduced drastically till fourth day and then maintained a slow decline till tenth day in 7, 9, 11 and 13 week fruits.

Fig. 4.11: Changes in total chlorophyll of *Spondias mangifera fruits* of different maturities during storage at ambient temperature.



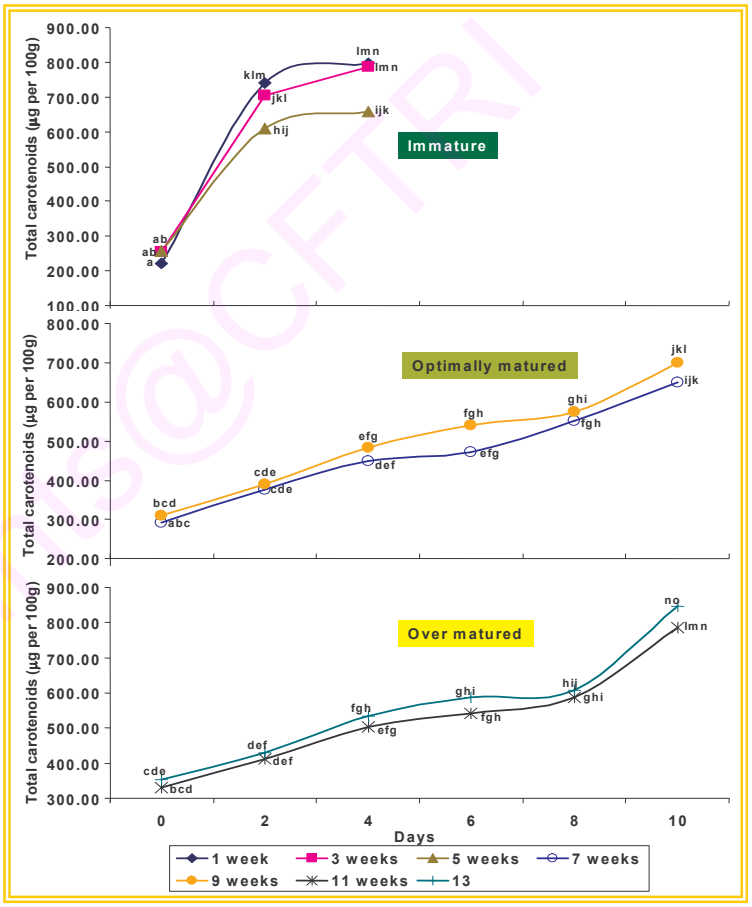
Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Contrary, an opposite trend was observed in the accumulation of the carotenoids during storage **[Fig. 4.12]**. Early Harvesting induced dramatic accumulation of carotenoids from 220.35, 255.56 and 257.30 µg/ 100g on the day of harvest to 796.85, 785.73 and

657.27 µg/ 100g on fourth day in immature fruits. There was a gradual accumulation of carotenoids from 291.23, 309.71 to 650.46, 700.48 in optimally matured fruits and in case of over matured fruits, from 331.14, 352.58 µg/ 100g on the day of harvest to 785.45, 847.12 µg/ 100g at the end of ten days of storage.

Peak accumulation of total carotenoids by 60-70% was noted in immature fruits of *Spondias mangifera* within 4days of storage whereas, optimally matured and over matured fruits recorded only 60% increase over comparatively longer period of storage for ten days from the time of harvest. During which, there was gradual and steady accumulation of total carotenoids.

Fig. 4.12: Changes in carotenoids of *Spondias mangifera fruits* of different maturities during storage at ambient temperature.



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

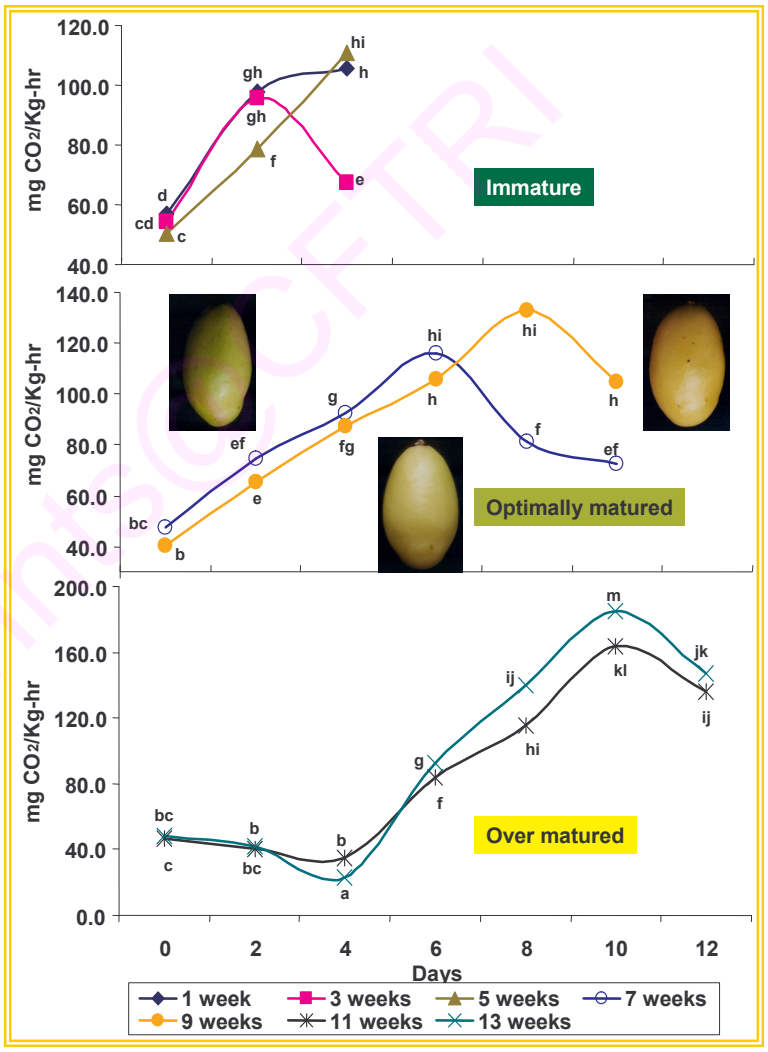
The constant transformation of certain pigments into others demonstrates the biosynthetic activity of carotenoids during the fruit growth stage. Further, the reason may be due to difference that affects the processes of formation and degradation of each carotenoid. The balance depends between variation in anabolic and catabolic with different maturity. Therefore, there must be some factor/s besides the carotenogenic process, individually and thus the synthesis of this pigment continues, confirming an earlier study (Watanabe and Takahashi, 1999, Roca and Mínguez-Mosquera, 2003).

PHYSIOLOGICAL

Rate of Respiration

Spondias mangifera fruits harvested at distinct phase reached climacteric peak on 6th (115.9 mg CO_2/ kg-hr) and 8th day with peak CO_2 output of 132.6 mg CO_2/ kg-hr during storage. While fruits harvested at Immature phase, showed climacteric peak on 2nd day, with output of 95.6-97.7 mg CO_2/ kg-hr,. Fruits harvested at over matured phase reached climacteric on 10th day with CO_2 outburst measuring 163.4 - 184.6 mg CO_2/ kg-hr., during storage. Climacteric peak in rate of respiration was observed on 2nd, 6th, 8th and on 10th day after harvest, in immature, optimum mature and over mature phase fruits **[Fig. 4.13].**

Fig. 4.13: Changes in rate of respiration of *Spondias mangifera* fruits of different maturities during storage at ambient temperature.



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

Delay in respiratory climacteric with increase in maturity of the Indian hog plum fruit was observed. Similar results were obtained with other stone fruits (Aharoni, 1968, Zauberman and Schiffmann-Nadel, 1972). Further, early harvested fruits are smaller in size resulting higher surface area per unit weight, which forms the

deciding factor for the increase in the rate of respiration. The rate of respiration is inversely proportional to the surface area as observed in other fruits (Aharoni, 1968, Blanpied, 1972). Intrinsic factors that may affect the rate of respiration in fruits appear to be cellular density, inter and intracellular status of oxygen, carbon dioxide and ethylene and other meatabolites status of the fruits (Eaks, 1970, Burg and Dijkman, 1967, Bain, 1958).

Increase in the rate of respiration during storage leading to ripening is noted in fruits harvested at all the stages of harvest (Kidd and West, 1930, Millerd et al., 1953, Pratt and Goeschl, 1969). Indian hog plum showed climacteric type of respiration, indicated that the fruit respiration increased with the fruit ripening. Accompanied by increased respiration and yellow surface coloration was observed during storage similar to mango and other fruits (Young and Olmstead, 1954, Al-Niami et al., 1992, Wang et al., 2001).

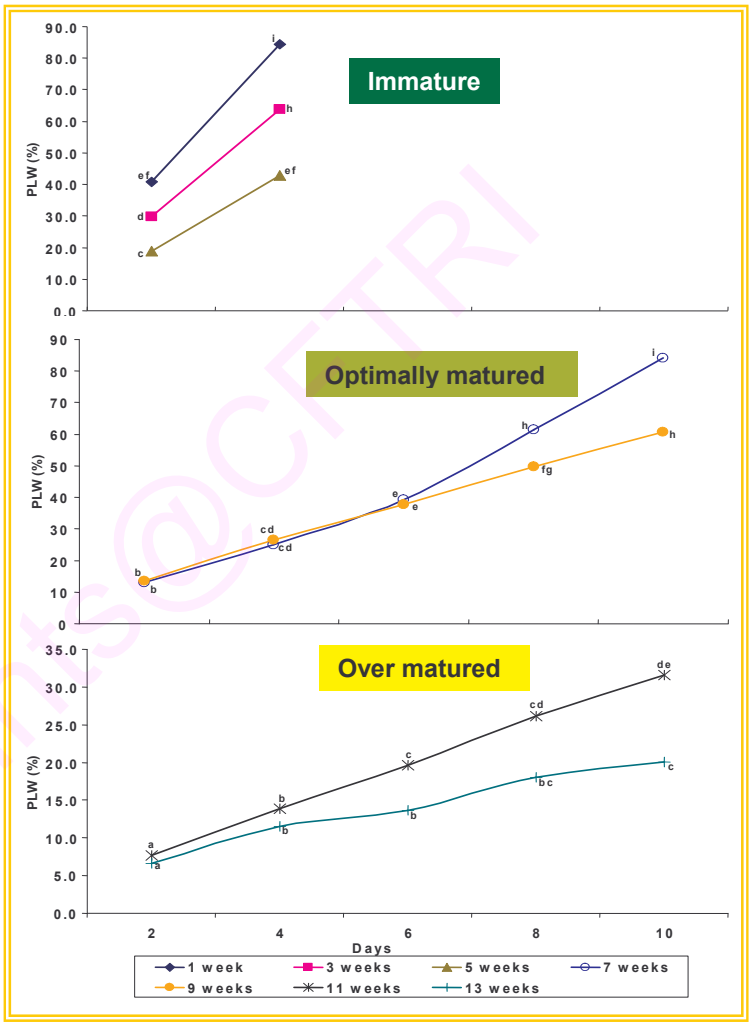
Fruit ripening

Indian hog plum fruits normally attain full ripe stage on the tree and drop. In the present study optimum maturity fruits were studied for ripening characteristic of fruit. The ripening changes like change in surface colour and associated biochemical changes coincides with the respiratory pattern. The fruits were green hard till 4th day coinciding with climacteric rise in optimum matured fruits. While, breaker stage i.e. de-greening and appearance of yellow colour precedes the climacteric peak in optimum and over matured fruits. But in 7th week fruits the breaker stage coincides with climacteric peak of respiration **[Fig 4.13]**. Complete change in colour from green to yellow and onset of senescence was observed after climacteric peak of respiration.

Physiological loss of water, shriveling and spoilage

Highest percentage (20-50 %) of physiological loss of water was observed in immature fruits of *Spondias mangifera*, followed by 12-13 % in optimally matured and 6-7 % in over matured fruits, on second day of storage at ambient temperature **[Fig. 4.14]**. This per cent of water loss was exponentially doubled on the fourth day both in optimally matured (9th week) and over matured (11th week) fruits. Immature fruits, due to their higher surface area per unit volume of the fruit lost 43-85% PLW. Whereas, over matured fruits with lower surface per unit volume of the fruit lost 20-30 % PLW at the end of storage period. Hence, loss of water was observed to be a function of ratio of surface area to the unit volume, which in turn is corresponding to the maturity stage of the fruit.

Fig. 4.14: Changes in physiological loss of water (PLW) of *Spondias mangifera* fruits of different maturities during storage at ambient temperature.

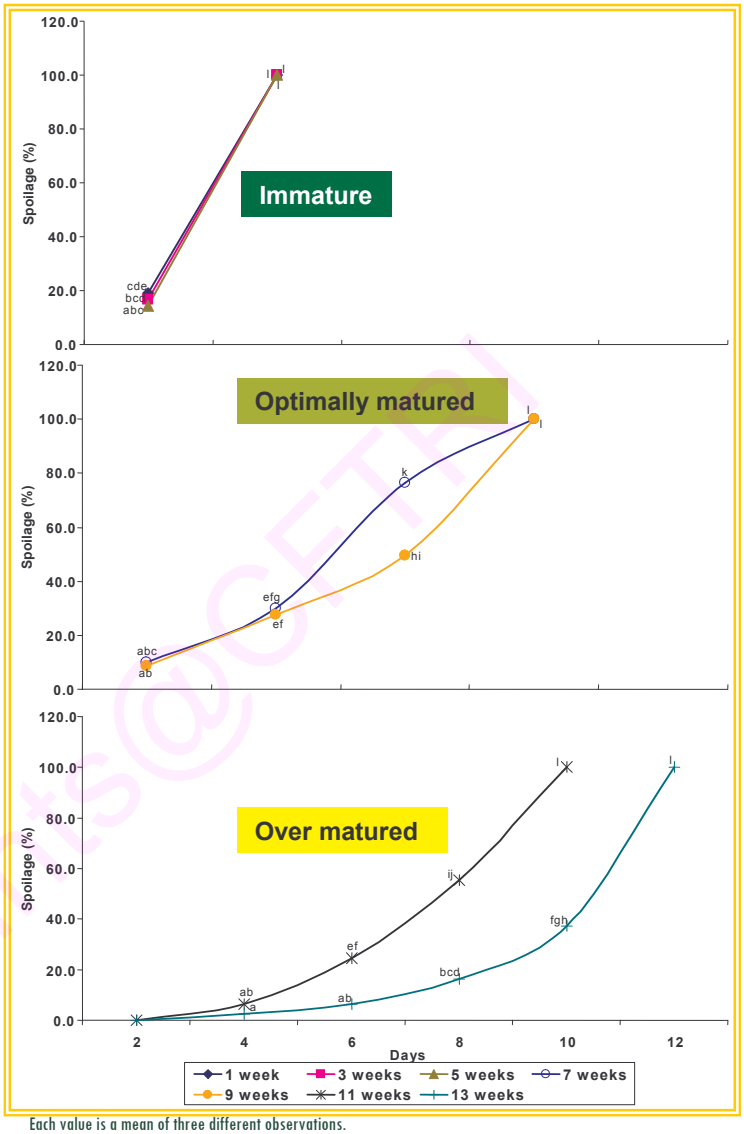


Each value is a mean of three different observations.
Values showed by different letters for each line are significantly different at $p < 0.05$.

Visible shriveling was observed earliest (3-5 days) in immature fruits, followed by optimally matured fruits on 7 & 10th day and over matured on 11 & 13th day during storage. The rate of weight loss, resulting from shriveling varies with variation in size and maturity of spondia mangifera fruit this may due to the high surface/weight ratio as

observed in other stone fruits (Fernandez-Trujilo and Artes 1998). Further water loss leads to a commercially objectionable level of shriveling and fruits become unsalable. Many vegetables become unsalable and manifest shriveling after loosing 7-20 % of their weight [Ben-Yehoshua and Rodov, 2003]. The pivotal physiological role of water in spondias mangifera fruit is maintenance of turgidity, freshness and quality as is the case for most perishables [Herppich *et al.,* 2000].

Fig. 4.15: Percentage of spoilage in *Spondias mangifera* fruits of different maturities during storage at ambient temperature.



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

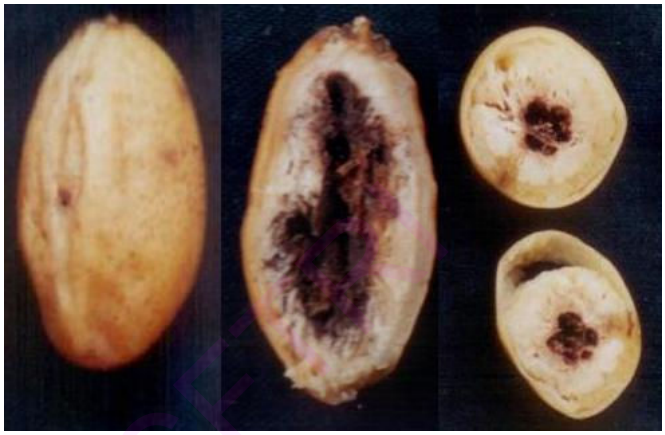
Cumulative percent of spoilage in terms of visible shriveling, browning, softening and incidence of fungal decay was observed to be highest in the immature fruits (14-19 %) followed by optimally matured fruits (8-10 %) and no spoilage in over matured fruits after 2 days of storage **[Fig. 4.15]**. Due to the higher rate of respiration and PLW, there was complete spoilage of *Spondias mangifera* fruits on fourth day. Lower rate of respiration and PLW caused delay in the spoilage of optimally matured and over matured fruits.

Physiological disorders

Browning of fruit

Browning of fruit is more predominant in *Spondias mangifera*. It was rapid and decay was prevalent after 2-3 days at room temperature 25 ±°C. Browning symptoms of the *Spondias mangifera* fruits were characterized by the presence of reddish brown coloration of the pericarp followed by shriveling and separation of pericarp from the mesocarp **[Fig. 4.16].** Further, browning symptoms were also observed in mesocarp and also in endocarp with shrive Initiation of browning. The bruised portion exhibited more browning at physically damaged or bruised region. Bruising damage in *Spondias mangifera* fruit is caused due to dropping of the fruit onto a surface, rubbing the fruit against other fruit or some other surface (vibration or abrasion bruising), or by pressing fruit against each other or a hard surface (compression bruising). This physical damage can occur during harvesting, hauling, and packing operations, and as a result of fruit movement in the package during transit to market as observed in other fruits (Sommer et al., 1960). This result suggests that flesh tissues and endocarp tissue are equally prone to browning. Alternatively, desiccation induced pericarp browning begins on the stalk end of the pericarp and subsequently spreads over the entire pericarp after 1-2 days as observed in other drupe fruits (Tongdee et al., 1982; Underhill and Critchley, 1995). Pericarp browning is the major post-harvest problem of Spondias mangifera. Pericarp browning renders the fruit unmarketable. In *Spondias mangifera* fruits browning was found to be associated with rapid loss of water like litchi fruits (Scott et al., 1982), although physical impact stress, senescence, fruit development pest or pathogen attack can also induce it. It was found that the mesocarp cells were the first to turn brown, followed by the endocarp in *Spondias mangifera* fruits. Browning has been attributed to pigments breakdown, PPO and peroxidase activity and enzymatic oxidation of ascorbic acid in many fruits (Underhill, 1992). Browning of tissues is generally attributed to oxidation of phenolics (Kulkarni and Aradhya, 2005). In the present study browning was

Fig. 4.16: Internal browning in fruits of *Spondias mangifera* causing spoilage during storage.

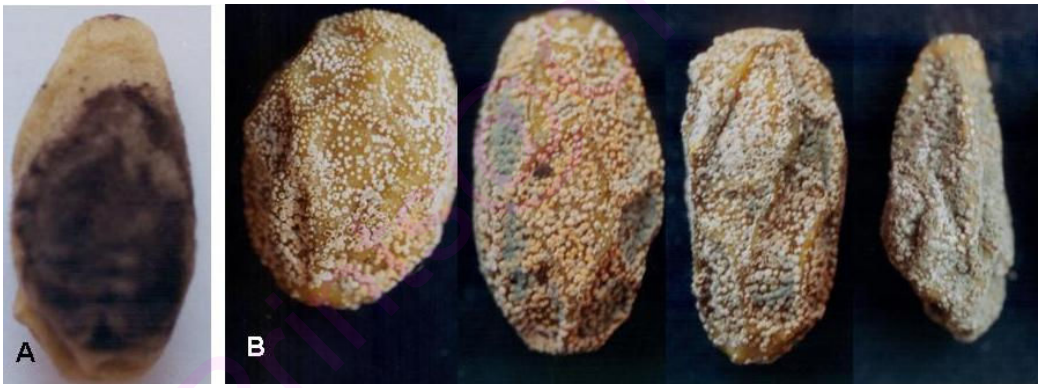


observed in late harvest or fruits at dominant phase of growth. Harvest maturity appear to influence quality and the susceptible browning disorders during storage life of Indian Hog plum fruit as observed in other fresh fruits (Prabhu Desai, 1989).

Pathological diseases

Pathogenic fungi isolated from the diseased fruits were cultured. Pathogenic spores inoculated on the healthy *Spondias mangifera* fruits showed the symptoms characterized by the diseased fruit. The taxonomic identification of the pathogenic fungi culture was carried out by its colony characteristic, morphological and reproductive structures. Based on the microscopic observations pathogens have been identified *Aspergillus niger* **[Fig. 4.17 A]** and *Pencillium expansum* **[Fig. 4.17 B]** by using manual (Paulin-mahady *et al*., 2002).

Fig. 4.17: Pathological diseases A: *Aspergillus niger* and B: *Pencillium expansum* of *Spondias mangifera* fruit causing spoilage during storage.



Indian Hog plum fruit are very susceptible to postharvest decay due to fungi during storage. Organisms associated with postharvest decay of *Spondias mangifera* being *Aspergillus niger* **[Fig. 4.17 A]** and *Pencillium expansum* **[Fig. 4.17 B]**. They are reported for the first time on *Spondias mangifera* fruits. *Aspergillus niger* appear to be opportunistic, facultative pathogen. It colonizes on physically damaged tissues of the fruits that inflict during harvesting and handling of fruits. Further in Indian hog plum fruits are harvested by shaking the branches, which causes physical injury or crack due to fall from the height. Microscopic cracks are present at harvest and increase after harvest as observed in many fruits (Underhill and Simons, 1993). The fungus gain entry through natural openings like lenticels to penetration is enhanced in over-mature fruit, during storage, or by bruising or puncturing as observed in other pome fruits (Eckert, 1978).

In case of *Penicillum expansum* the fungus colonize in natural opening like stomata and lenticels. The conidia-bearing conidiophores, grouped to form coremia, are formed on the surface of the lesion. As the conidia mature, they become blue-green and form masses which give the decay its typical color. Decay can progress rapidly at ambient storage temperature. With advance of infection the fruit shrivels and ultimately mummifies **[Fig 4.17B]**. The fungus can spread sound fruit, forming 'nests' of decay. The fungus is reported for the first time on *Spondias mangifera* fruit. Several strains of P. *expansum* can produce the mycotoxin patulin may be highly toxic to animal tissue and may also display carcinogenic and mutagenic properties.

Shelf-life

There is an increased concern about the quality of fruit during development and prior to harvest aimed at minimizing post-harvest deterioration. In the present study, changes in the major physical, bioactive and biochemical composition, during ambient storage temperature was carried out, with three distinct maturity stages of fruit. Immature *Spondias mangifera* fruits intensified and hastened the physiological and biochemical processes such as high rate of respiration, water loss, loss of ascorbic acid, phenols, appearance of visible shriveling, yellowing, browning, softening and incidence of fungal decay. Thus, these changes hampered the acceptability and reduced their shelf to four days. Over matured fruits, though, found to have higher shelf life of 12 days, due to the hardening of the endocarp, yellowing of the fruit peel and its easy separation from the endocarp hinders its fresh consumption and also utilization for processing industry or for pickles or culinary preparations. Early harvest may impede the development of the characteristic colour, taste, texture and aroma, while late-harvested fruits exhibit a reduced shelf life; early deterioration, which was also observed in apple, mango and other tropical fruits (Fellman et al., 2003, Medlicott et al., 1988). Optimally matured fruit were found to be the most favorable for storage and also in terms of consumer acceptance for processing and culinary preparations. These fruits, with moderate rate of climacteric respiration and decelerate loss of the ascorbic acid, phenols, chlorophyll, water loss, soft endocarp, and delayed appearance of inferiority parameters such as shriveling, yellowing, browning, softening, fungal decay had a shelf life up to 10 days. Thus, physiological, biochemical changes, consumer preferred quality of *Spondias mangifera* fruits candidly

administered by the maturity and storage. The shelf life of the fruit was found to be a function of maturity and storage temperature in *Spondias mangifera* fruits. However, there is an ample scope to increase the shelf life by using various post harvest technology that is commonly employed for other fruits.

CONCLUSION

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