Studies on inositols in *Hevea* Latex: Isolation, characterization and their role in drought tolerance

Thesis submitted to
MAHATMA GANDHI UNIVERSITY
For the award of the degree of
DOCTOR OF PHILOSOPHY
in
BIOCHEMISTRY
(Faculty of Science)

By

JAYASREE GOPALAKRISHNAN

Under the supervision and guidance of

Dr. Molly Thomas

RUBBER RESEARCH INSTITUTE OF INDIA
KOTTAYAM, KERALA,
INDIA - 686 009

MAY 2015

Dedicated to my Father

DECLARATION

I hereby declare that the thesis entitled **"Studies on inositols in *Hevea* Latex: Isolation, characterization and their role in drought tolerance"** is an authentic record of original research carried out by me under the supervision and guidance of Dr. Molly Thomas, Principal Scientist, Crop Physiology Division, Rubber Research Institute of India, Kottayam-9 in partial fulfilment of the requirement for the degree of DOCTOR OF PHILOSOPHY of the Mahatma Gandhi University and no part of this work has been presented for any degree or any other similar titles of any university.

RRII
2nd May 2015

Jayasree Gopalakrishnan

The Rubber Research Institute of India
Rubber Board, Ministry of Commerce and Industry, Govt. of India
Kottayam – 686 009, Kerala, INDIA

CERTIFICATE

This is to certify that the thesis entitled **"Studies on inositols in *Hevea* Latex: Isolation, characterization and their role in drought tolerance"** is an authentic record of original research work carried out by Smt. Jayasree Gopalakrishnan, at Rubber Research Institute of India, Kottayam-9, under my supervision and guidance for the award of the degree of **Doctor of Philosophy in Biochemistry,** under the faculty of Science, Mahatma Gandhi University, Kottayam. It is also certified that the work presented in this thesis has not been presented or submitted earlier for any degree, diploma or any other similar titles of any university.

Kottayam
2-5-2015

Dr. Molly Thomas,
Principal Scientist
Crop Physiology Division
Rubber Research Institute of India,
Kottayam-9

Tele Phone: 91-481-2353311 to 2353320 Fax: 91-481-2353327

ACKNOWLEDGEMENT

I wish to express my deep sense of gratitude and sincere thanks to my respected guide ***Dr. Molly Thomas,*** *Principal Scientist, Crop Physiology Division, Rubber Research Institute of India, Kottayam for her learned counsel, meticulous and gracious guidance, sustained interest, constant and inspiring encouragement sympathy and generosity much beyond her formal obligations, affectionate advice, constructive criticisms and forbearance all through the research work all of which contributed the most to the completion of the study and the critical scrutiny of the manuscript.*

I sincerely express my deep sense of gratitude for ***Dr. James Jacob,*** *Director of Research, Rubber Research Institute of India for his rare brilliance, keen and unstinted interest and critical comments and ever willing help rendered to me at all stages of this endeavor.*

I extent my sincere thanks to ***Dr. K, Annamalainathan,*** *Joint Director, Crop Physiology Division, Rubber Research Institute of India, Kottayam for his creative suggestions, supports and constant encouragement which aided me to complete the thesis.*

I would like to thank ***Dr Luxmi Varma,*** *R, Sr. Principal Scientist & Head, Organic Chemistry, NIIST, Thiruvananthapuram, for her help in the spectral analysis (FT-IR and NMR) of the samples and the interpretation of the data.*

I wish to express my sincere thanks to ***Dr. R, Krishnakumar,*** *Joint Director, Climate Change and Ecosystem Studies Division, RRII, Kottayam, for his valuable suggestion and support throughout the work.*

I am very much obliged to ***Dr. Rekha K,*** *Senior Scientist, Biotechnology Division, RRII, Kottayam for her critical comments, encouraging and valuable suggestions, advice and ever willing help rendered to me at various stages of my thesis work.*

I am grateful to ***Sri. Madhusoodhanan,*** *Principal Scientist, Rubber Technology Division, Rubber Research Institute of India for timely and propitious support and help rendered to me in the preparation of illustrations of this thesis work.*

It is of great pleasure to express my deep sense of gratitude to my colleagues in Crop Physiology Division ***Dr. D. Bhuvanendran Nair, Dr. S. Sreelatha, Dr. K.V. Sumesh, Dr. Jayanta Sankar*** *and* ***Dr. Sheela P. Simon*** *for their support and suggestions throughout the work.*

I convey my thanks to Mr. ***Jayasooran, Dr. Shammi Raj, Smt. Shalaja Devi*** *and* ***Mr. Pradeep,*** *Climate Change and Ecosystem Studies division, RRII, Kottayam for their valuable suggestions and comments.*

I convey my thanks to ***Mr. P. Anish*** *and* ***Smt. Suma George,*** *Statistics and Computer Division, RRII, Kottayam for their support in statistical data analysis and computer work.*

I owe my thanks to ***Benny K.K (Publicity & Public relations Division),***

Rajan Mathew, K.R. Harikumar, M.B. Rajan, P. M. Sebastin *and all other members of Crop Physiology Division for their timely help in different stages of this work.*

I wish to thank cooperations of the PhD students of the Crop Physiology Division is thankfully acknowledged.

This dissertation has been possible with the guidance and the help of several individuals who in one way or another contributed and extended their valuable assistance in the preparation and completion of this study. Here, I would like to express my appreciation to all of them, and in particular to those who supported me throughout this journey.

I duly acknowledge with full heart the personal scarifices, timely permission, incessant encouragement and moral support of my husband ***Sri. Prasannakumar. R.,*** *Mother* ***Smt. Sarasamma.*** *and my daughter* ***Megha Prasannakumar*** *and son* ***AdithyaKrishna,*** *P.K, which I believe enabled me to complete this venture successfully.*

I sincerely thank all my family members for their moral support and constant encouragement throughout the work.

My sincere thanks to M/s Minitek Computers, opp. M.G. University, Kottayam for neat documentation of this thesis.

Above all, I bow my head before the ***God Almighty*** *whose blessings were always with me enabling to undertake this endeavor successfully.*

Jayasree Gopalakrishanan

ABSTRACT

Plants synthesize numerous secondary metabolites from primary metabolites as critical adaptation strategies against adverse environments such as biotic and abiotic stresses. They are beneficial to mankind in many ways. Rubber (*cis*-polyisoprene) is one such compound of latex synthesized within the laticiferous tissues of the rubber plant, *Hevea brasiliensis*. Latex contains a variety of non rubber components along with rubber particles. Among these, inositols (cyclitols) form a large group having commercial importance. Among the inositols L-quebrachitol is the most abundant non-rubber component present in *Hevea brasiliensis* latex. It has immense application in pharmaceutical, medical and cosmetic fields. Inositols/ cyclitols are thought to be the major contributor of osmotic pressure and cryoprotection of plant. Thus inositols play a critical role in osmotic potential regulation of laticiferous system in response to drought.

Hence, an attempt was made towards the isolation of L-quebrachitol from *Hevea* latex. Its extraction potential was studied through different extraction methods from the latex of different *Hevea* clones. Clonal and seasonal variations of total inositol content in the latex of different *Hevea* clones were evaluated. The association of inositols and other osmotically important solutes to water relation of latex and their role in drought tolerance was established. For this, the contents of total inositols, sugar, reducing sugars, free amino acids and different ions such as K^+, Mg^{2+}, Ca^{2+} and P were estimated in the latex of different *Hevea* clones during peak yielding and stress seasons.

In the present study, the isolation of L-quebrachitol from *Hevea* latex was successfully accomplished. Identification of the isolated compound was confirmed by HPLC, FTIR, LC/MS/ESI and ^1H and ^{13}C-NMR analyses in

comparison with that of the standard L-quebrachitol (Sigma). A protocol was developed for the isolation of L-quebrachitol from natural rubber latex (IP 238511). The protocol developed was found suitable for the isolation of quebrachitol from sera obtained through any type of latex extraction methods. The extraction potential of L-quebrachitol from natural rubber latex varied with respect to clone and method of extraction. The highest rate of recovery was observed in C-serum (1.41% w/w) followed by A- serum (0.48% w/w) and the lowest in factory effluent. An average rate of recovery of quebrachitol was obtained from alcoholic extraction (0.27% w/w) and cold treatment (0.22% w/w).

Total inositol content of latex was found higher in high yielding and metabolically active clones. There was significant clonal and seasonal variation in inositol contents. Similarly significant clonal, seasonal and clone x season interaction effects were observed for latex organic and inorganic solutes. Osmotic concentration of latex varied among the clones which had significant seasonal and clone x season effects. It was significantly high under stress season in all the clones. Osmotic concentration influences the latex flow in *Hevea* trees and there by latex yield. Contribution of solutes including ionic components to the latex osmotic concentration showed significant variation between peak yielding and stress seasons. Interestingly, the major solute contributing to the total measured osmolality of latex is found to be inositols (cyclitols). Significant varietal and seasonal variations were observed for its contribution to total osmolality. Inositols alone accounted for 28 to 41% of the osmotic potential during stress season. Under stress season clones such as RRII 105, PB 311, RRII 118 and GT1 maintained relatively better latex osmotic potential and physiological status than other clones. .

Key words: Secondary metabolites, Inositols, *Hevea brasiliensis*, L-quebrachitol, Osmotic potential, Osmoregulation

PREFACE

Inositols or polyols are cyclohexane hexols that occur widely in many different forms in nature. They are low molecular weight polar compounds and chemically very stable. In response to the environmental stresses such as drought, salinity, cold, *etc*, various organisms accumulate these organic solutes at substantially higher intracellular concentrations. The physically benevolent properties of inositols allow cells to sustain major changes in their concentration without affecting the cellular functions. Compatible solutes including various polyols stabilize macromolecules of cells during environmental perturbations. Under diverse environments various eukaryotes use different types of inositols and inositol derived cyclitols as compatible solutes such as methyl inositols in plants.

Myo-inositol, the most common form of inositol is present in plants as phytic acid and as phosphoinositide in microbes and animals. Myo-inositol possesses essential growth factor and vitamin like properties. Myo-inositol and various inositol derivatives functions as secondary messenger, thus play an important role in cellular signalling. These have application in drug development *e.g.* myo-inositol esters for liver diseases, aluminum and ammonium salt of myo-inositols in chemotherapy. The precursor of amino-cyclitols based antibiotics is myo-inositols. Naturally occurring inositol such as L-quebrachitol has been used as raw material for the synthesis of various inositol derivatives of potential application in pharmaceutical and medical research field because of simple and cheaper procedures. The significance of the plant *Hevea brasiliensis* is that, its latex contains relatively large amount of inositol in L-quebrachitol form. Thus an attempt was undertaken for the isolation of L-quebrachitol from latex. Studies were also focused on rate recovery from the latex serum obtained

through different methods, clonal and seasonal variation of inositol contents and its involvement in osmotic potential along with other latex solutes.

In the present study, isolation of L-quebrachitol, a major inositol from latex was attempted with an objective to develop an accurate and effective method for its isolation from latex. Recovery of this compound from serum collected through different extraction methods and different *Hevea* clones, identification and characterization of the isolated compound from the latex were illustrated in this thesis. Clonal and seasonal variation and physiological significance of inositol and other latex solutes to the water relations of latex were evaluated. The general concept of the research topic, its significance and objectives of the study, review of literature, methodologies, results and implication are described in five different chapters of the thesis.

CONTENTS

Chapter 3 - Isolation, purification and quantification of L-quebrachitol from *Hevea* latex

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

^{13}C- NMR	Carbon nuclear magnetic resonance
DMSO–d6	Duteriated dimethyl sulphoxide
DRC	Dry rubber content
ESI	Electrospray ionization
FT-IR	Fourier Transform Infrared
g	Gram
H. brasiliensis	*Hevea brasiliensis*
^{1}H –NMR	Proton nuclear magnetic resonance
HPLC	High Performance liquid Chromatography
LC/MS	High Performance Liquid Chromatography-Mass Spectrometry
µg	Microgram
Mg	Milligram
MHz	Megahertz
ml	milli litre
MPa	Mega Pascal
Nm	Nanometer
NMR	Nuclear Magnetic Resonance
NR	Natural rubber
NRS	Natural rubber serum
mOsmol	milliosmols
OA	Osmotic adjustment

PES	Polyethersulfone
RBD	Randonized block design
RI	Refractive Index
RRII	Rubber Research Institute of India
Rpm	Revolutions per minute
RWC	Relative water Content
TCA	Trichloroacetic acid
TFAA	Total free aminoacid
TMS	Tetramethyl silane
V/V	volume/volume
W/V	weight/volume
W/W	weight/weight

Chapter 1

General Introduction

1.1 The Rubber Tree (*Hevea brasiliensis*)

Natural rubber is synthesized by over 2500 plant species confined to 300 genera of seven families *viz.*, Euphorbiaceae, Apocynaceae, Asclepiadaceae, Asteraceae, Moraceae, Papaveraceae and Sapotaceae (Cornish *et al.,* 1993). Rubber tree which (*Hevea brasiliensis* Muell. Arg.) belongs to the family Euphorbiaceae, is an important perennial crop that produces natural rubber (*cis* 1, 4-polyisoprene). Other major rubber producing plants include *Manihot dichotoma* (Jeque rubber), *Castilla elastica* (Panama rubber), *Ficus elastica* (India rubber), *Cryptostegia grandiflora* (Madagascar rubber), *Parthenium argentatum* (Guayule), *Taraxacum kok-saghyz* (Russian dandelion), and *Palaquin gutta* (Gutta percha). Of these Guayule and Russian dandelion are known for producing large amounts of rubber with high molecular weight. Guayule provides 10 percent of the world's natural rubber (NR). Other species are not commonly exploited as commercial source. Among the rubber producing plants, *Hevea brasiliensis* has been established as the key source of natural rubber amounted to 99% of total NR (Fig. 1.1). There are ten species under the genus *Hevea viz, H. benthamiana, H. brasiliensis, H. camargoana, H. comporum, H. guianensis, H. microphylla, H. milida, H. pauciflora, H. rigidifolia,* and *H. spruceana* (Schultes, 1970; 1977; 1987; 1990; Wycherley, 1992). Among various species of the genus, *H. brasiliensis* is the only one grown commercially for the source of NR because of its high yield and rubber quality (Asawatreratanakul *et al.*, 2003).

Fig. 1.1 Rubber plantation

Hevea brasiliensis grows wild in the Amazon River basin in the Para region of Brazil South America. The major producers of NR are Thailand, Indonesia, Malaysia, India, China and Vietnam. In India, rubber growing regions are classified as traditional and non-traditional. The traditional rubber growing regions include Kanyakumari district of Tamil Nadu, whole of Kerala and Dakshin Kannada and Coorg districts of Karnataka State. The non traditional regions cover North Eastern states, West Bengal, Konkan region of Goa and Maharashtra, parts of Andhra Pradesh, Madhya Pradesh and Odissha. Majority of rubber plantations are concentrated in South Kerala in the districts of Trivandrum, Kollam, Kottayam and Pathanamthitta. More than 90% of the rubber production in India is from traditional rubber growing tracts of Kerala and parts of Tamil Nadu and Karnataka.

Natural rubber is the constituent of latex of the rubber tree. It is synthesised in specialized cells or tissue called laticiferous tissue. Latex is present in almost all parts of the plant but laticifers of the trunk are commercially utilized. Latex is harvested from *Hevea* trees through controlled wounding of the bark by the process called tapping (Fig.1.1). During this process the latex vessels are opened up and the latex exudes from the cut end of the vessels and it is collected and further processed to obtain rubber.

1.2 Latex composition

Latex is a mixture of *cis* 1, 4 polyisoprene (rubber), proteins, sugars, resins and water with small quantities of stabilizing components. The composition of *Hevea* latex is given in table.1.1

Table 1.1 General composition of *Hevea* latex

Components	Percentage
Rubber	30 – 40
Protein	2 – 2.5
Resins	1 – 2
Sugars	1 – 1.5
Minerals	0.7 – 0.9
Water	55 - 60

The fresh latex is a polydisperse system which can be separated into four main fractions by ultra centrifugation. The upper white fraction entirely of rubber particles (30-40%), below an orange or yellow layer containing Frey-Wyssling particles (1-3%), a middle aqueous phase of latex called C-serum and bottom fraction containing predominantly the lutoid particles (10-20%) (Jacob *et al.*, 1993; Nair, 2000).

1.2.1 Rubber particles

The rubber particles, constitutes 30-40% of the volume of the fresh latex and their size range in diameter from 0.02 to 3 μm (Southorn and Yip, 1968; Gomez and Moir, 1979). The hydrophobic rubber molecules are protected from the hydrophilic medium by a complex film of proteins and lipids (Ho *et al.*, 1975). The rubber particles comprise of sphere consisting of rubber and are surrounded by spherical shells containing phospholipids and proteins (Gomez and Moir, 1979). Besides the role of phospholipids in rubber particle membrane integrity, it is also associated with chemical branching of natural rubber molecules (Rojruthai *et al.*, 2009). The lipoprotein membrane complex is considered to impart stability and colloidal charge to rubber particles (Yip and Gomez, 1980; Claramma *et al.*, 1995; Ho *et al.*, 1996; Wtitsuwannakul and Wtitsuwannakul, 2001).

1.2.2 Lutoid particles

Lutoids are the most abundant non-rubber particles in *Hevea* latex. These are vacuoles having spherical membrane-bounded bodies with a diameter of 2 to 5 μm (Southorn and Yip, 1968; Dickenson, 1969). Lutoids are lysosomal in nature, which contains acid hydrolases. They swell and burst in a hypotonic solution (Ruinen, 1950; Jacob and Sontag, 1974). The membrane surrounding the lutoids is very osmosensitive (Pujarniscle, 1968; 1969). It has a strong negative charge because of the presence of equal

proportions of saturated and unsaturated fatty acids (Jacob *et al.*, 1993; Nair, 2000). The lutoid (B- serum) contains a wide range of metabolites, proteins and hydrolytic enzymes which is capable of bringing about flocculation, creaming, and coagulation of rubber particles (Southorn, 1969). The major protein in B-serum is hevein which accounts for about 70% of the water soluble proteins in the bottom fraction (Archer *et al.*, 1969; Soedjanaatmadja *et al.*, 1995). Lutoids are directly involved in cellular homeostasis of lactiferous system and play a major role in latex coagulation.

1.2.3 Frey-Wyssling complexes

The Frey-Wyssling complexes are spherical, 4 - 6 μm in diameter and bound with a double membrane (Dickenson, 1964; 1969). It is a composite organelle containing small particles of lipids and carotenoids. The yellow color is due to the accumulation of lipid globules, isoprenic compounds, plastochromanols, plastoquinones and carotenoids (Jacob *et al.*, 1993; d'Auzac *et al.*, 1997). It is usually larger in size than rubber particles (Webster and Paardekooper, 1989). The highly complicated structure of the Frey-Wyssling complex suggests that it has an important function in the metabolism of *Hevea* latex. The role of Fray-Wyssling particles suggested being the supply of isopentanyl pyrophosphate supply (IPP) through tubular thread reticulum (Yoonram *et al.*, 2008).

1.3 Natural rubber serum (NRS)

The natural rubber serum (NRS) is the aqueous portion of NR latex. After removing the rubber, the NRS is usually discarded. NRS contain a number of non rubber particles. Among the components of NRS, nitrogenous materials constitute a major fraction followed by quebrachitol (Fig.1.2).

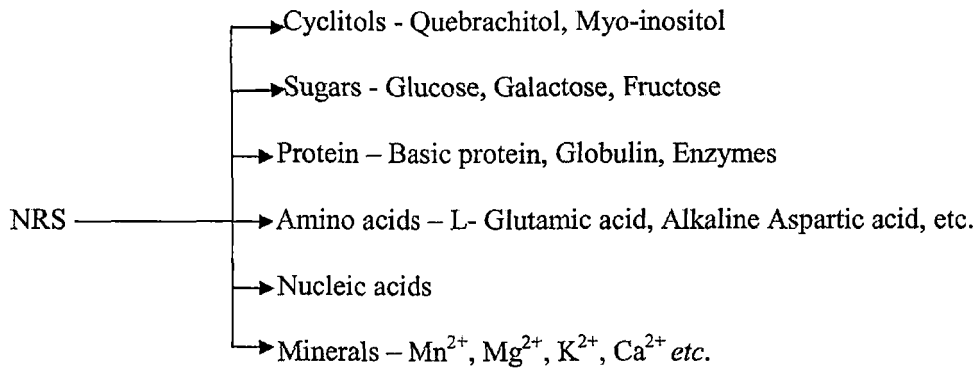


Fig. 1.2 Components of natural rubber serum

1.4 Inositols in natural rubber latex

Major soluble carbohydrates in latex are cyclitols (inositols), sucrose and glucose (Low, 1978). Cyclitol concentration is higher than sugars in the latex (Bealing, 1969; 1981). Carbohydrate level of the bark was affected by tappings. Different harvesting system *i.e.* intensive tapping system and repeated stimulation have a negative impact on latex cyclitols and found a low level of total cyclitols in latex (Low and Gomez, 1982). Sucrose concentration and total cyclitol concentration of latex were found altering

with tapping intensity (Bealing and Chua, 1972). The most abundant polyol in *Hevea brasiliensis* (rubber tree) latex is quebrachitol (mono-methyl L-inositol). The quebrachitol content in latex is reported to be 1-3% (Bealing, 1981; Gopalakrishnan *et al.*, 2008; 2011).

1.5 Quebrachitol

Quebrachitol is an interesting inositol with a substituted methyl group. It is considered as a major factor determining the turgor of the latex vessels and there by influencing the latex yield. Its synthesis in *Hevea* is through methylation of myo-inositol. Quebrachitol synthesis involves two pathways; i) through L-chiro inositol and ii) through D-bornesitol pathway (Scholda *et al.*, 1964; Bealing, 1981). The L-chiro inositol pathway of synthesis is reported in *Hevea* latex (Bealing, 1981). The Fig.1.3 depicts the schematic representation of L-quebrachitol synthesis in *Hevea* latex.

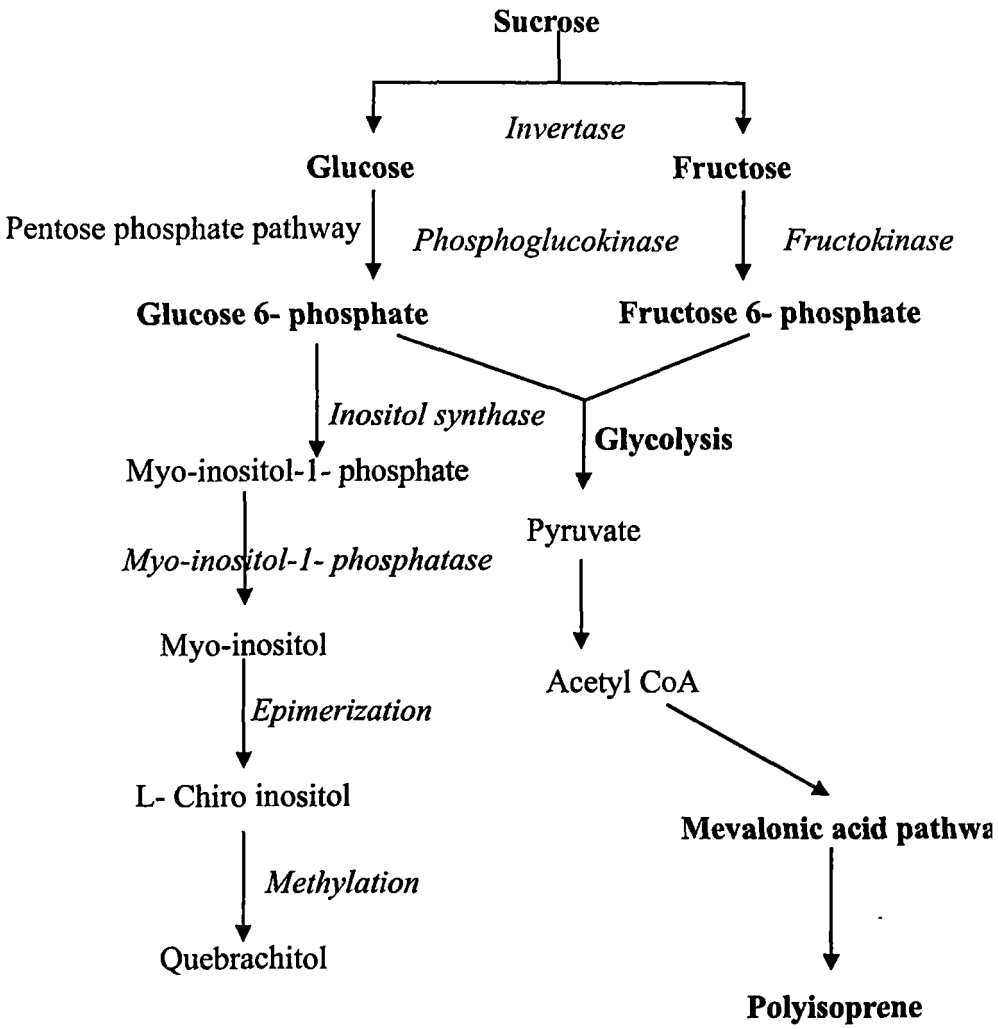


Fig.1. 3 Schematic representation of L-quebrachitol biosynthetic pathway in *Hevea* latex

Quebrachitol is the most abundant inositol present in rubber latex with smaller amounts of other components like L- and myo-inositol (Bealing, 1969; Anderson, 1972; d'Auzac *et al.*, 1989). Quebrachitol has a sugar like chemical structure as shown in Fig.1.4.

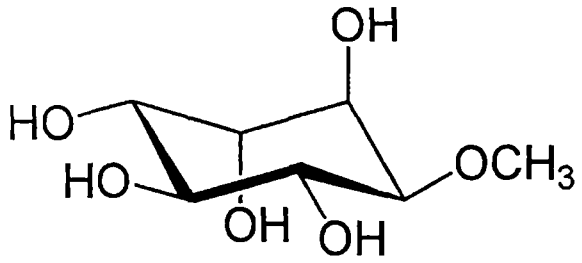


Fig. 1.4 Chemical structure of L- quebrachitol

It is optically active and can be easily converted into biologically important inositol derivatives *e.g.* inositol phosphates and fluorinated esters of inositol (Kozikowki *et al.*, 1989). It is a high value compound with several commercial applications. Special inositol derivatives of potential application such as anti cancer drugs, antibiotics and enzyme inhibitors can be synthesised from this compound (Lau, 1993; 1996; Yaojun, 1999). Cell signalling research involving various inositol derivatives is slow because only very small quantity of these compounds are available from natural sources and are extremely difficult and expensive to extract them in their original form. The chemical synthetic routes to obtain these compounds through myo-inositol as the starting material are long, tedious and require expensive chemical resolution procedures. Researchers have found that quebrachitol as the starting material instead of myo-inositol was successful using simpler and cheaper procedures (Kozikowki *et al.*, 1989; 1993; Kiddle, *et al.*, 1995). Considering the production cost of drugs, chiral drugs are expected to dominate the pharmaceutical market in the future. In this

context, quebrachitol is one such compound in the chiral pool that is ecofreindly and available from the natural rubber industry.

1.6 Objective of the Study

Hevea latex contains 30-40% rubber, which is being used for natural rubber production and the remaining non-rubber components, is discarded as waste. The non-rubber components include water, carbohydrates, proteins, inorganic acids, lipids, *etc.* Among the non-rubber components, inositols form a rich group of carbohydrates, especially quebrachitol. It can be easily harvested through tapping from the tree. Inositols and their phosphate derivatives are of commercial importance. There is a demand for inositols including those which have optical properties *e.g.* L-quebrachitol. Because of its chirality, it can be used as a starting material for other valuable chemicals like antibiotics, rare sugars, fungicides, pesticides *etc.*

Quebrachitol is a substituted inositol with optical activity. This optical property enables the conversion of this into various inositol derivatives of biological importance. Inositol and its derivatives have potential applications in the areas of pharmaceutical and medical research. By suitable chemical modifications, several inositols of potential commercial applications can be synthesized, including various inositol phosphates and fluorinated isomers of inositol. It has application as a chemical feed stock for pharmaceutical and medical products, chiral building block for organic

chemical synthesis and as tool for pharmacological research to study the cell signalling mechanism and cellular responses.

Myo-inositol is an important membrane constituent, a reserve substance, and a cofactor in galactose metabolism and is a starting material for the synthesis of other cyclitols. It acts as an intermediate in carbohydrate metabolism and as precursor of certain structural carbohydrates. Inositol 1, 4, 5 triphsophate and inositol 1, 4 diphosphate are involved in signal transduction and functions as second messengers in cell signalling mechanisms. These inositol derivatives have been synthesized from myo-inositol (Sureshan *et al.,* 2008). But it is a long and cumbersome process. An alternative method is the use of quebrachitol as the starting material because of its optical properties. By suitable chemical modifications products from it can be used as anticancer drug, antibiotics, antifungal, or as enzyme inhibitors.

In adverse environmental conditions plants accumulates solutes/ osmolytes during natural and induced hardening. These compounds are called compatible solutes *e.g.* sugars, polyols, amino acids *etc.* Accumulation of these solutes can be an adaptive mechanism to environmental stresses like water deficit, salinity and extreme temperatures. In general, the function of these solutes is not only to maintain turgor in dehydrating cells, but also to protect macromolecules. Sugar alcohols/ cyclitols have a role in scavenging of toxic oxygen species. They prevent peroxidation of lipids and cell

damage. They maintain the cell turgor through osmotic adjustment, thereby provides osmoprotection. Even though sucrose is the predominant sugar in *Hevea* latex, the total cyclitol (inositol) concentration is higher than sugars. *Hevea* latex contains about 1 % quebrachitol (methylated inositol).

L-quebrachitol has been isolated from plants like *Allophylus edulis, Cannabis sativa, Paullina pinnata, Acalypha indica,* Sea-buckthorn, natural rubber serum (aqueous phase left after removing rubber) and recently from *Artimesia sodiroi.* Leaves, stem, root, fruits, flowers and aerial parts of these plants are being used for isolation of L-quebrachitol (Diaz *et al.*, 2008). In this context, the advantage of *Hevea* as a source of quebrachitol is that there is no need to harvest the plant or plant parts for the isolation of L-quebrachitol. By the usual practice of tapping the tree for latex, it can be readily harvested along with rubber. Even though different methods were attempted for the extraction of L-quebrachitol from NRS (Lau, 1993; Yaojun, 1999), a precise method and suitable serum source is very much essential for the isolation of this compound.

The rubber tree is a prominent plantation crop of considerable significance to Indian economy. In India, at the end of 2013-2014, 7.76 lakh hectares of area is under rubber cultivation and production is 8.44 lakh tons. Small holdings comprise around 1.2 million units and dominate the production sector. 95 % of total small holdings sector is occupied by high

yielding clones. As the latex of high yielding rubber clones is a rich source of inositols (Bealing, 1969; 1981; Gopalakrishnan *et al.*, 2008), the raw material is available in abundant quantities for the isolation of such high value compounds from the latex of *Hevea*. The potential applications of inositols particularly L-quebrachitol to drug industry, pharmacological and medical research field, relatively high abundance in *Hevea* latex in optically active form, involvement in water relation of latex and thereby latex flow and yield prompted to carryout the present work with the following objectives.

Objectives:

1. Isolation and characterization of L-quebrachitol from *Hevea* latex.
2. Quantification of inositol content of *Hevea* latex from different clones and analysis of seasonal variation.
3. Studies on water relations of *Hevea* latex (osmotic potential of latex) in relation to the contents of inositols and other solutes (osmoprotectants).

Chapter 2

Review of Literature

2.1 The Rubber Tree

Natural rubber tree (*Hevea brasiliensis* Muell. Arg.) is a perennial tropical tree indigenous to the tropical rainforests of the Great Amazon Basin of South America. *Hevea brasiliensis* is one of the most recently domesticated crop species in the world. It belongs to the Euphorbiaceae family and is the most commercially important member of the genus *Hevea.* It is of major economic importance because of the milky white latex extracted from the tree, which the primary source of natural rubber (Dall' Antonia *et al.*, 2006: Obianga *et al.*, 2009). The genus *Hevea* has 10 species, which are inter-crossable (Clement-Demange *et al.,* 2000). Only three species of the genus yield usable rubber, *Hevea brasiliensis*, *H. guianensis* and *H. benthamiana.* Other species have high ratio of resin to rubber in their latex. *Hevea brasiliensis* is the only commercially cultivated species and it gives the best natural rubber (Clément-Demange *et al.*, 2000; de Faÿ *et al.*, 2010: Venkatachalam *et al.*, 2013). It is now cultivated on large scale plantation in Southeast Asia and Africa.

Natural habitat of rubber is situated within 5° latitudes at altitudes below 200 m. The climate of this region is equatorial monsoon type characterized by mean monthly temperature of 25-28°C and with abundant rainfall of more than 2000 mm per year without any real dry season. The trees evolved in this environment have developed a preference for warm,

humid weather. The regions closely resembling the climates of original habitat are best for commercial cultivation of rubber tree (Rao and Vijayakumar, 1992). The climatic conditions essential for optimum growth of rubber trees are rainfall of 2000 mm or more which is evenly distributed without any marked dry season and with 125-150 rainy days per annum, maximum temperature of about 29-34°C and minimum of about 20°C or more with a monthly mean of 25-28°C, increased atmospheric humidity of about 80 percent with moderate wind and bright sunshine amounting to about 2000 h/annum at the mean rate of 6 h per day throughout all the months (Webster and Paardekooper, 1989). Although not ideally suited, rubber is successfully cultivated even up to latitude of 25°, well beyond the traditional latitude, in countries such as India and China. Rubber is planted at a typical density of 450-500 trees per hectare. Depending up on climate, soil conditions and management practices, the initial growth phase of rubber generally varies from 5-7 years.

2.2 Physiology of *Hevea* latex

Natural rubber is a constituent of latex, a milky white substance produced in the cytoplasm of laticiferous cells. Latex is synthesized in specialized tissue *i.e.* laticiferous tissue, which is present in bark, leaves and other parts of the plant (Li *et al.*, 2010). Laticifers or latex vessels are the ducts in the phloem of the rubber tree (bark). Laticifer cells are

arranged as concentric rings around cambium and forms a ubiquitous network of tubes in rubber tree (Gomez and Moir, 1979; Hao and Wu, 2000; Kongsawadworakul and Chrestin, 2003; Chow *et al.*, 2007). Laticifer characters such as number of rows, density of vessels per row, diameter of latex vessels and intensity of anastomosis are significant clonal characters (Premakumari and Saraswathyamma, 2000). These parameters along with some physiological and biochemical factors influences volume of latex produced by a plant (Nair, 2000).

Prior to tapping, the latex is at a high hydrostatic pressure in the vessels. When the latex vessels are severed during tapping, due to the turgor pressure, latex exudes out and continuous latex flow occurs by water entering from surrounding cells into laticiferous cells (Adiwilga and Kush, 1996; Thomas *et al.*, 1999; Hao and Wu, 2000). The severing of the latex vessels with the consequent loss of turgor disturbs the original osmotic equilibrium throughout the outflow area which creates a suction pressure resulting in the influx of water from the neighbouring cells into the latex vessels (Gomez, 1983). Latex flow is an energy involved process. Sufficient energy is provided by active metabolic processes for the flow and enhanced latex production. Strong positive relationship between yield and ATP, lutoid membrane ATPase and C serum (aqueous phase of latex) pH had been reported (Amalou *et al.*, 1992; Sreelatha *et al.*, 2004).

Natural rubber latex is a specialized form of cytoplasm. It contains 30-40% rubber (*cis*- 1, 4-polyisoprene) and has a specific gravity of 0.96 to 0.98 and a pH range of 6.5 to 7.0. Polyisoprene with an average molecular weight as high as $1x10^6$ Da occur in *Hevea* latex. Latex contains all the subcellular organelles of non-photosynthetic cells such as vacuoles, plastids, mitochondria, nuclei, endoplasmic reticulum and polysomes (d' Auzac and Jacob 1989; de Fay *et al.*, 1989). Latex properties are influenced by environmental and soil factors (Ebi and Kolawole, 1992). The total solid composition of latex denotes the rubber and non-rubber content of the latex. Based on various physiological and physical parameters, the composition and concentration of latex organic non-rubber components may vary (Gelling and Porter, 1988; Pakianathan *et al.*, 1992). It was reported that, compared to traditional regions the ash content of latex was high in non-traditional region (George *et al.*, 2006).

Sucrose is the primary precursor and functions as fundamental building block of natural rubber (Chow *et al.*, 2007; Sando *et al.*, 2008). Latex production and sucrose shows both positive and negative association. A higher level of sucrose indicates a better loading to the laticifers and also less metabolic utilisation. The high level of sucrose content in the latex denotes an increased sugar supply (Tupy and Primot, 1976) or low metabolic efficiency of the laticiferous tissue (Prevot *et al.*, 1984). Higher sink demand

and metabolic activity in tapped trees compared to untapped tress was denoted by increased sugar concentration (Annamalainathan *et al.*, 2008). Sugar uptake is an active process across the plasma membrane of laticifers (Tupy, 1973; Eschbach *et al.*, 1986; Silpi *et al.*, 2007). The metabolic activities of laticifers were sufficient to regenerate and compensate the loss of cytoplasm upon each tapping. Gohet *et al.,* (2005) reported that for sustainable latex regeneration, the laticiferous tissue require sufficient sucrose loading.

Sugar import into the laticifer has been an important limiting factor of latex production. Sugar transporters (SUTs) facilitate the movement of sucrose across the plasma membrane of the cell. Within the laticifers, sucrose move across the plasma membrane through specific transporters before being metabolised (Bouteau *et al.*, 1999; Dusotoit-Coucaud *et al.*, 2009). Seven sucrose transporters (Dusotoit-Coucaud *et al.*, 2009), one putative hexose transporter and one polyol transporter were identified in *Hevea* latex. SUT gene expression varies in latex and inner bark tissues. HbSUT1A and HbSUT1B were predominant forms of SUTs. Under various physiological conditions, the expressions of SUTs and hexose transporters have been enhanced in laticifers. Most abundant isoform of SUTs having higher induction by ethylene were HbSUT1A and HbSUT1B. They have been highly expressed in laticifers and inner bark tissues and positively

correlated with latex production (Dusotoit-Coucaud *et al.*, 2010). Laticifers are symplastically isolated from other adjacent cells of soft bark tissue suggesting its specificity to membrane transporters. Sugar transport into laticifers is complex as evidenced by the large number of sugar transporters compared to polyol and hexose transporters (Dusotoit-Coucaud *et al.*, 2009; 2010). Based on the laticifer functioning and physiological parameters, clonal characterisation of *Hevea* has been performed (Gohet *et al.*, 2003; Nair, 2003). Metabolic characterisation (Nair *et al.*, 2001) and mechanisms related to summer yield drop (Sreelatha, 2003) was reported for the clone RRII 105.

On commercial basis, latex production is enhanced through hormonal stimulation. Thomas *et al.,* (1999) reported that the yield increment by ethephon treatment was due to the loss of semipermeability of lutoid membrane leading to the loss of osmotic gradient and avoidance of continued water flux into the lutoid particles. Ethylene stimulation destabilized the lutoid content (B-serum) through the increment of buffer value of C-serum and lead to latex stability (Sreelatha, 2003). Yield enhancement was associated with the concomitant increase in C-serum pH. Significant changes with respect to total solid content, free acid phosphatases activity, inorganic phosphorus, sucrose and protein content of B and C-serum by stimulation have been observed (Sreelatha, 2003). Stimulation resulted in

latex production concomitant with reduction in total solid content mainly through water flux in laticifers. Zhu *et al.,* (2009) demonstrated that ethylene influences rubber biosynthesis through prolonged latex flow and accelerated sucrose metabolism. *Hevea* species contains cyanogenic glucosides in bark tissues. Kangsawadworakul *et al.,* (2009) established that cyanogenic glucosides of bark tissues were associated with latex production as a source buffering nitrogen and glucose.

2.3 Latex /Rubber yield

Latex harvesting is performed through out the year. Rubber yield is determined by the total volume of latex obtained through tapping. Genetical, environmental and the interactions of a large number of major and minor components of latex governs yield (Jayasekara *et al.,* 1997). Rubber yield is influenced by climatic factors. In India, the period of peak yielding is from September - January (non-stress period) and dry period is from February - April (stress period). The latex yield is generally reduced at low soil moisture levels prevalent in summer months. The soil moisture remarkably altered the pattern of latex flow. The duration of flow as well as the amount of latex was reduced during water stress conditions (Sethuraj *et al.,* 1984). According to Sethuraj and George (1976), the drop in latex yield under soil moisture stress was due to fast rate of plugging and restricted drainage area.

Annual yield depends upon the average values of initial flow rate, plugging index and rubber content through different seasons of a year. Rubber yield is directly proportional to the dry rubber content (DRC) and total volume of latex (Nair *et al.*, 1993). Rubber yield is reported as a highly heritable character (Licy *et al.*, 1993a; 1993b; Mydin and Mercykutty, 2007). Clones of *Hevea* vary in their sensitivity to water stress (Saraswathyamma and Sethuraj, 1975). Physiological characteristics of latex yield are prone to environmental alterations. The two main determinants of yield are the initial flow rate and the latex vessel plugging during latex flow (Yeang and Paranjothy, 1982). The extent of damage to lutoids in the latex is an important factor determining seasonal latex vessel plugging. In low yielders latex yield was limited through impairment of latex flow by the activity of small rubber particles in the latex (Ruderman *et al.*, 2012). C-serum contains lectin like proteins (HLL) binding to lectin-binding protein (CS-HLLBP) with anticoagulant properties. A strong correlation was reported between rubber yield per tapping and CS-HLLBP (Wititsuwannakul *et al.*, 2008a). *Hevea* lectin binding protein bound to the rubber particles (RP-HLLBP) act as ligand for HLL like proteins and cause aggregation of rubber particles. CS-HLLBP competes for HLL and prevents aggregation of rubber particles and maintains the colloidal stability of latex. Accordingly HLLB protein of C-serum functions as anticoagulant factor and have major role in latex coagulation (Wititsuwannakul *et al.*, 2008b). Water status of the bark tissue

is reflected in the panel turgor. Initial flow rate of latex was influenced by panel turgor which in turn influenced by the moisture status of the tree and could be reduced by water stress (Yeang and Paranjothy, 1982).

In the regulation of the final yield output from the tree on a seasonal basis, the duration of flow is relatively more important than the severity of the initial decrease in flow rate. Prolonged flow of latex depends partly on the influx of water from the surrounding cells. Aquaporins are integral membrane proteins that facilitate the movement of water in and out of cells. They have significant role in water uptake and movement (Zhao *et al.*, 2008). As the mature latex vessels are devoid of plasmodesmata (de Faÿ *et al.*, 1989) these intrinsic membrane proteins (water channel or aquaporins) were found associated with water flux into laticifers. Stimulation enhances latex yield through the activation of aquaporins. Expression of aquaporins (HbPIP2;1 and HbTIP1;1) envisaged the increased latex yield brought by stimulation through water flux between laticifers and their neighbouring cells and maintenance of soft bark turgor pressure (Tungngoen *et al.*, 2009). Hormonal (ethylene, abscisic acid and salicylic acid) induction of HbPIP2;1 and HbTIP1;1 expression confirmed their role in increased latex production (Tungngoen *et al.*, 2011). Any conditions contributing to good supply of water to tissue or reduction in the loss of water by evapo-transpiration are favorable for latex flow and high production. It was emphasized that super

productivity of rubber plants has been imparted by improved sucrose loading capacity and utilization for rubber synthesis, enhancement in the general metabolism and timely stress evasion competence (Tang *et al.*, 2010).

2.4 Water relations in *Hevea*

Rubber biosynthesis and latex flow characters upon tapping are closely associated with soil moisture conditions (Devakumar *et al.,* 1988; Jiang, 1988; Raj *et al.*, 2005). There is a typical seasonal change in rubber yielding pattern in *Hevea* clones. The physiological mechanisms responsible for latex exudation when the tree is tapped and for the subsequent decline in the rate of latex flow and its final stoppage have been related to water status of the tree trunk (Southorn, 1969; Sethuraj and George 1976; Yeang and Paranjothy, 1982; Gomez, 1983; Sethuraj *et al.*, 1984; d'Auzac and Jacob, 1989; Hao and Wu, 2000). At tapping, the high turgor pressure expels latex from the cut vessels that experience an elastic collapse near the cut ends. Subsequent to the loss in turgor after tapping, latex flow is retarded and ceases eventually by the mechanism of latex vessel plugging (An *et al.*, 2014a). The plugging of latex vessels involves many physiological and biochemical reactions (Wititsuwannakul *et al.*, 2008a; 2008b; Priyadarshan, 2011; Ruderman *et al.*, 2012).

Water deficit adversely influence growth and metabolism of many crop species and the responses depend on severity and duration of the stress,

plant genotype, development stage and environmental factors (Bray, 1993). The plant water status of rubber trees in dry season was associated with climatic parameters and soil drought. Latex flow was favoured by high soil moisture and low vapour pressure deficit (Rao *et al.*, 1990; 1998; Devakumar *et al.*, 1998). Yield reduction was resulted by latex vessel plugging through variation of soil moisture content (Dey *et al.*, 1999). As sap flux density and stomatal conductance were reduced in dry season during tapping (Kunjet *et al.*, 2013), care is very essential in drought prone areas. Under water limited conditions there was reduction in transpiration (Sinclair, 2005; Breda *et al.*, 2006). Intermittent drought during rainy season resulted in reduction of whole-tree transpiration (E_T). There was no change in the midday leaf water potential. It was suggested that the whole-tree hydraulic conductance (K_T) was responsible for the reduction of E_T. Sharp decrease in K_T takes place under water limiting situation and remained constant for a wide range of environmental conditions. A hydraulic model incorporating all critical parameters of water relations of rubber plants demonstrate the involvement of hydraulic conductance limitation in water use regulations of mature rubber trees under water deficient conditions (Isarangkool *et al.*, 2010; 2011). E_T was directly as well as indirectly influenced by leaf water potential, one of the major components of water relations. Leaf water potential directly regulates E_T through leaf turgor pressure (Cochard *et al.*, 2002) or by the interaction of stomatal sensitivity and abscisic acid (Tardieu

and Simonneau, 1998) where as indirectly through the relationship with xylem water potential and xylem cavitation it influences E_T (Sperry *et al.*, 1998; Cochard *et al.*, 2002).

The conservative stomatal behaviour of *Hevea* clone, RRIM 600 indicated the transpirational response to high evaporative demand leading to short term reduction in growth, productivity and long term improvement in survival (Silpi *et al.* 2006; Isarangkool *et al.*, 2011). Based on the changes in the xylem sap flow rate there was diurnal variation in *Hevea* phloem turgor pressure. It was decreased immediately by tapping and gradually recovered through water flow from the adjacent tissues and laticifer plugging (An *et al.*, 2014a; 2014b). Under water stress xylem vessels of rubber tree are vulnerable to cavitation and can be limited by stomatal closure. Clonal variation was observed for xylem cavitation in *Hevea* (Carr, 2012). All the variations in the water potential might be attributed to the variation in osmotic potential, the osmotic component of water potential (Siddque *et al.*, 2008). A clone with high osmoregulation will maintain turgor pressure at low water potential. Phloem turgor pressure is considered as an indicator of phloem development and latex yield potential in rubber trees (An *et al.*, 2014b). Total sugar and potassium were the major contributors for osmoregulation (Karyudi, 2004). Potassium is one of the major osmotic factor contributing changes in osmotic potential under drought stress in

Hevea. Significant clonal, seasonal and clone x season effect was observed with respect to its relation to the osmoticum (Gopalakrishnan *et al.*, 2010). Rubber clone vary in the extent of osmoregulation suggesting the differences in adaptation to water stress (Karyudi, 2004; Gopalakrishnan *et al.*, 2010). Osmotic pressure or turgor pressure of laticifers and surroundings cells was maintained by sugars, polyols and potassium (Buttery and Boatman, 1966). It was found that at whole plant level, loading of osmolytes, aquaporin expressions and leaf stomatal control of transpiration contribute to the daily growth maintenance and dehydration avoidance (Junjittakarn *et al.*, 2012).

Flow of latex from the tree is influenced by latex water relations (Buttery and Boatman, 1966). Devakumar *et al.*, (1988) found that summer yield drops were low in *Hevea* clones RRII 105 and GL 1. High latex vessel turgor and low solute potentials in RRII 105 in the dry season indicated the presence of osmotic adjustment. Higher plant water status and lower transpiration in this clone might have helped in better turgor. Generally latex production is reduced under low soil moisture level, *i.e.* during summer months. Soil moisture altered the pattern of flow during water stress which resulted in reduced flow duration and the amount of latex (Sethuraj and Raghavendra, 1987). Satheesan *et al.*, (1982) reported that drought tolerant clones maintained high solute potential in their C-serum *i.e.* the cytoplasmic serum (to keep the lutoids intact) even in summer months. It has been

reported that mineral cations like calcium and magnesium do not seem to contribute to the seasonal trend in latex vessel plugging (Yeang and Paranjothy, 1982). Latex yield during summer or rainy periods was influenced by osmotic potential of B (serum of lutoid particles) or C-serum. During rainy period the osmotic concentration of C-serum is less in drought tolerant than drought susceptible clones and during summer season drought tolerant clones had higher osmotic concentration in their C-serum than susceptible clones. The higher C-serum osmotic concentration prolongs flow of water into the latex and thereby facilitates continuous latex flow (Satheesan *et al.*, 1982). Soil drought and tapping resulted in a reduction of plant water status and sap flux density during dry season in *Hevea* plants (Kunjet, *et al.,* 2013).

2.5 Plants under adverse environments

Plant growth performance is affected by abiotic stresses, *i.e.* drought, low temperature and salinity. Environmental stresses that limit plant growth and development play a role in determining the geographic distribution of plant species. Environmental stress can disrupt cellular structures and impair key physiological functions (Larcher, 2003). Optimum and harsh environmental conditions varies with plant species, *i.e.* a condition harmful for one plant species might not be stressful to another (Larcher, 2003; Munns and Tester, 2008). Thereby different stress response mechanisms exist in

plants. Responses to stresses occur at all levels of organisation. Modifications of cell wall architecture, changes in cell cycle and division, adjustment of the membrane system are the common cellular responses to stress. Metabolic alterations in combination with compatible solute synthesis to stabilize protein, cellular structures, maintain turgor by osmotic adjustment, redox metabolism to remove excess reactive oxygen species and re-establishment of cellular redox balance are some of the mechanisms related to water stress in plants (Bartles and Sunkar, 2005; Valliyodan and Nguyen, 2006; Munns and Tester, 2008; Janska *et al.*, 2010).

Among the abiotic stresses, drought is the most severe factor limiting plant growth and production. Plants respond and adapt in order to survive during stress conditions. Water deficit induces various biochemical and physiological responses in *Hevea* (Gopalakrishnan *et al.*, 2011; Sumesh *et al.*, 2011). Plants growing in extreme conditions are adapted to those severe conditions with changes in their growth and development. Gene expression is modified under stress condition (Chinnuswamy *et al.*, 2007; Shinozaki and Shinozaki, 2007: Thomas *et al.*, 2011; 2012). In response to environmental stress the epigenetic regulation plays a vital role in gene expression (Hauser *et al.*, 2011; Khraiwesh, *et al.,* 2012). Genes induced by stress includes genes that provide direct protection, synthesis of osmoprotectants, detoxifying

enzymes, transporters and regulatory proteins such as transcription factors, protein kinases and phosphatases.

Water stress directly affects cellular processes, plant growth, development and finally yield. There are some traits that are responsible for tolerance of plants to drought. Osmotic adjustment (OA) is one such physiological trait which imparts tolerance to stress condition through osmolyte accumulation (Chen and Jiang, 2010). It is an important plant tolerance mechanism to progressive drought which allows water maintenance and there by cell turgor. It can be defined as the active accumulation of solutes within the plant tissue in response to lowering of soil water potential. During osmotic adjustment there is a net increase in solute concentration which is an important survival mechanism under drought stress (Turner, 1997; Farouk and Abdul Qados, 2013) and contributed to yield stability in dry environments (Blum, 1997; Babita *et al.*, 2010). As water is removed from the plant, its osmotic potential is reduced due to the simple effect of solute concentration. But during cellular water loss, solutes are actively accumulated and results in reduction of osmotic potential. Under drought stress in *Eucalyptus* the osmotic potential decreased in response to stress (White *et al.*, 2000). This was accomplished through active solute accumulation or constitutive increase of solute through cellular water reduction (Chaves *et al.*, 2003). Osmotic adaptation in *Eucalyptus* plants was

reported to be associated with quercitol and sucrose concentration (Merchant *et al.*, 2006a; 2006b). OA has no adverse effect on water use efficiency and contributes to the grain yield by increasing the water use from the sub-soil during the reproductive phase in several crop plants (Singh *et al.*, 1990; Ludlow and Muchow, 1990).

2.6 Osmoprotectants

In nature, plants are subjected to all type of biotic and abiotic stresses. One of the mechanisms of responding to stress like salinity, drought, low temperature *etc.* is by accumulation of intracellular solutes. Plants accumulate a variety of osmoprotective solutes under unfavourable environments (Bartles and Nelson, 1994; Ingram and Bartles, 1996; Verslues and Sharp, 1999; Zinselmeier *et al.*, 1999; Quan *et al.*, 2004; Chen and Murata, 2011) as an adaptive mechanism. Osmolyte accumulation is a common mechanism found in all organisms during osmotic stress (Dhir *et al.*, 2012; Padmavathi and Rao, 2013; Ajithkumar and Paneerselvam, 2013). Advantages of osmolytes are their compatibility to micro molecular structures and functions at high or variable osmotic concentrations, little effect on functions of protein. Osmolytes protect cells by maintaining membrane integrity, preventing protein denaturation and protective effect against oxidative damage by scavenging free radicals. Generally osmolytes function to stabilize protein, protein complexes and membrane during

environmental stresses (Hare *et al.*, 1998; Diamant *et al.*, 2001; Jaindl and Popp, 2006; Singh *et al.*, 2011).

Plant regulates ionic concentration and accumulates a variety of solutes such as organic acids, amino acids, carbohydrates, quaternary ammonium compounds and cyclitols (Hasegawa *et al.*, 2000). These solutes do not interact with cellular enzymes. They protect cell membranes and metabolic processes under stress (Popp and Smirnoff, 1995; Jaindl and Popp, 2006). Accumulation of non-structural carbohydrates like sucrose, hexoses and polyols under stress among many plant species was widely reported. During stress periods there was a strong correlation between carbohydrate accumulation and stress tolerance (Bartels and Sunkar, 2005).

Cyclitols are considered as compatible solutes and are accumulated during stress (Morgans, 1984; Nguyen and Lamont, 1988; Evans *et al.*, 1992; Bohnert *et al.*, 1995; Wanek and Richter, 1997; Mundree *et al.*, 2000; Streeter, 2001b; Merchant *et al.*, 2006a; 2006b; 2007a). Pinitol, quebrachitol and quercitol can accumulate in relatively large amounts in response to drought (Nguyen and Lamont, 1988; Popp *et al* 1997; Merchant *et al.*, 2006a) and salinity (Richter *et al.*, 1990) and revealed the role of cyclitols in stress tolerance. During abiotic stress such as drought, low temperature and salinity plants accumulate many kinds of inositol derivative metabolites where as animals accumulate only myo-inositol. Cyclitols act as

cryoprotectants (Drew, 1984; Loewus, 1990; Loewus and Murthy, 2000). It has been reported that compounds such as pinitol, quebrachitol, quercitol, *O*-methyl muco-inositol accumulate at low temperatures (Diamantoglon, 1974; Ericsson, 1979; Popp *et al.*, 1997). At the onset of cold season a number of tree species induces enhanced storage of cyclitols in bark tissue and buds (Popp and Smirnoff, 1995; Popp *et al.*, 1997). Their opinion is that the presence of these cryoprotectants diminished the mechanical stress by decreasing the osmotic potential and there by reduced the freeze induced shrinkage. Studies using *Eucalyptus* species have shown an increase in the concentration of quercitol under salinity and drought (Adams *et al.*, 2005; Merchant and Adams, 2005). *Eucalyptus* plants growing under rainfed area produced significantly higher levels of quercitol in leaves than the mesic forms indicated the species distribution based on water availability (Merchant and Adams, 2005). Sugar alcohols are acyclic polyols and the simplest form is glycerol. Sugar alcohol that has been studied in detail at physiological, biochemical and molecular levels is mannitol and is widely distributed among plant families.

Chemically osmoprotetcants fall into three major groups *viz.*, amino acids (*e.g.* proline), quaternary ammonium compounds (*e.g.* glycine betaine) and sugars and polyols (Sucrose, glucose, fructan, trehalose, mannitol, pinitol,

ononitol, quebrachitol, quercitol, *etc.*). Among these osmoprotectants, proline, glycine betaine and mannitol are commonly found in plants.

2.6.1 Sugars

Sugars have dual role as metabolic resource and constituents of cell wall. They have critical function in energy, carbon transport, signalling as hormones, source for building essential compounds such as amino acids, nucleic acids, proteins, *etc.* Their level was greatly affected by the plant genotype and environment (Halford *et al.*, 2010) and thereby regulates various processes associated with growth and development. Sugar molecules interact with sensor molecules (protein) and influences metabolic process. A number of molecular and genetic studies supported the concept of sugar signalling (Smeekens, 2000; Rolland *et al.*, 2002; Halford and Paul, 2003). There are reports that sugars regulated the production of secondary metabolites such as anthocyanin (Vitrac *et al.*, 2000), artemisinin, (Patrick *et al.*, 2010), *etc.* Strong correlations between soluble sugars and stress tolerance have been revealed through ecological and agronomic studies. The key component of stress tolerance mechanism is the source-sink partitioning between different organs (Ho, 1988; Krapp and Stitt, 1995). Sucrose and glucose act as respiratory substrates or as osmolytes for the maintaninence of cellular homeostasis (Gupta and Kaur, 2005). During seed development and maturation, soluble sugars are involved in desiccation tolerance (Obendrof,

1997; Hoekstra *et al.*, 2001). Studies on metabolic engineering of compatible solutes indicated that optimum to increased level of plant tolerance to abiotic stresses can be achieved through an elevated level of sugars or other osmolytes. Sugars accumulate in response to stress and function as osmoprotectants to maintain cell turgor and protect membranes and proteins from damage (Madden *et al.*, 1985; Murakeozy *et al.*, 2002; 2003; Kaplan and Guy, 2004). Carbohydrtaes coming under osmoprotectants mainly include sucrose, glucose, polyols (like *Myo*-inositol, mannitol, ononitol, pinitol, quebrachitol, quercitol) and complex sugars like trehalose and fructans (Yancey *et al.*, 1982; Popp *et al* 1997; Sheveleva *et al.*, 1997; Serraj and Sinclair, 2002; Bartels and Sunkar, 2005; Yancey, 2005; Merchant *et al.*, 2006a).

Hevea laticifers are strong sink for sucrose so as to meet the high demand of carbon and energy (Silpi *et al.* 2007). In *Hevea,* sucrose supply and metabolism were found to play major role in latex regeneration. Sucrose is involved in many cellular processes such as energy production, cellular components, rubber biosynthesis, cell differentiation, development, response to pathogen and environmental challenges (Koch, 2004).

2.6.2 Polyols

Polyols are important class of biologically active compounds (Takahashi *et al.*, 2001) and exists in two forms *i.e.* acyclic and cyclic forms.

The common acyclic forms found in plants are mannitol, glycerol and sorbitol and cyclic forms are ononitol and pinitol. Cyclic forms of polyols are also known as cyclitols. Polyols are compatible solutes that occur in reasonable amounts in a wide range of plant species (Popp *et al.,* 1997). The adverse effects of stress conditions across a range of stress types can be ameliorated by polyols. The polyol concentration was significantly higher in grape berry mesocarp under water stress and functions as physiological strategy for stress tolerance (Conde *et al.*, 2014). They function as sink for carbon. Changes in plant growth pattern in response to the altering environmental conditions reflect the alteration in allocation of carbon from carbohydrates to polyols.

2.6.2.1 Mannitol and sorbitol

These are straight chain forms of sugar alcohols involved in osmoregulation of plant tissue. Mannitol and sorbitol have functions similar to sucrose in translocation of assimilated carbon (Lewis, 1984; Loescher, 1987). Mannitol is formed from fructose 6-phospahte. Fructose-6-phosphate is first converted to mannitol-1-phosphate by the enzyme mannitol-1-phosphate dehydrogenase which is then converted into mannitol by a non specific phosphatase (Tarczynki *et al.*, 1992; 1993). *Arabidopsis* plants transformed with celery's mannose-6-phospahte gene produced mannitol and

found better adapted under salt stress compared to wild plants (Sickler *et al.*, 2007).

2.6.2.2 Inositols

Inositols constitute an important group of naturally occurring cyclitols. It is a six carbon compound which is essential for the development of plants, animals and other organisms. There are nine stereoisomers, myo-inositol, cis-inositol, allo-inositol, epi-inositol, muco-inositol, neo-inositol, scyllo-inositol and DL pair of chiro-inositol (Loewus *et al.*, 1987; Michell, 2008) (Fig. 2.1).

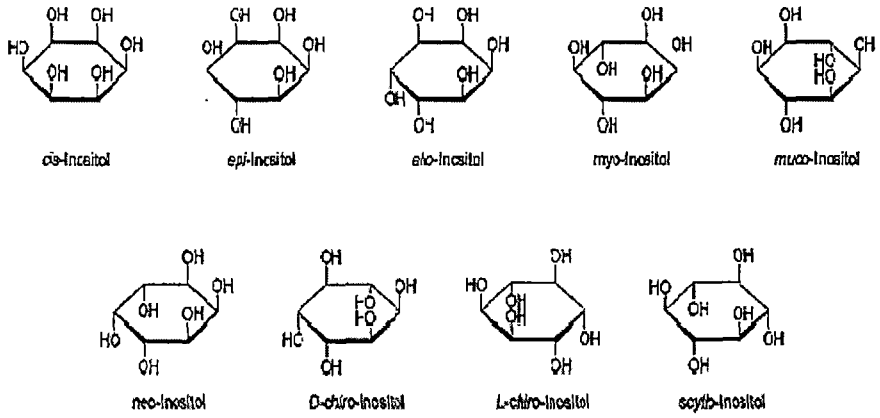


Fig. 2.1 Nine stereoisomers of inositols

The naturally occurring isomers of inositols are myo, chiro, scyllo, muco and neo-inositol with myo-inositol being the most abundant (Wang *et al.*, 1990) (Fig. 2.2). Several fruit extracts contain myo-inositol but not chiro-inositol or scyllo-inositol (Sanz *et al.*, 2004). They are soluble and are widely distributed throughout various tissues and organs.

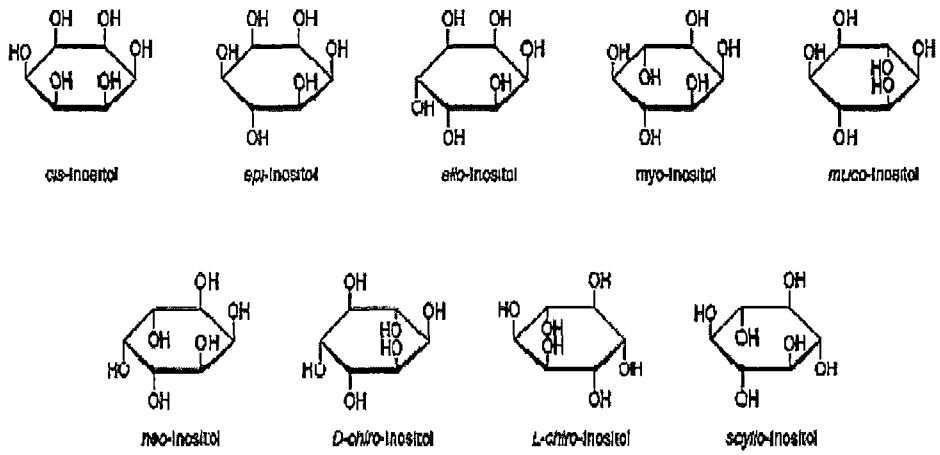


Fig.2.2 Naturally occurring isomers of inositols

As an essential component for growth and development, plants maintain inositol pool at basal level throughout their life cycle. Inositols and its derivatives have been involved in growth regulation, membrane biogenesis, hormone regulation, signal transduction, pathogen resistance and stress adaptation in higher plants (Stevenson *et al.*, 2000; Michell, 2007).

Free inositols occur in various types of plants. The most commonly present form is as phytic acid (hexakis phosphate). Phytic acid constitutes 80% of total phosphorus content in seeds and cereal grains. Phosphatidyl inositol is a normal constitute of plant phospholipids, which accounts 10-12% of the phospholipids in mitochondria and chloroplast.

2.6.2.2.1 Myo-inositol

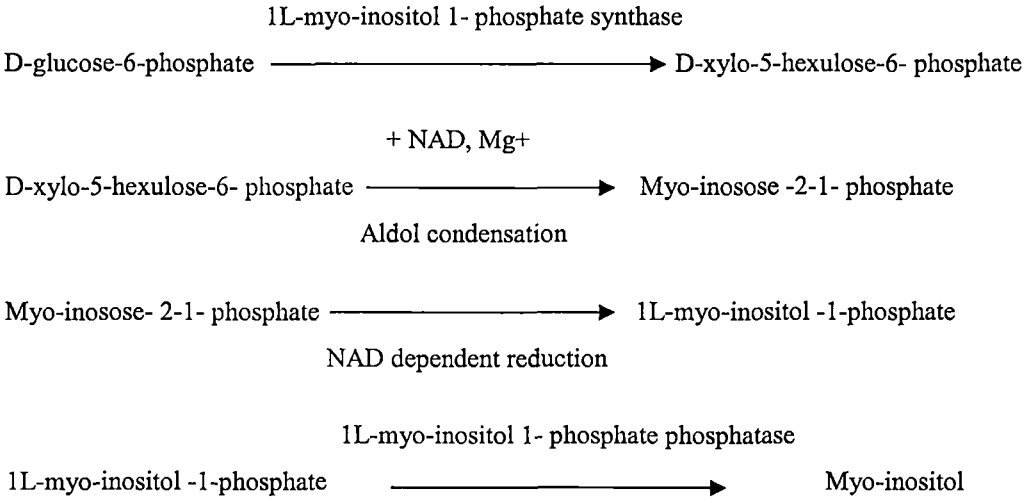
Myo-inositol is a naturally occurring form of inositol. It is essential for plant growth, seed storage, nitrogen fixation and protection during stresses.

Plants defense reaction involves cellular inositol and inositol triphosphate pathway. This pathway is involved in cell division, growth and elongation (Stevenson *et al.*, 2000; Klink *et al.*, 2009). Chiro-inositol is another naturally occurring form of inositol. It exists as two enantiomers, D and L-chiro inositol and are biologically active. Myo-inositol is a central component of several biochemical pathways and functions mainly in four ways. Firstly, as inositol phosphates which are essential for signalling in almost all organisms and in plants, inositol hexaphosphate provides as phosphate storage (Hübel and Beck, 1996). Secondly as the lipid components of membranes (Mathews and Van Holde, 1990) and thirdly as the basic substrate for the raffinose series of sugars in plants. These sugars have been implicated in stress tolerance and also in carbohydrate transport (Loewus *et al.*, 1982). Lastly, inositol may be conjugated to auxins, preventing biological activity and allowing long-distance transport within the plant and the regeneration of inositol without significant *de novo* synthesis (Cohen and Bandurski, 1982).

Inositol synthesis is the first step leading to other biochemical pathways for the synthesis of many compounds which are critical for cell wall formation, glycoproteins and compounds in response to stresses. Inositol has important role in cell wall synthesis (Bohnert *et al.*, 1995).

2.6.2.2.2 Biosynthesis of myo-inositol

Biosynthesis of inositol is by the direct cyclization of D-glucose derivative, an NAD-dependent internal oxidation reduction reaction. Cyclization process uses glucose-6-phosphate as substrate and requires cofactors like Nicotinamide adenine dinucleotide (NAD) and magnesium (Mg^{2+}). Glucose-6-phosphate is the starting material for the synthesis of myo-inositol. Biologically myo-inositol is synthesised in a two step conversion of D-glucose-6-phosphte (Hoffmann-Ostenhof and Pittner, 1982; Stieglitz, 2005). The enzyme 1L-myo-inositol-1-phosphate synthase (MIPS) catalyzes the reaction which produces 1L-myo-inositol-phosphate (Hoffmann-Ostenhof and Pittner, 1982). Dephosphorylation of this intermediate by specific Mg^{2+} dependent inositol-1-phosphatase results in the production of myo-inositol (Loewus and Murthy, 2000). The enzyme involved in this step is L-myo-inositol 1-phosphatase (Stieglitz, 2005). This mechanism is conserved among all myo-inositol producing organisms (Majumdar *et al.*, 1997). *INO1* is the gene coding for cytosolic MIPS and was first identified in the yeast *Saccharomyces* (Klig and Henry, 1984; Majumdar *et al.*, 1981). 1L myo-inositol 1-phophate synthase was identified in *Hevea* latex C-serum (Loewus *et al.*, 1986).



2.6.2.2.3 Role of inositols

In higher plants inositols have a range of functions such as carbon storage, translocation, stress tolerance and secondary messengers in signal transduction pathways. Myo-inositol is required for proper growth and development as well as suppression of spontaneous cell death. They function from protein stabilization in higher plants and lower organisms (Lozano *et al.*, 1994; Salvucci, 2000) to the protection of plants from biotic and abiotic stresses (Stoop *et al.*, 1996). The myo-inositol level has been higher in tolerant genotypes than sensitive ones in tomato during salinity (Sacher and Staple, 1985). The most common polyols found in plants are acyclic forms like mannitol, glycerol, sorbitol and cyclic forms such as ononitol and pinitol (Ashraf and Harris, 2004). Myo-inositol significantly increased under drought stress in *Vigna umbellate* (Wanck and Richter, 1997) and in *Cicer* (Boominathan, 2004). Drought stress induced accumulation of pinitol in

leaves of *pigeon pea* (Gorham and Jones, 1981; Keller and Ludlow, 1993). Sorbitol and mannitol are major translocated sugars in some higher plants; such a role is not attributed to myo-inositol (Klages *et al.*, 1998).

Sugar alcohols are produced and accumulated during stress conditions so as to overcome the adverse situation (Yancey *et al.*, 1982; Adams *et al.*, 1992; Kelavker and Chhatpar, 1993; Popp and Smirnoff, 1995; Zhu, *et al.*, 2005; Noiraud *et al.*, 2000; Conde *et al.*, 2014). Under stress condition there was a substantial enhancement in sugar alcohol concentration (Briens and Larher, 1983; Hare *et al.*, 1998; Lewis, 1984; Nguyen and Lamant, 1988; Vernon and Bohnert, 1992; Wang and Stutte, 1992). They act as osmoprotectants to stabilize membranes and also as carbon storage compounds (Schobest, 1977; Loewus and Loewus, 1983; Paul and Cockburn, 1989). The most important role is that they serve as osmolytes in response to water, salt and other abiotic stresses (Nguyen and Lamant, 1988; Tarczynski *et al.*, 1993; Keller and Ludlow, 1993; Wang *et al.*, 1996; Liu, and Grieve, 2009).

Plants have different adaptive mechanisms to respond to the changing environmental conditions. The environmental perturbations was perceived and transported through signal transduction cascade system. Phosphoinositides signalling pathway is one such signal transduction cascade in plants (Munnik *et al.*, 1998; 2010). Inositol is an essential component in this pathway. It has

been shown to involve in a variety of plant responses such as variation in turgor pressure of stomatal guard cells (Cote and Crain, 1993) and gravitropism in *Zea mays* (Perera *et al.*, 1999). Inositol has a role in hormone transportation from the site of production to its target site. Storage and transport of auxin as inositol conjugates of IAA may regulate the available IAA for physiological changes in plant tissues. Inositol is an important molecule in cell wall synthesis (Loewus and Loewus, 1983).

Alterations in biosynthesis of inositols and its derivatives may confer salt tolerance to crop plants (Bohnert *et al.*, 1995). It has been reported that in higher plants mannitol metabolism occurs as a mode for coping the stressful condition (Stoop, *et al.*, 1996). Polyols act as signals (Steinitz, 1999) and as free oxygen species quenchers (Shen *et al.*, 1977a, 1997b, Jenning *et al.*, 1998; Nelson *et al.*, 1998). Polyol accumulation influence water relations and cellular processes and take part in osmotic adjustment. Sugar alcohols help the cell to gain more water under water stress condition thus maintaining cell turgor through osmotic adjustment (Tuner and Jones, 1980; Morgan, 1984; Evans *et al.*, 1992) and function as osmoprotectants (Le Rudulier *et al.*, 1984; Bohnert *et al.*, 1995). It has been noticed that compatible solutes stabilize membranes and liposomes under stress (Galinski 1993; Papageorgion and Murata, 1995; Ortabuer and Popp, 2008). There was no short term variation in their content, suggests their ability in stabilizing

cell structure. Hydration of cellular structures through preferential exclusion was attributed to many polyols (Anderson *et al.*, 2011). During drought stress sorbitol level was high in *Xerophyta viscose* (Mundree, *et al.*, 2000). Accumulation of inositols particularly *O*-methyl inositols occurs in higher plants in response to abiotic stresses (Ford, 1982; Yancey *et al.*, 1982). *O*-methyl inositols reported to serve the role of improving tolerance to heat, drought and salt conditions (Ford, 1984; Guo and Oosterhius, 1997; Sheveleva *et al.*, 1997; Streeter *et al.*, 2001b; Manchanda and Garg, 2008). Lo Bianco *et al.*, (2000) observed an accumulation of sorbitol in drought stressed Nemaguard peach plants. The level of polyols and carbohydrates increases in plants under low temperature. The cryoprotection by polyols may be due to the coordinated function of freezing point depression and stabilization of enzymes and membranes (Wimmer, 1997; Suthanbawa and Li-Chan, 1999; Ford *et al.*, 2000).

Mesembryanthem crystallium (ice plant), a halophyte acclimatized to drought, cold, and salinity via synthesis and accumulation of pinitol. Under stress conditions the ononitol produced are epimerized and large amount of D-pinitol was accumulated (Paul and Cockburn, 1989; Adam *et al.*, 1992; Vernon *et al.*, 1993). Ononitol and pinitol, expected to lower the cytoplasmic osmotic potential and balance sodium accumulation in the plant vacuole. Loewus and Murthy (2000) reported that the inositols, D-ononitol and pinitol

function as (1) protectant of cellular structures from reactive oxygen species such as hydrogen peroxide and (2) control the turgor pressure. Accumulation of these osmolytes leads to an enhancement in turgor pressure. Methylation of polyols results in partial hydrophobicity and there by an increase in size of hydration shell (Nelson *et al.*, 1998; Michell, 2008). Methylation of myo-inositol to D-ononitol in plant tissues reduces hydrogen peroxide production through photorespiration (Hare *et al.*, 1998). This improves the capacity of the solute to interact with and protect protein structures. They stabilize proteins, nucleic acid, and other biological structures of cell (Wang *et al.*, 1995; 1996; Wimmer *et al.*, 1997; Kaushik and Bhat, 1998, Del Vecchio, *et al.*, 1999). Compatible solutes also provide the function of antioxidants (Smirnoff and Cumbes, 1989: Orthen *et al.*, 1994; Shen *et al.*, 1997a; 1997b). Recent research works on polyols revealed that species like olive trees cope with drought and salinity by co-ordinating mannitol transport with intracellular metabolism (Conde *et al.*, 2011a, 2011b).

Inositols and its derivatives are an emerging family of compounds that are crucial for development and signalling in plants. They essentially function as either metabolic mediators or participate in various signalling pathways in response to stress, hormones and nutrients by transcriptional regulation of the elicitor responsive genes. More over, metabolites coming

in the downstream and the associated pathways function in a highly co-ordinated manner contributing towards stress tolerance in plants. Inositol derived galactinol and raffinose are emerging as essential compounds in plant stress tolerance, as antioxidants or signal mediators under stress to cellular functions.

2.6.2.2.4 Applications of inositols

Inositols are very essential nutrients for plants and animals (Holubus, 1986; Loewus and Murthy, 2000). They are chemically and physiologically very similar to sugars to such extent that some of them (*i.e.* sorbitol, mannitol, xylitol, erythritol, maltiol, lactitol) are used as sweeteners in food industry (Bieleski, 1982; Loescher, 1987). L-chiro inositols have nutraceutic and theraptic application because of their hypoglycaemic property. *Suringodium flotsam*, a sea grass which has high concentration of L-chiro inositol has become a cheap source of L-chiro inositol (Nuissier *et al.*, 2008). The dietary form of myo-inositol is inositol hexaphosphate (phytic acid). It was widely found in cereals and legumes. Phytic acid has been reported for its antiproliferative, anticancer properties and was described as natural cancer fighter (Narayanan, 1987; Nurul-Husna *et al.*, 2010; Abbas *et al.*, 2014). Inositols are used for the treatment of liver problem, depression, panic disorder and diabetes (Narayanan, 1987). Inositols are very essential for the proper function of brain neurotransmitters. Inositols eliminate the damages

caused by diabetes (Gregeresen *et al.,* 1978; 1983). Due to the calming effects of inositols on the central nervous system, it is useful to insomnia patients.

Myo-inositol is essential for the synthesis of many metabolites. Myo-inositol and its derivatives are well known for their antitumor properties (Somasundar *et al.*, 2005) and their antidepressant activity in humans (Einat *et al.*, 2001). Inositol deficiency can lead to depression and other mental disorders (Levine *et al.*, 1995; Benjamin *et al.*, 1995; Fux *et al.*, 1996). The polycystic ovary syndrome is associated with deficiency in dietary inositols (Gerli *et al.*, 2003; 2007). The dietary merits and demerits of other forms of inositols are under active evaluation.

2.6.2.2.5 Molecular studies on inositols and other sugar alcohols

Plants usually accumulate low molecular weight compatible solutes in response to osmotic stress to maintain cell turgor. Inositols and other acyclic polyols are compatible solutes and are accumulated during stress (Ford, 1982; Klig and Henry, 1984; Nguyen and Lamant, 1988; Wanek and Richter, 1997; Streeter, 2001b; Merchant *et al.*, 2006a; 2007a; 2007b). Stress induced enhancement in inositol accumulation and distribution in various organs has been reported (Saxena *et al.*, 2013). Genetic engineering for increased osmoprotectant synthesis is one of the best approaches for the enhancement of abiotic stress tolerance in plants (Bhatnagar-Mathur *et al.*,

2008). Over expression of polyols was a potential way to enhance abiotic stress tolerance in plants (Merchant and Richter, 2011).

Shen *et al.*, (1997a) utilized manntiol-1-phosphate dehydrogenase gene (*mtlD*) a bacterial gene for stress tolerance in tobacco plants and increased resistance was found against oxidative stress as a result of high mannitol production in the chloroplast of the transgenic tobacco plants. Mannitol accumulation reported to increase the metabolic path ways that are normally involved in stress tolerance and there by afford protection (Tarczynski *et al.*, 1993; Prabavathi *et al.*, 2002; Maheswari *et al.*, 2010). Salinity tolerance was enhanced by significant increase of mannitol accumulation in transgenic potato plants with *mtlD* expression. This increase in tolerance is resulted mainly from the osmoprotectant action of mannitol (Maheswari *et al.*, 2010; Rahnama *et al.*, 2011). Over expression of myo-inositol *O*-methyl transferase found to occur in transgenic *Mesembryanthemum crystallinum* (ice plant). Salinity induces gene *Imt1* that encodes the myo-inositol *O*-methyl transferase and is known to be involved in the biosynthesis of pinitol (Posternak, 1965; Vernon *et al.*, 1993; Tarczynski *et al.*, 1993). Transgenic tobacco plants showed elevated levels of total inositol and the capacity to withstand higher salt stress (Patra *et al.*, 2010).

In order to impart abiotic stress tolerance particularly for drought, genetic transformation was carried out in *Hevea* using sorbitol-6-phosphate

dehydrogenase (Jayasree *et al* in press). ALDRXV4, aldose reductase was found expressed in *Xerophyta viscose* leaves during water deficit (Mundree *et al.*, 2000). Gene encoding myo-inositol 1-phosphate synthase (MIPS) was established and isolated in *Arabidopsis* through genetic complementation of yeast *ino1*. Yeast *ino1* mutant can be rescued by *AtMIPs* emphasising their role as the functional counter part of yeast *INO1* in *Arabidopsis* (Luo *et al.*, 2011). Ray *et al.*, (2010) identified two new *Pc INO1* genes and peptide sequences in wild rice related to salinity tolerance. The level of myo-inositol and MIPS transcripts was found increased during cold acclimtation and was associated with cold tolerance of *M. falcata*. Over expression of *MfMIPS* in tobacco resulted in increased level of myo-inositol, galactinol and raffinose (Tan *et al.*, 2013).

MIPS gene expression is suggested to be organ specific and induced by stress. Gene expression and transcription increases after 16 hrs. of heat stress at 37^0C in *Passiflora edulis* (Abreu and Araga, 2007). *Arabidopsis* MIPS genes have been expressed through out development in various tissues of which *AtMIPS1* have the crucial role (Donabue *et al.*, 2010). In many plants, MIPS were represented by a multiple gene family (Abid *et al.*, 2009). Plant inositol monophosphatase (IMP) is encoded by multiple genes. These genes exhibit differences in their expression, which may be attributed to IMP isoform functions in different cell types (Gillaspy *et al.*, 1995). The

physiological function of MIPS isoforms differ according to the developmental and type of abiotic stress (Yoshida *et al.*, 1999; 2002; Mitsuhashi *et al.*, 2008). Characterisation of three MIPS genes in *Arabidopsis* has been conducted and found that *MIPS1* is more significantly expressed than other forms of MIPS (*MIPS2* and 3). It is expressed in all types of cells where as others mainly restricted to vascular or related tissues. MIPS genes were expressed in wheat, rice and Arabidopsis under heat stress and flowers developed after ten hours of stress (Khurana *et al.*, 2012).

2.6.2.2.6 Metabolism of inositols

Myo-inositol occupies a central position in inositol metabolism. It can be channeled through various metabolic pathways and different inositols and inositol derivatives are produced (Loewus and Murthy, 2000; Stevenson *et al.*, 2000). Inositols (myo, scyllo, chiro, muco and neo) through isomerisation and methylation form *O*-methyl inositols (sequoyitol, bornesitol, quebrachitol, pinitol and ononitol, *etc.* Sequoyitol by epimerization form D-pinitol which through demethylation converted into D-chiro inositol with the help of NADP specific D-chiro-pinitol dehydrogenase (Stiegliz *et al.*, 2005). *O*-methyl inositols participated in stress related responses, storage products of seeds and glycosides, *etc.* Some plant species utilize inositol for the production and accumulation of D-ononitol and D-pinitol. Myo-inositol is

converted to D-ononitol through methylation and epimerised to D-pinitol (Ishitani *et al.*, 1996; Streeter *et al*, 2001b).

2.6.2.3 D-ononitol and pinitol

Myo-inositol and its derivatives are generally associated with cell signalling, membrane biogenesis and also involved in stress response (Nelson *et al.*, 1998). The accumulation of methylated derivatives of myo-inositol such as ononitol and pinitol is correlated with abiotic stress tolerance in many lower and higher plants and animals. In salt tolerant plant species, myo-inositol is the precursor for the production of cyclic sugar alcohols, pinitol and ononitol, which accumulate and help to lower the cytoplasmic osmotic potential so as to balance the vacuolar sodium concentration.

D-ononitol is the product of the S-adenosyl L-methionine dependent methylation of inositol. The enzyme myo-inositol 6-*O*-methyltransferase (m6*O*MT) is highly specific for six hydroxyl group of myo-inositol (Wanek and Richter, 1997). Pinitol has been found in a number of plants naturally or in response to stress. The most abundant *O*-methyl inositol in soyabean is pinitol and ononitol is its precursor (Philips and smith, 1974; Streeter, 1980; Loewus and Murthy, 2000; Chiera *et al.*, 2006). The increased pinitol level in plants may be associated with improved stress tolerance. *O*-methyl inositols have role in improving tolerance to heat, drought and salt stresses (Ford, 1984; Guo and Oosterhmis, 1997; Sheveleva *et al.*, 1997; Streeter *et*

al., 2001a; 2001b; Manchanda and Garg, 2009). Ahn *et al.*, (2011) reported that methylation of myo-inositol yields *O*-methyl inositols when plants are exposed to abiotic stress.

2.6.2.4 Galactosyl cyclitols

Galactosyl cyclitols are inositols with glycoside group. Most common form is galactinol and is formed from myo-inositol in the presence of the enzyme galactinol synthase (E.C.2.4.123). UDP-galactose is the donor of glycoside moiety. Galactopinitol and galactosylononitol are other galactosyl cyclitols (Chien, *et al.*, 1996; Peterbauer *et al.*, 1998). Studies in *Lupin* species showed that soil drought caused an increase in the accumulation of cyclitols and galactosyl cyclitols (Piotrowicz-Cieslak *et al.*, 2007). Under drought stress, galactinol and raffinose was found accumulated in pea seedlings (Lahuta *et al.*, 2014).

2.6.2.5 Quebrachitol

Quebrachitol is the 2-methyl ether of L-chiro-inositol. The latter is a cyclitol whose isomers occur in various plant sources (Proteaceae, Apocynaceae, Elaeguaceae, and Sapindaceae) (Diaz *et al.*, 2008). Inositol methyl transferase catalyses the methylation of L-chiro inositol into quebrachitol (Bealing, 1981). Quebrachitol, was first identified as natural product in *Asdosperma quebracho* bark (Tanret, 1889). It occurs in various species of Sapindaceae, *Acer pseudoplastanus* L., *A. platanoides* L.,

Cardiospermum halicacabum L., *Alectryon excelsus* Gaertn. *Harpullia pendula* Planch., and members of Aceraceae and Euphorbiaceae. In Euphorbiaceae family *Hevea brasiliensis* Muell. Arg. is a rich source of quebrachitol (Jan van Halphen, 1951). It has been isolated from the leaves of *Allophylus cobbe* L. in Vietnam (Nguyen and Lieu, 2006). Quebrachitol is suggested to be the major contributor of osmotic pressure (Richter and Popp, 1992) and cryoprotection of plants (Orthen and Popp 2000).

Quebrachitol is a naturally occurring optically active cyclitol synthesised from myo-inositol. It was found in *Allophylus edulis* (Diaz *et al.*, 2008) and in the serum of *Hevea brasileinsis* latex (Bealing, 1969). *A. edulis* is used as an infusion to treat diabetes because of the presence of considerable amount of quebrachitol. It was also found in *Cannabis sativa* and sea buckthorn (Kallio *et al.*, 2009; Stinson *et al.*, 1967). Quebrachitol is a starting material in the synthesis of several pharmaceuticals. Naturally occurring bioactive materials are synthesised using quebrachitol as building blocks *e.g.* Oudemansin X (Lau, 1993). Inositol and methyl inositols are bioactive compounds essential for regulating physiological processes of plants and humans. Quebrachitol has similar physiological properties as inositol. Inositol is essential for structural organization of cell membrane. It participates or takes part in transmembrane signalling mechanism (De Almeida *et al.*, 2003). It stabilizes the cell membrane and thus act as

cryoprotectant (Orthen and Popp, 2000; Hincha and Hagemann, 2004). Quebrachitol also have free radical scavenging ability (Lemos *et al.*, 2006) and cytoprotection against cell death in cell culture (Nobre *et al.*, 2006).

L-Quebrachitol (2-O-methyl inositol) is one of the main carbohydrates in *Hevea* latex. Synthesis occurs in cytoplasm of laticifers and enhanced by stimulation with ethylene (d'Auzac and Ribaillier, 1969). Latex contains about 1.2% w/v of quebrachitol in comparison with 0.4% of w/v sucrose (Bealing, 1969; 1981). Clonal variation is observed for its concentration. It ranges from 1-3% of the whole latex (Gopalakrishnan *et al.*, 2008; 2011). Quebrachitol is involved in the formation and maintenance of cytosolic osmotic pressure of laticifers. It is very much essential for latex flow after each tapping (Sheldrake, 1978; Chong, 1981). Laticifer metabolic status is also evaluated by its intracellular concentration (d'Auzac and Jacob, 1989). Polyol transporters are considered to have a role in latex production in *Hevea* plant to different stress stimuli. The up regulation of polyol transporters upon hormonal treatment can be associated with turgor status of soft bark tissue. It was shown that quebrachitol accumulation by quebrachitol transporter HbPLT2 in the soft bark tissue created osmotic forces which lead to the flow of water from the adjacent xylem to the blast region (Tungngoen *et al.*, 2009; 2011). Dusotoit-Coucaud *et al.*, (2010) reported that ethylene stimulated latex production is related to the transportation of quebrachitol to

laticifers as it is involved in regulating turgor pressure of soft bark tissue and laticifers. It was hypothesed that two internal limiting factors coordinate the regulation of ethylene stimulated latex yield in virgin trees. Latex dilution that determines the duration of latex flow is the first factor which is dependent on the concomitant up regulation of quebrachitol transporter HbPLT2 and two aquaporin transporters (HbPIP2 and HbTIP1). The second one is rubber biosynthesis via the stimulation of two putative sucrose transporters (HBSUT1A and HBSUT2A)

The synthesis and occurrence of polyols offer considerable promise as bioindicators of plant health and biomarkers for use as selective traits for plant improvement programmes. Studies of plant metabolism within and the core reactions of cell, *i.e.* carbohydrates and derived metabolites may offer significant knowledge into alterations in plant metabolism in response to environmental changes. Rubber yield in *Hevea* is positively related to carbohydrate metabolism in latex as well as the ability to convert the substrate to rubber particles.

There have been earlier studies in the direction of isolation of value added compounds from *Hevea* latex. Currently China, Japan, India and the United States are involved in studies on separation of L-quebrachitol from rubber latex and some patents have been registered in this area from other countries. These patents utilized serum obtained through centrifugation, acid

coagulation and creaming for the isolation. The procedures involved were, first of all treating the serum with lime and boiling so as to remove the proteinaceous contaminants. There after the serum is passed through carbon dioxide or phosphoric acid. The pH of the solution kept decreased with the help of acetic acid, formic acid or hydrochloric acid. Further purification was carried out by passing through anionic exchange resins and evaporation under pressure (Hart, 1945). Mcgavack and Binmore (1930) reported a method which involved extraction of latex serum in a heating chamber and there after with hot ethanol. The resultant serum after treating with bone black / animal charcoal was crystallized. Ogawa *et al.*, (1990) demonstrated a procedure which involved acetylation of latex serum using acetic anhydride at 30-120^{0}C for 5 hours with stirring. A base such as pyridine and dehydrating agent such as concentrated sulphuric acid or zinc chloride were used as reaction auxiliaries. The resulted serum, washed with alkali and passed through silica column, acetylated quebrachitol was obtained. This was then deacetylated by an acid (sulphuric acid) or a base (Na_2OCH_3) hydrolysis in an oil bath at 100-150^{0}C for 10-20 hours. Udagawa (1991) described pulverisation of natural rubber serum to get a solid form and further processing for recovering quebrachitol from rubber latex serum. The dry material was dissolved in methyl alcohol for extraction and concentrated by vaccum and kept overnight at room temperature for crystallization. These crystals were dissolved in distilled water and treated with active carbon for

discolouration. After, all these steps serum was passed through suitable ion exchange resin. The crystals were developed through ethanol precipitation and low temperature was imposed for crystal growth. All the procedures were lengthy, difficult and the quality of the isolated compound was not mentioned. Moreover, these methods utilized hot alcohol, propanol or butanol *etc*, concentrated acids and alkalies for extraction from serum solids followed by deacetylation, discoloration, cooling and precipitation. The method of recovery was hazardous and liable to contain eluates other than quebrachitol which adversely affects the purity of the final compound.

Research on identification and isolation of quebrachitol was preferred due to its application in medical and pharmaceutical industry. L-quebrachitol is a chiral precursor of inositol derivatives. Levorotatory forms of inositol derivatives are very much useful for the development of drugs for the treatment of cancer, diabetes, acquired immunity deficiency syndrome (AIDS) and other diseases. It was reported (CN 102516041 A) that, for the research activities and production of quebrachitol, a pharmaceutical company in Hebi is investing 260 million Yuan from 2011 to 2014. *Hevea* latex is a rich source of L-quebrachitol. The composition of latex serum is complicated as it contains lot of nitrogenous compounds, cofactors, lipids, carbohydrates, minerals and other components. Separation of quebrachitol through conventional separation methods is very difficult. Hence an

appropriate method is very much needed for the isolation of this compound. In the present study, experiments were carried out for developing a refined and more precise method for the isolation and quantification of L-quebrachitol and characterization of the purified compound by different standard techniques. The possibility of effect of different latex extraction methods for the isolation of L-quebrachitol from different *Hevea* clones was explored. Clonal and seasonal variations in total inositol content in different *Hevea* clones were analysed. The influence of osmolytes to latex water relation and there by latex yield in different *Hevea* clones were also determined.

Chapter 3

Isolation, purification and quantification of L-quebrachitol from *Hevea* latex

3.1 Introduction

The latex of *Hevea brasilensis* Muell. Arg. is a rich source of quebrachitol. It was found in the serum left after the coagulation of the *Hevea* latex. Among the inositols quebrachitol is the most abundant inositol in latex. It is reported that quebrachitol is a major factor determining the turgor pressure of latex vessels, thus involved in latex yield (Bealing *et al.*, 1981). Quebrachitol was first identified as natural product of *Asdosperma quebracho* (Apocynaceae) by Tanret (1889). It was found in various species of Sapindaceae, *Allophylus edulis* (Diaz *et al.*, 2008), *Hippophae rhamnoides* (Yang *et al.*, 2009), *Mitrephora vulpina* (Moharam *et al.*, 2010), *Artemisia sodiroi* (Briceño, 2011) and members of Aceraceae and Euphorbiaceae (Bealing, 1969). Quebrachitol is chemically 2-methyl ether of L-chiro-inositol, with its unique structure, useful in the pharmaceutical industry and synthesis of a range of bioactive compounds. Isomers of L-chiro-inositol occur in various plant sources *i.e.* Proteaceae (Bieleski and Briggs, 2005), Apocynaceae (Nishibe *et al.*, 2001), Elaeagnaceae and Sapindaceae (Jan van Alphen, 1951).

L-quebrachitol (1L-2-*O*-methyl-chiro-inositol) is a naturally occurring optically active inositol (cyclitol). It is a high value compound with several commercial applications and is mainly used in pharmaceutical industry and in medical research (Lau, 1993; 1996; Yaojun, 1999). L-quebrachitol was used

as the raw material for the synthesis of biologically active compounds by many groups of researchers. The first report on the synthesis of a natural compound from L-quebrachitol is L-mannitol (Kuhn and Klesse, 1958; Angyal and Hoskinson, 1963). Later on it has been used for the synthesis of inositol and its derivatives, a number of inositol phosphates and related structures, other carbohydrates, various other natural and potential bioactive products such as (-)- conduritol F, (+)- conduritol B, cyclophellitol and (-)- ovalicin. Naturally occurring bioactive material are made using quebrachitol as building blocks *eg.* Oudemansin X (Lau, 1993; Kiddle, 1995). Chida *et al.*, (1998) synthesised glycosyl phosphatidyl inositol (GPI) using quebrachitol as the starting material. GPI is present on the surface of the eukaryotic cell and have a role in cellular interaction and differentiation. Quebrachitol has been used for the synthesis of optically active antibiotics and anticancer drugs (Sakdapipanich, 2005). It has gained much attention because of its optical properties and also due to its derivatives which are involved in cell signalling mechanisms and in medical field (Lau, 1993; Akiyama, 1996; Ningjian, 2005). The usefulness of this compound to mankind triggered the development of methods for the extraction of L-quebrachitol from natural rubber latex serum (Yaojun, 1999; Deng and Deng, 2000; Gopalakrishnan *et al.*, 2010) and recently from rubber processing factory waste water (Wu *et al.*, 2012; Jiang *et al.*, 2014).

Plant secondary metabolites are currently the subject of much research interest. Phytochemical constituents are the basic sources which add value to the crop species. Separation of bioactive compounds from plant extract remains to be a big challenge for the process of identification and characterization, as it contains various compounds with different proportions/polarities. Development of a quick, reliable and efficient extraction protocol for a particular class of compound is highly essential. As a result of the present interest in quebrachitol and apparent usefulness of the compound, its recovery from latex serum (NRS) has been given importance.

As cited in literature review a few isolation procedures for L-quebrachitol from *Hevea* natural rubber serum have been published. The present study was aimed at standardization of an appropriate protocol/ technology for the isolation, purification and quantification of L-quebrachitol from *Hevea brasiliensis* latex serum. The study was also focussed on the rate of recovery of quebrachitol from different *Hevea* clones and varying latex extraction methods.

3.2 Experimental details

3.2.1 Identification and quantification of individual inositols from *Hevea* latex serum

Latex samples from two *Hevea* clones (RRII 105 and RRIM 600) were collected on icc and were transported from field to the laboratory for the

study. Serum was prepared for the identification and quantification of individual inositols from the latex.

3.2.1.1 Preparation of latex serum

3.2.1.1.1 C- serum preparation

Fresh latex samples from RRII 105 and RRIM 600 were transferred to pre-weighed centrifuge tubes. These samples were centrifuged at 23,000 rpm for 45 minutes at 4^0C using Sorvall OTDB Ultra centrifuge. On centrifugation latex samples separated out into three layers, an upper rubber cream, middle cytoplasm portion and lower bottom fraction containing the lutoid particles. The middle cytoplasmic portion *i.e.* the C-serum was collected using a syringe and stored for further processing.

3.2.1.1.2 A- serum preparation

Rubber phase was separated from the latex as in the case of sheet making. The latex samples were coagulated with 3% acetic acid (10:1 v/v) and kept for 4-5 hours. The coagulated rubber particles were removed and the serum was collected.

3.2.1.2 Processing of C - and A - sera

The C and A-sera were deproteinised by adding 100% ice cold acetone (1:2 v/v) and kept overnight in a refrigerator (4^0C). Next day these serum samples were centrifuged at 8000 rpm for 30 minutes at 4^0C. The clear supernatant was collected into an already weighed bottle. The supernatant

was kept at -80^{0}C for 12-24 hrs. These freezed samples were dried in a lyophiliser (Speed Vac, Savant Instrument).

3.2.1.3 Identification of the compounds

3.2.1.3.1 High Performance Liquid Chromatography (HPLC)

Separation of inositols and other sugars from the lyophilised serum samples (C and A sera) was performed using an Aminopropyl column (Waters Spherisorb NH_2 column, 250 x 4.6 mm ID, 5μm). Standardization of a method was attempted to obtain the best condition for separation of all components. The freeze dried samples and standards were dissolved in 5 ml of solution containing water and acetonitrile in the ratio 1: 4 (v/v). The injection volume was 20 μl. The column was equilibrated with solvent system prior to the injection of sample. The effect of flow rate and change in mobile phase on separation of components was monitored. Flow rate checked was 1 ml/min. and 0.5 ml/ min. The mobile phase was changed by using different ratios of solvents *i.e.* acetonitrile and water. The total run time for sample elution was 25 minutes. Detection was done by refractive index detector (Shimadzu 10A series). Sugar alcohol such as quebrachitol, myo-inositol, sorbitol, mannitol and sugars such as sucrose, glucose and fructose were used as standards.

3.2.2 Isolation, identification and quantification of L-quebrachitol from *Hevea* latex serum

In the present study, fresh latex from the field collection was used. Serum was extracted out from the latex samples without any delay to reduce bacterial contamination. Latex samples from two *Hevea* clones (RRII 105 and RRIM 600) were collected on ice for the study.

3.2.2.1 Column preparation

3.2.2.1.1 Silica gel column

A glass column of 2 cm diameter is used for preparing the Silica column. 10-20 g of silica gel is equilibrated with occasional stirring and washing for 15 minutes in acetone. The slurry is poured into the column and allowed to settle down to required height. The column was repeatedly washed with acetone at least for three times.

3.2.2.1.2 Amberlite ion exchange resin column

Amberlite MB 150 ion exchange resin was used to make the ion exchange column. A glass column of 1 cm and 50 cm height was taken for preparing the column. The dry resin, approximately 10-20 g was transferred to a 500 ml beaker. Sufficient amount of distilled deionized water was added to cover the resin bed by 1-2 inches. Stirred the resin gently for a minute to ensure complete mixing and allowed the material to stand for 15 minutes. The process was repeated thrice and the water - slurry made was used to make the column as above. The column was washed with milliQ water.

3.2.2.2 Extraction of serum

Approximately 100g (100 ml) of latex sample was mixed with 200 ml of 80% alcohol and serum was collected. The solid rubber phase was again mixed with 80% alcohol heated on a water bath at 80^0C for 30 minutes. The extraction procedure was repeated for four times. The extract was pooled and collected in a conical flask.

3.2.2.3 Purification of serum

Two different protocols were tried for the purification of the serum.

3.2.2.3.1 Method -1

Pooled latex serum extract was deprotenised by adding 50% cold acetone and kept overnight in cold condition (refrigerator). It was centrifuged at 8,000 rpm at 4^0C for 30 minutes. The supernatant was collected into a beaker/conical flask and completely evaporated using rotary evaporator at 40^0C. The sample was suspended in acetone and was loaded on an equilibrated glass column made of silica. Sample was allowed to remain in the column for 15-30 minutes and eluted out with appropriate amount of acetone. The eluate was evaporated completely and then resuspended in a solution containing 24 ml distilled water and 10 ml chloroform. Kept the solution overnight at 2^0C for phase separation and the aqueous layer was collected and lyophilized.

The lyophilised sample was reconstituted in minimum volume of milliQ water and treated with Amberlite resins. The sample material was

allowed to remain in the resin column for 15 minutes. There after the sample was eluted out using small quantity of milliQ water followed by wash with 100 ml milliQ water. The eluates were combined and freeze dried.

3.2.2.3.2 Method -2

The volume of the pooled latex serum (alcohol extract) was reduced up to 10% of its total volume by heating at 80^0C and deproteinised with 50% acetone. The deproteinised serum was filtered and the filtrate was collected. Further the filtrate was concentrated by heating at 70-80^0 C for 30 minutes. The concentrate was dissolved in small amount of acetone and passed through a silica column. The sample was eluted out with acetone and the eluate was concentrated by heating at 50-60^0C for 30 minutes. The extract eluate was dissolved in minimum amount of milliQ water and passed through mixed bed ion exchange resin. The eluate (about 150 ml) was concentrated by heating at 80^0C. The steps of the protocol followed for isolation of L-quebrachitol form *Hevea* latex serum is shown in Table 3.1.

3.2.2.4 Crystallization of the isolated compound

Crystals of quebrachitol were obtained from the eluate by following any of three methods.

1. The concentrated eluate was mixed with 2 ml solution of alcohol: water (1:2) and warmed for 30 minutes. The mixture was kept at room temperature for one day and thereafter kept in a refrigerator (4^0C) for 3 to 4 hrs. Under low temperature crystals were developed.

2. The concentrated eluate was dissolved in milliQ water and warmed for 2 minutes at 60^0C. Kept at room temperature for 2-3 hours and cooled for crystallization.

3. The concentrated eluate was evaporated completely (80^0C). Evaporation of the aqueous solution itself mediates crystal growth. Further, it was speeded up by giving a cold shock.

Table. 3.1. Protocol followed for the isolation of L-quebrachitol

Method 1	Method 2
Latex- Extraction of latex with 80% ethyl alcohol	Latex- Extraction of latex with 80% ethyl alcohol
Deproteinisation and centrifugation	Concentration by boiling (80^0C) Deproteinisation and filtration
Concentration (Vacum evaporation)	Concentration (boiling at 80^0C)
Column chromatography with Silica	Column chromatography with Silica
Concentration of eluate and liquid - liquid solvent extraction (overnight at 4^0C)	-
Concentration of eluate (lyophilization) of aqueous layer and column chromatography with ion exchange resin	Concentration of eluate (boiling at 80^0C) and column chromatography with ion exchange resin
Concentration of eluate (lyophilization)	Concentration of eluate (boiling at 80^0C) and cooling (Crystallization)
Dissolution in milliQ water, concentration by heating, cooling and crystalization	-
Crystals of L-quebrachitol	Crystals of L-quebrachitol

3.2.2.5 Identification of the isolated compound

3.2.2.5.1 High Performance Liquid Chromatography (HPLC)

The crystals of the isolated compound were tested for L-quebrachitol. The samples were dissolved in a minimum amount of water (milliQ water), filtered using PES sterile 0.22 µm syringe filters and analysed using a HPLC system (Waters, USA) with Rhyodene sample injector on a Shodex sugar SC1011 column (8 mm ID x 300 mm, 5µm, Showa Denko, Tokyo) and with RI (Waters deferential refractive Index detector, W2996) detector. The mobile phase was milliQ water and the running phase was in isocratic mode. The sample (20 µl) was allowed to elute through the column for 60 minutes at a flow rate of 0.6 ml/min. The column and RI detector temperature were set at 30^0C. L-quebrachitol purchased from Sigma was used as the standard. A set of filtered serum sample without purification and lyophilisation was also analyzed as per the conditions.

HPLC system conditions

Mobile phase	MilliQ water
Diluent	MilliQ water
Column	Shodex SC1011 (8 mm ID x 300 mm, 5µm)
Column temperature	30^0C
Flow rate	0.6 ml/min.
Detection	Refractive Index (W2996) at 30^0C and sensitivity 64
Injection volume	20 µl
Run time	60 minutes

3.2.2.6 Characterization of the isolated compound

3.2.2.6.1 Fourier Transform Infrared analysis (FT-IR)

The isolated compound from latex serum (alcoholic extract) was characterized using FT-IR. FT-IR spectra were recorded on Bomem MB series FTIR spectrometer as KBr pellet by mixing approximately 1 mg of sample with 100 mg of KBr. Absorbance was recorded in cm^{-1}. The spectra were collected in the range of 4200 to 400 cm^{-1}.

3.2.2.6.2 High Performance Liquid Chromatography-Mass Spectrometry (LC/MS/ESI)

LC-MS analyses of the isolated compound were carried out (Waters Lab, Banglore) with Alliance HPLC system and Waters Quattro Premier XE Mass detector. For liquid chromatography Shodex SC1011 (8mm ID x 300mm) HPLC column was used. Standard and isolated compound at a concentration of 100 ppm were prepared using HPLC grade water and were resolved with the above mentioned column. The compounds were ionized in the mass spectrometer using electrospray ionization in the negative mode. The mass spectrometer was tuned to achieve the highest sensitivity of the molecule of interest *i.e.* L-quebrachitol which has an atomic mass of 194.13 Daltons. All other parameters were adjusted accordingly, to attain the highest sensitivity for L-quebrachitol. The peak of total ion content was identified in comparison with the standard. The target component as observed in the MS total ion count has been scanned for their mass spectrum in the range of

50 m/z to 250 m/z. The mass data obtained for the isolated compound and standard were processed using the Mass Lynx software.

LC-MS System Conditions

Mobile phase	HPLC grade water
Diluent	HPLC grade water
Column	Shodex SC1011 (8 mm ID x 300 mm, 5µm)
Column temperature	30^0 C
Sample temperature	4^0C
Flow rate	1.2 ml/min.
Detection	Mass detector in ESI negative mode
Injection volume	10 µl
Run time	15 minutes

3.2.2.6.3 Nuclear Magnetic Resonance (NMR)

The identity and structure of the isolated compound was confirmed by NMR. The NMR spectrum was recorded using 300 MHz spectrometer (DPX 300, FTNMR Spectrometer, Bruker). ^{1}H-NMR and ^{13}C-NMR spectra of the isolated compound and standard was carried out in deturiated DMSO (DMSO–d6). Tetramethyl silane (TMS) was used as an internal standard in NMR analyses. The spectrometer operating at 300 MHz for proton and 75 MHz for carbon was used for the analyses of the isolated compound and

standard. The data were obtained at 25^0C with 5 mm probe. The chemical shifts (δ) are quoted in parts per million relative to the internal standard. The following abbreviations are used to describe the NMR signals m- multiplet, s - singlet, d - doublet, and dd- double doublet.

3.2.3 Recovery of L-quebrachitol from different *Hevea* clones and latex serum extraction methods

The suitability of the protocol developed for the isolation of L-quebrachitol from different clones and types of sera was studied. The recovery of a desired substance from a complex mixture is referred as extraction. Therefore recovery of L-quebrachitol from serum obtained through different extraction methods was also determined. Latex samples were collected from four different *Hevea clones*, *viz.* RRII 105, RRIM 600, RRII 430 and RRII 414 with three replications. Fresh latex without preservatives such as ammonia was used in this study. Serum from the latex samples obtained through the following extraction methods was used as source material for quebrachitol isolation.

3.2.3.1 Methods of Extraction

(1) Coagulation of latex with acetic acid (A- serum)

Fresh latex samples (50 ml) were diluted with water (1:2 v/v) and 3 percent acetic acid (5 ml) was added for coagulation. After coagulation, the serum was collected by removing the coagulated rubber particles.

(2) Coagulation of latex with alcohol

Fresh latex samples (50 ml) were mixed with 100 ml 80% alcohol and kept at 80^0 C for 30 minutes, cooled and filtered to collect the serum. This step was repeated thrice with the remaining coagulam. The serum samples were pooled and used as source material for quebrachitol extraction.

3) Centrifugation of latex (C-serum)

Latex samples (50 ml) were centrifuged using an Ultracentrifuge (Sorvall OTD 55B) at 23,000 rpm for 45 minutes at 4^0C. The latex was separated into three layers and 10 ml of the middle layer (C-serum) was used for extraction of quebrachitol.

(4) Cold treatment of latex

Fresh latex samples (50 ml) collected were frozen immediately at 2-0^0C for 3 to 4 days. Coagulated samples were squeezed and the serum was collected for isolation of quebrachitol.

(5) Effluent from latex processing factory

In addition to the sera of the four clones obtained through four different extraction methods, effluent from a latex processing factory was also collected and tested as a source material for quebrachitol extraction. In the present study effluent was collected from a processing factory where preserved latex (ammoniated) was coagulated using formic acid. The effluent contained latex serum left after coagulation, large quantities of water used for

diluting the field latex (*i.e.* dilution to a standard drc of 12.5%) and for washing coagulum and machineries.

3.2.3.2 Purification of serum

Serum samples obtained through different methods were processed as per the protocol developed for the extraction of quebrachitol from *Hevea* latex (Gopalakrishnan *et al.*, 2010). The serum samples collected were concentrated and then deproteinised using acetone and kept overnight in cold condition. Next day, the samples were filtered and the filtrate was collected. It was then evaporated and the residue was dissolved in acetone and passed through a silica column (Silica gel) for removing lipids and related impurities. After concentrating and resuspending in milliQ water, the resultant solution was passed through mixed bed ion exchange resin column (Amberlite MB 150) for further purification. The eluate thus obtained was concentrated to obtain L-quebrachitol crystals.

3.2.3.3 HPLC analysis

The crystals (quebrachitol) obtained from different serum samples were analyzed by HPLC (Waters). A known quantity of quebrachitol was dissolved in water (milli Q) and filtered through a 0.45 μm syringe filter. The filtrates were analyzed using a Ca loaded cation exchange column of Shodex Sugar SC1011 (8 mm X 300 mm, 5μm, Showa Denko, Tokyo) with a refractive index detector. The column and RI detector were kept at 30^0C and

elution was carried out using milliQ water. The isolates were identified based on the retention time of standard L-quebrachitol (Sigma). Standard techniques like LC/MS/ESI, FT-IR and NMR were used for confirmation of the identity and structure of the isolated compound (L-quebrachitol).

3.3 Results

3.3.1 Identification and quantification of individual inositols

The deproteinised and lyophilised serum samples (C and A-sera) were used for separation and identification of different serum components. The HPLC analysis of the lyophilised C and A-sera of both the clones showed presence of L-quebrachitol and glucose (Fig.3.1). Standardisation of system condition was carried out to get the complete resolution of all components. The effect of changes in mobile phase and flow rate on retention time of the compounds (inositols) was evaluated so as to find the best separation condition. When the mobile phase was 100 % water with 1 ml /min flow rate all compounds were eluted out at 3.24 minutes. With same mobile phase at 0.5 ml/min also all compounds were eluted out at 6.56 min. Later on different ratios of mobile phase *i.e.* actonitrile and water was tried with 1 ml or 0.5 ml/ min. flow rate. The best resolution was achieved with the mobile phase acetonitrile: water (85:15 v/v) ratio and with 0.5 ml/min. Standards *viz*, quebrachitol, myo-inositol, sorbitol, mannitol, sucrose, glucose and fructose were eluted at retention times 8.511 min., 14.97 min., 8.944 min., 9.06 min.,

11.837 min., 7.894 min. and 7.222 min. respectively. Individual compounds were identified based on their retention times compared to the standards.

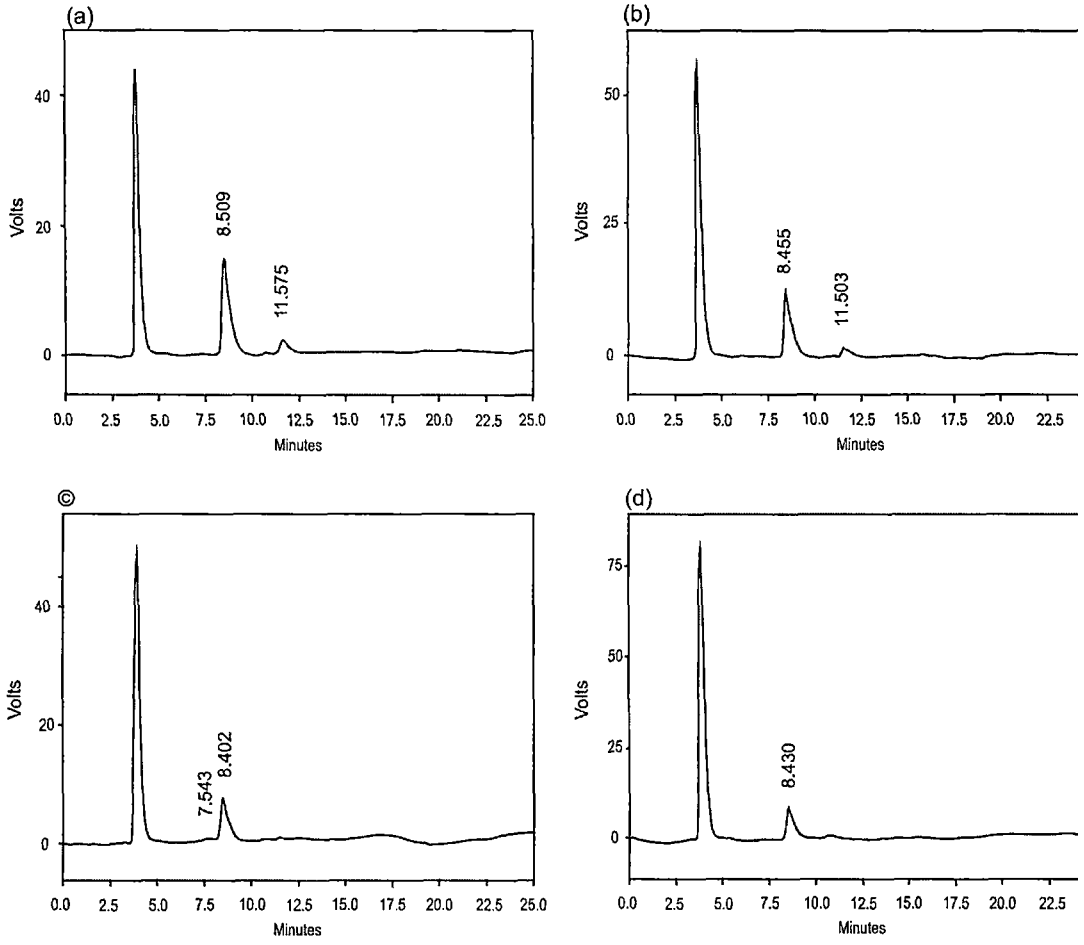


Fig.3.1. The chromatographic profile of *Hevea* clones. (a) &(b) C-serum of RRII 105 and RRIM 600, (c) & (d) A-serum of RRII 105 and RRIM 600.

From the peak area of the sample and the standard, the percentage of individual compounds was calculated based on the weight of the lyophilized sample (Table 3.2). It was found that there was fairly large amount of L-quebrachitol, sucrose and glucose in these samples. Myo-inositol, mannitol and sorbitol were not found in samples from both the *Hevea* clones.

Quebrachitol and sucrose were found in the C-serum of both the clones. A-serum of RRII 105 showed the presence of quebrachitol and glucose where as A-serum of RRIM 600 showed only quebrachitol.

Table.3.2. Percentage of quebrachitol, sucrose and glucose in C- and A-sera of *Hevea* clones, RRII 105 and RRIM 600

Compound	C-serum		A-serum	
	RRII 105	RRIM 600	RRII 105	RRIM 600
Quebrachitol	24.57	30.77	12.92	26.92
Sucrose	3.06	4.35	-	-
Glucose	-	-	1.22	-

3.3.2 Isolation, identification and quantification of L-quebrachitol from *Hevea* latex serum

The latex serum (alcoholic extract) was purified as per the methods developed (Table 3.1). Two methods (Table 3.1) were tried for the purification of L-quebrachitol from latex serum. There was no much difference in resolution of the isolated compound by these methods. Plant extracts are complex in nature as it contains various compounds likewise the latex serum is also complex in nature. The serum purification steps were efficient in both the methods however, the second method was better because of the reduction of the steps involved (Table 3.1).

The chromatogram developed using the latex serum without any purification steps showed resolution of a large number of compounds (Fig.3.2). A number of workers have reported the presence of various soluble

non-rubber components in latex serum (Bealing, 1969, 1981; Jacob *et al.*, 1989; Lau, 1993). This shows the complexity of the latex serum and the need of an appropriate purification method.

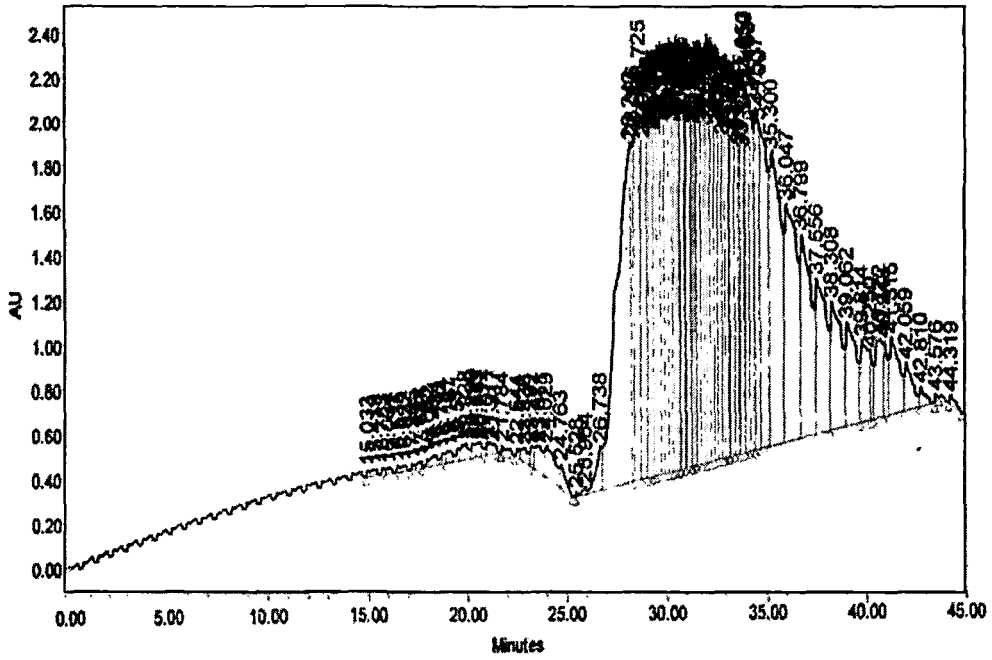
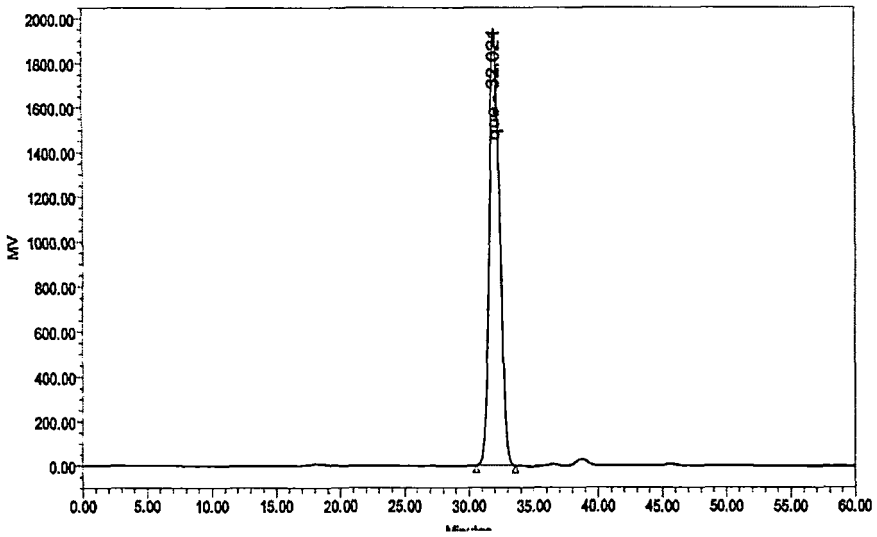


Fig.3.2. The chromatographic profile of the unpurified *Hevea* latex serum

A single peak was detected in the HPLC chromatogram (Fig.3.3) of samples processed through purification steps as in Table 3.1 indicating that the isolated compound does not contain any other components /contaminants. The isolated compound was identified as L-quebrachitol by comparing its retention time with that of the standard (Sigma). The chromatogram obtained for the sample and the standard by HPLC analysis was given in Fig.3.3. The retention time of standard L-quebrachitol (Sigma) was 32.021 minutes. The sample L-quebrachitol obtained from natural rubber latex serum was found to have a

retention time of 31.896 minutes. Isolation of L-quebrachitol from *Hevea* latex serum was successfully accomplished by following the method developed. The identity and purity of the compound was confirmed by standard techniques.

(a)



(b)

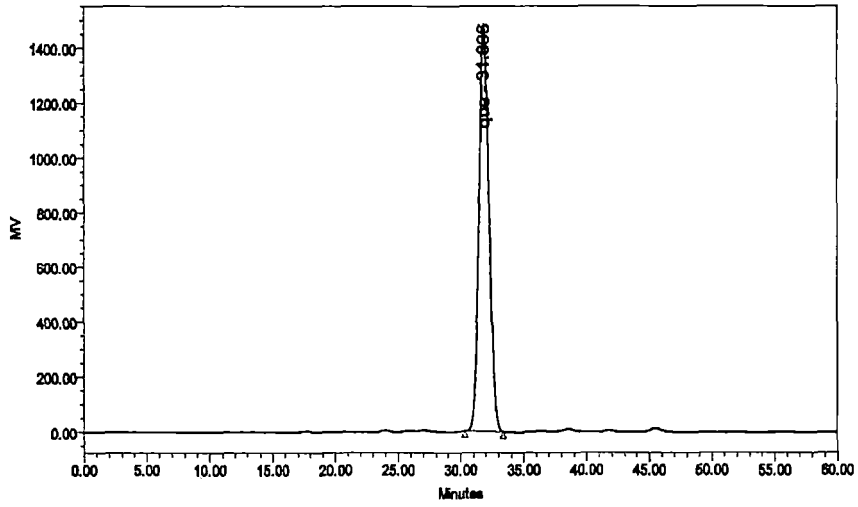


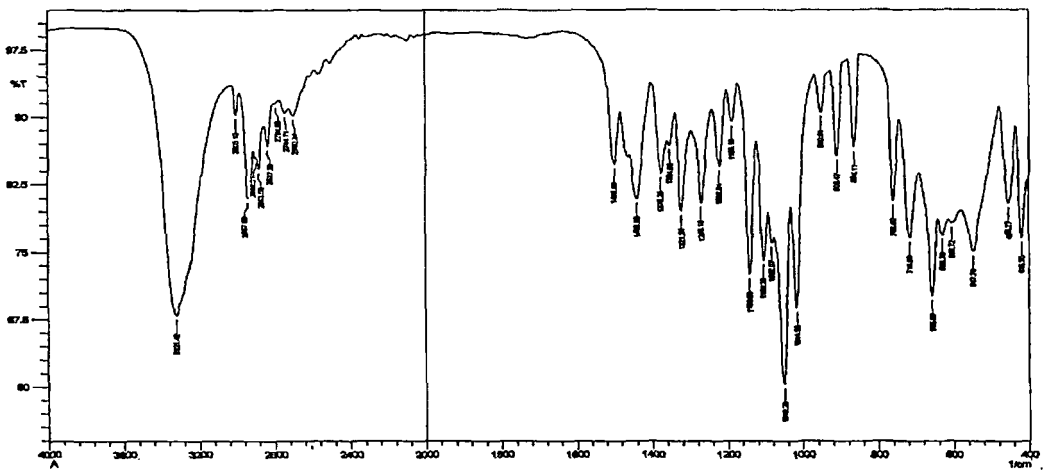
Fig.3.3. HPLC of L-quebrachitol (a) standard, (b) sample isolated from *Hevea* latex

3.3.3 Characterization of the isolated compound

3.3.3.1 FT-IR analysis

The identity and purity of the isolated compound was confirmed by FT-IR analysis. The spectrum of the standard (Fig.3.4a) and that of the compound isolated from *Hevea* latex (Fig.3.4b) showed the presence of identical absorption bands.

(a)



(b)

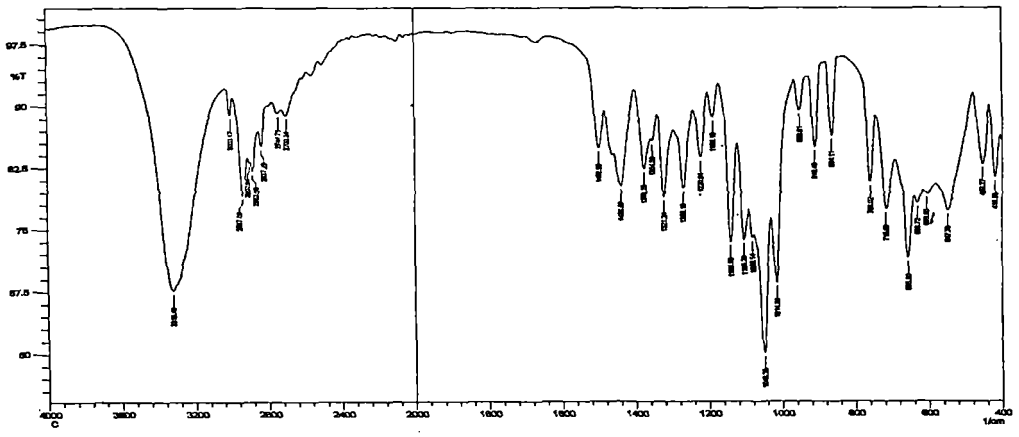


Fig.3.4. FT-IR Spectrum of L-quebrachitol (a) standard, (b) sample isolated from *Hevea* latex

The IR spectrum of the isolated compound was found super imposable with that of the standard L-quebrachitol. The characteristic absorption bands (3331 cm^{-1} representing the -OH stretching, 2939 cm^{-1}, 2928 cm^{-1}, 2901 cm^{-1}, 2882 cm^{-1}, 2835 cm^{-1} representing the six C-H bonds and one methyl group) and finger prints for L-quebrachitol exist in the spectrum of isolated compound from *Hevea* latex (Fig.3.4b).

3.3.3.2 High Performance Liquid Chromatography-Mass Spectrometry (LC/MS/ESI)

LC-MS analysis was done to find out the molecular weight of the isolated compound. LC-MS analysis was done using Alliance HPLC system with Quattro Premier XE Mass detector (Waters). The isolated compound was found to have a mass of 193.13 Dalton which was eluted at about 7.82 minutes and that of standard 7.75 minutes (Fig.3.5). The chromatograms of both standard and the isolated compound in continuum mode were shown in Fig.3.6. The LC-MS analysis (Fig.3.7) revealed that the molecular weight of the isolated compound was 193.10 and was matching with the molecular weight of the standard L-quebrachitol (193.13).

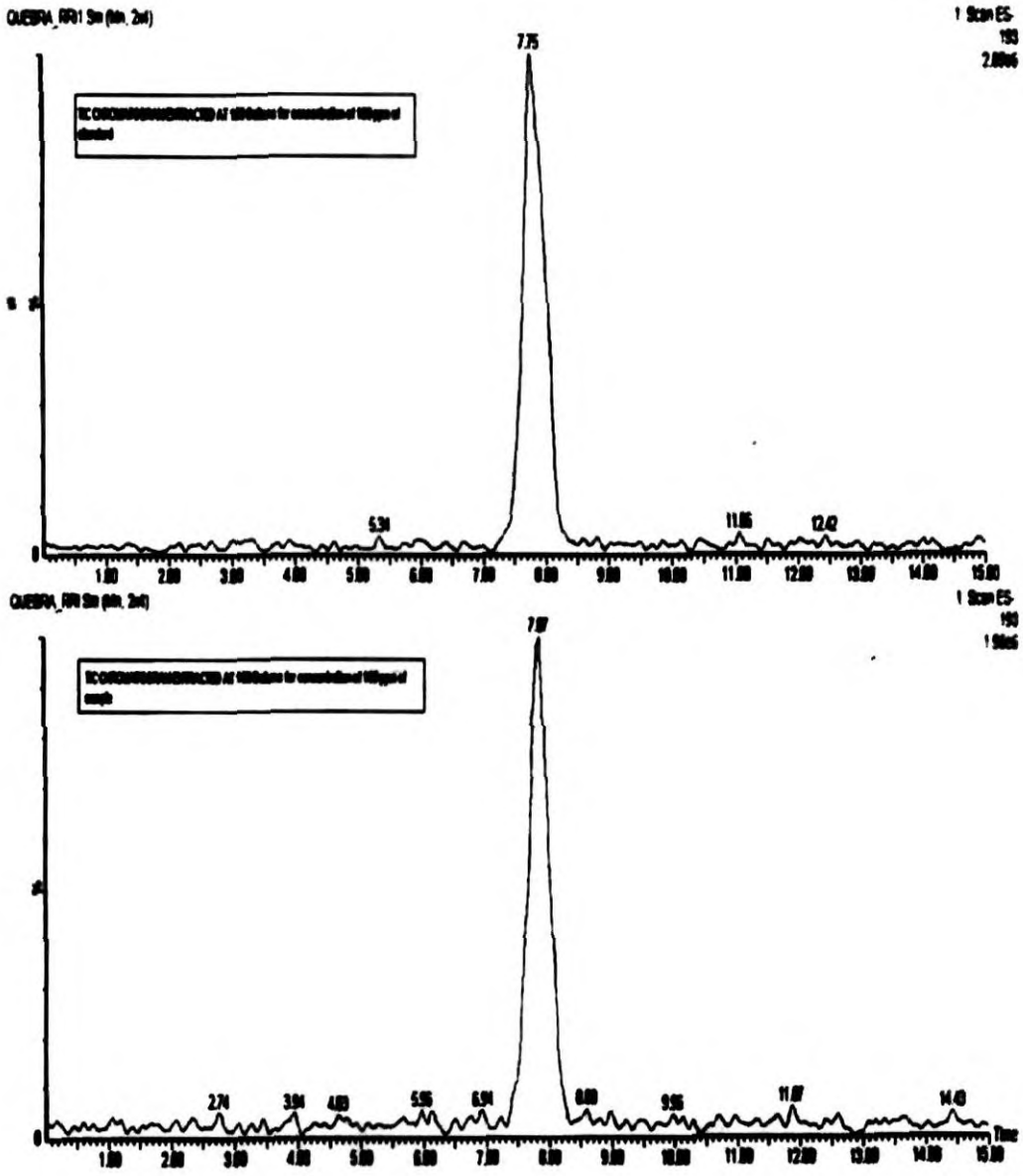


Fig.3.5. The chromatographic (LC/MS/ESI) profile of total ionic content of L-quebrachitol (a) standard, (b) sample isolated from *Hevea* latex

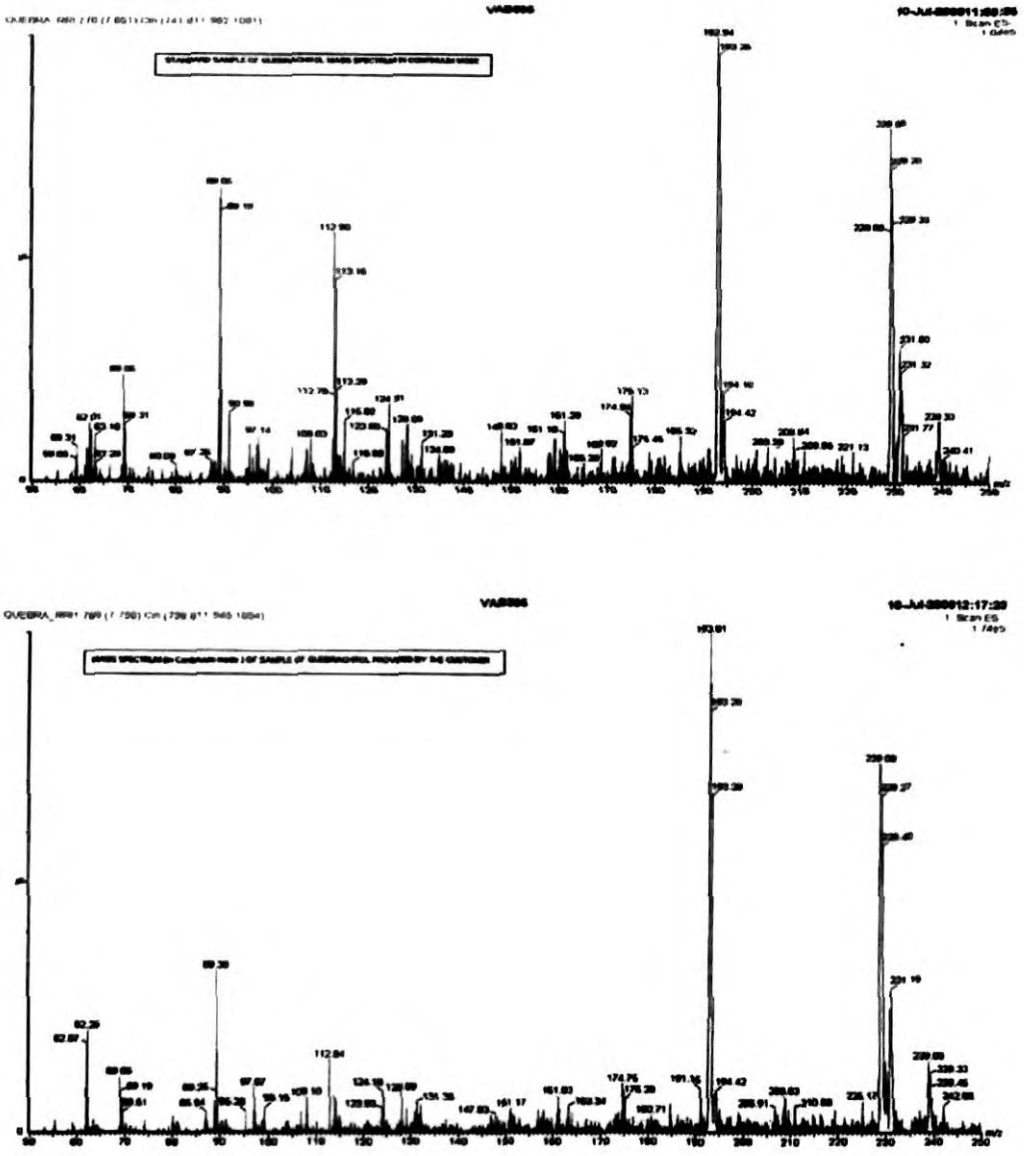


Fig.3.6. LC/MS/ESI spectrum of L-quebrachitol in continuum mode (a) standard, (b) sample isolated from *Hevea* latex.

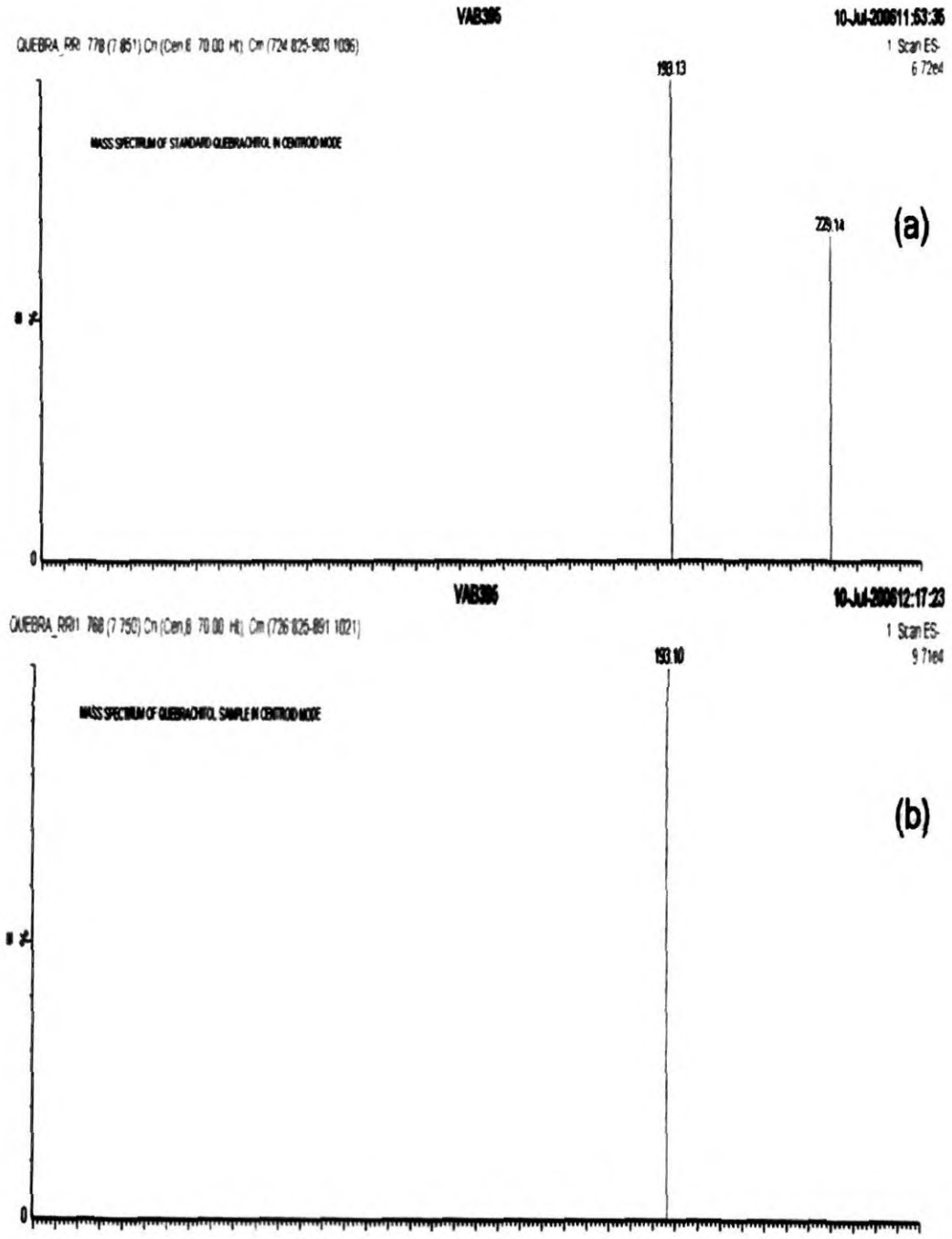
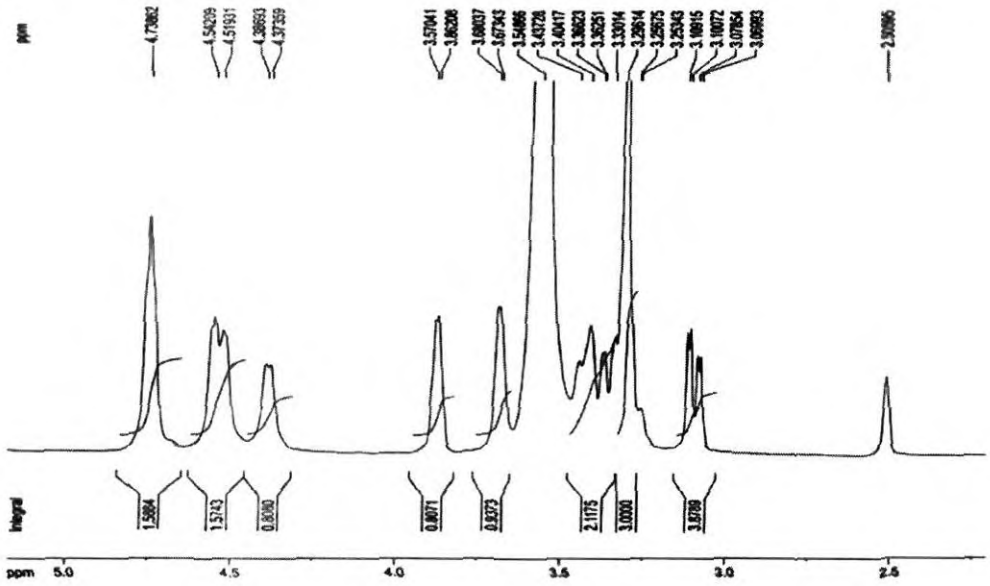


Fig.3.7. LC/MS/ESI spectrum of L-quebrachitol in centroid mode (a) standard, (b) sample isolated from *Hevea* latex.

3.3.3.3 NMR analysis

NMR characterisation of isolated compound was further done using ^{1}H-NMR and ^{13}C-NMR spectroscopy. The NMR spectra were obtained in a DPX 300, Bruker spectrometer operating at 300 Hz for ^{1}H and 75 Hz for ^{13}C using DMSO–d6 as solvent. The ^{1}H-NMR spectrum exhibited the signals as depicted by the chemical shifts similar to standard (Fig.3.8). The spectrum of the isolated compound was compared with spectrum of standard quebrachitol purchased from Sigma Aldrich. The spectrum showed presence of three protons at δ 4.35-4.33, δ 3.86-3.85 and δ 3.67-3.66 (doublet), δ 3.1-3.06 (double doublet), δ 4.51- 4.47, δ 4.70- 4.67 (multiple shifts). A singlet signal at δ3.3 indicated the presence of one methoxy group and the rest are six carbohydrate protons. The ^{1}H-NMR revealed that both the isolated compound and standard spectrum had identical chemical shifts. The ^{1}H- NMR spectra of the isolated compound coincided with those of authentic L-quebrachitol (Fig.3.8). Therefore, the isolated compound was identified as L-chiro methyl inosoitol (L-quebrachitol). The ^{13}C-NMR spectrum of the isolated compound showed the presence of 7 well resolved signals (Fig.3.9). δ 56.71 denote one C-O primary carbon and six anomeric carbons are represented by δ 68.09, δ 70.77, δ 72.19, δ 72.35, δ 73.38 and δ 81.21.

(a)



(b)

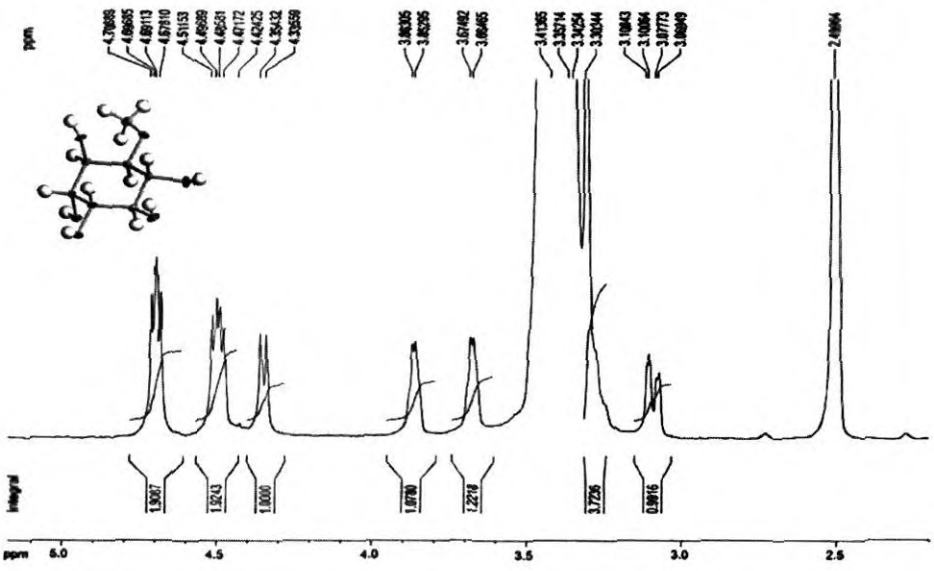


Fig.3.8. ^{1}**H-NMR spectrum of L-quebrachitol (a) standard, (b) sample isolated from *Hevea* latex**

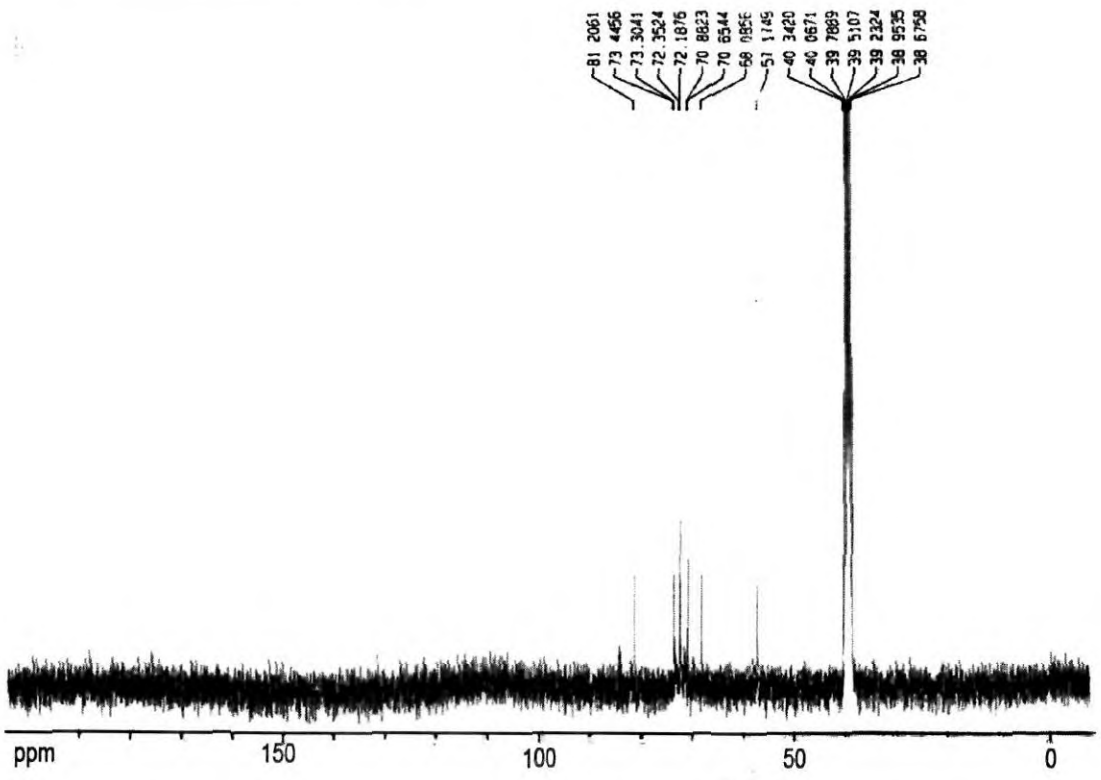


Fig.3.9. ^{13}C-NMR spectrum of L-quebrachitol isolated from *Hevea* latex

3.3.4 Recovery of L-quebrachitol from different *Hevea* clones and latex serum extraction methods

The results revealed that the protocol developed was suitable for the isolation of L-quebrachtiol from the sera obtained from different type of latex extraction methods. The chromatographic profiles obtained by HPLC analysis of quebrachitol isolated from different *Hevea* clones and serum sources are found to have good resolution (Fig.3.10). Similar chromatograms were also obtained for quebrachitol isolated from other serum sources. It was found that all the isolates have only one peak at the retention time obtained for the standard quebrachitol. Through these tested technique, it was proved

that isolates have only one pure compound and showed a similar trend for all other types of isolates.

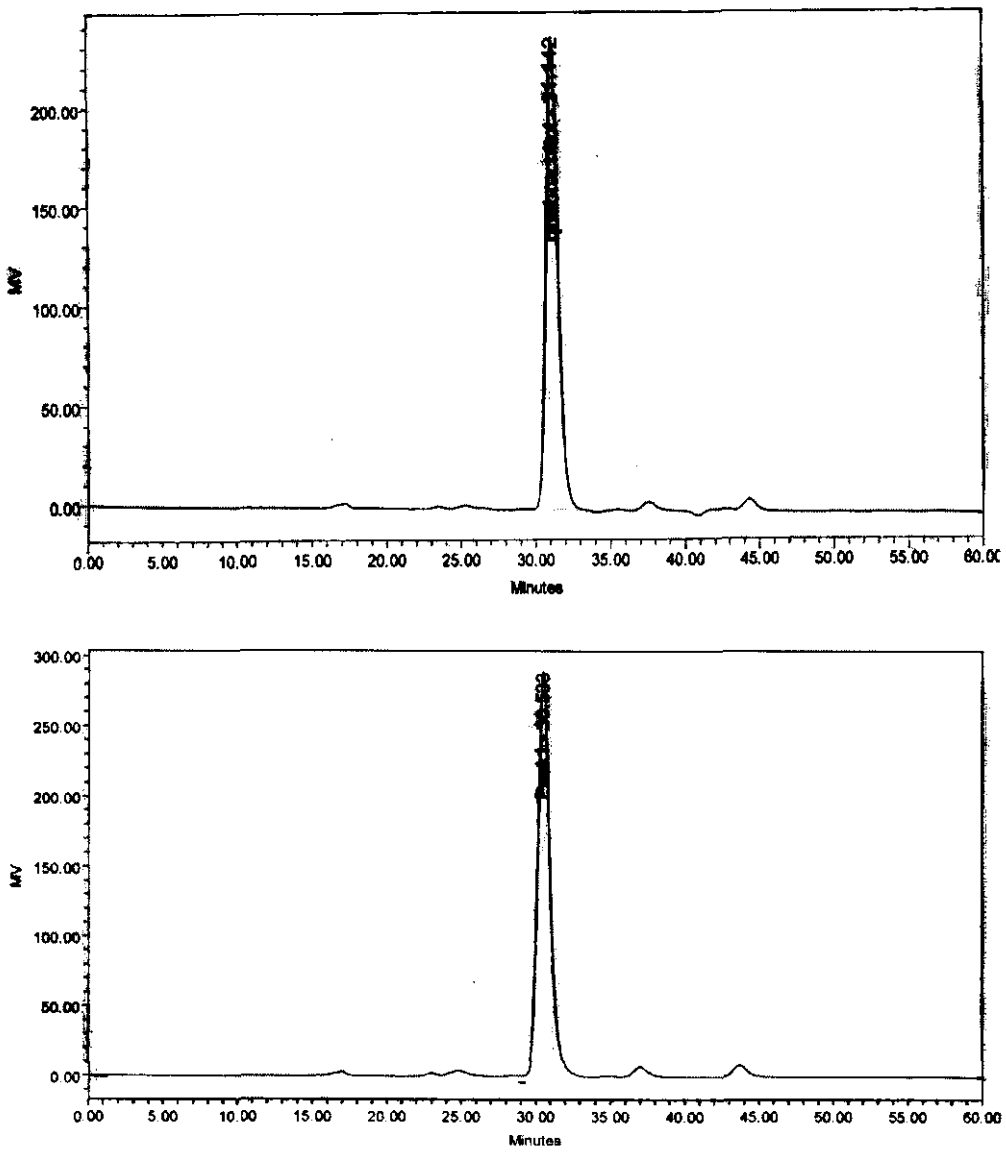


Fig.3.10. The chromatogram of L-quebrachitol isolated from *Hevea* latex of different clone (a) RRII 430 C-serum, (b) RRII 414 C-serum

Purity and identity of the isolated compound was further confirmed by standard techniques like FT-IR (Fig.3.11), LC/MS/ESI (Figs.3.12, 3.13 and 3.14) and NMR. It was found that all the isolates have only one peak at the retention time as that of the standard. Through standard techniques, it was proved that isolates have only one compound and showed a similar trend for all type of isolates.

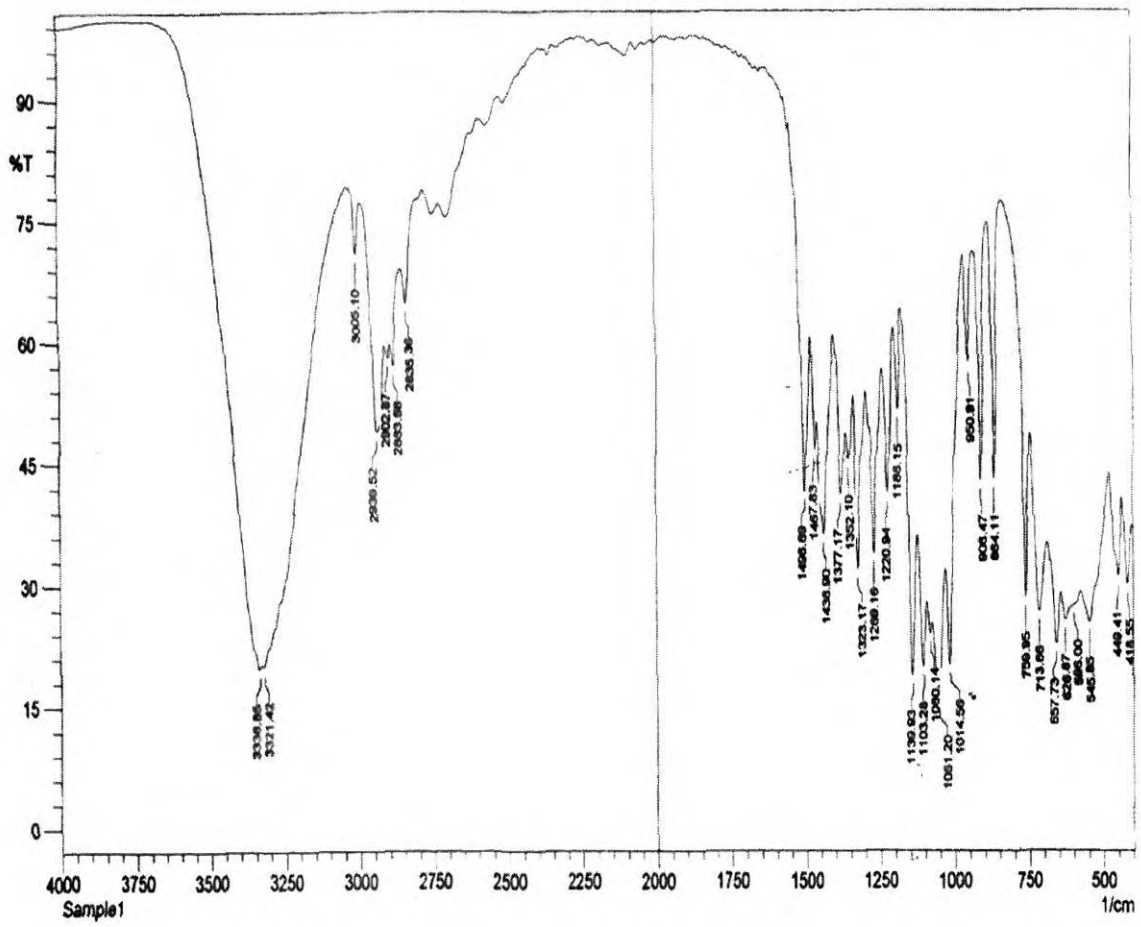


Fig.3.11. FT-IR Spectrum L-quebrachitol isolated from *Hevea* latex of RRII 430

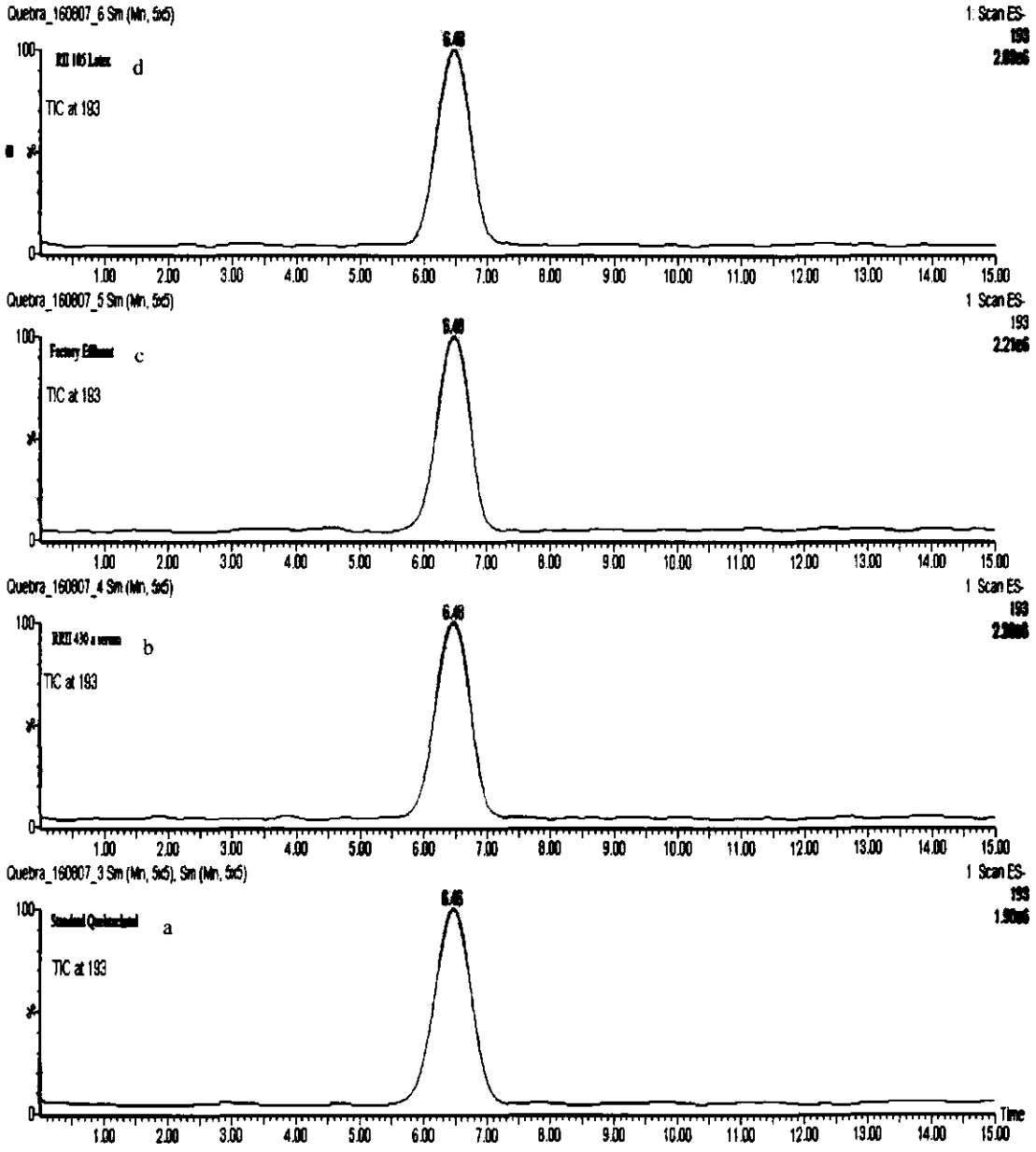


Fig.3.12. LC/MS/ESI profile of total ionic content (a) standard L-quebrachitol, samples isolated from (b) *Hevea* clone RRII 430 A-serum, (c), factory effluent, (d) RRII 105 latex

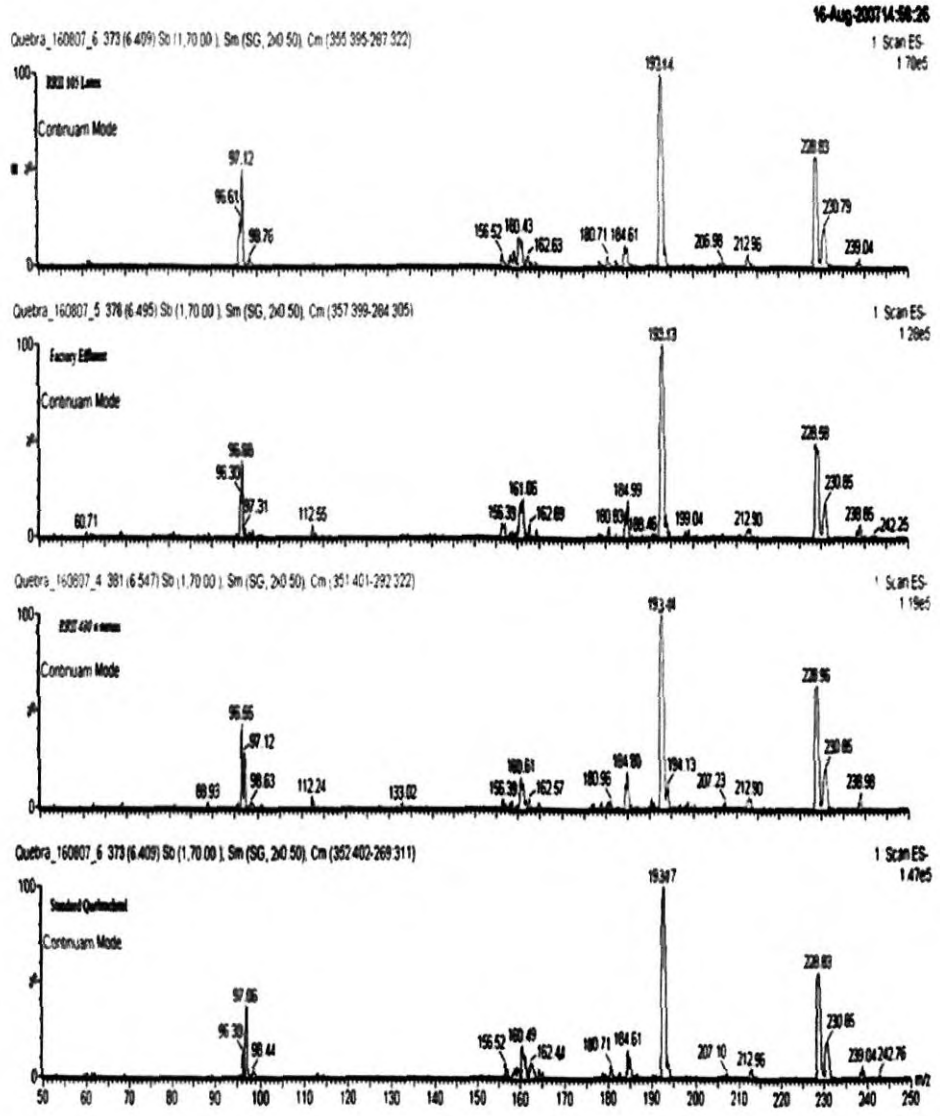


Fig.3.13. LC/MS/ESI spectrum of L-quebrachitol in continnum mode (a) standard, samples isolated from (b) *Hevea* clone RRII 430 A-serum, (c), factory effluent, (d) RRII 105 latex

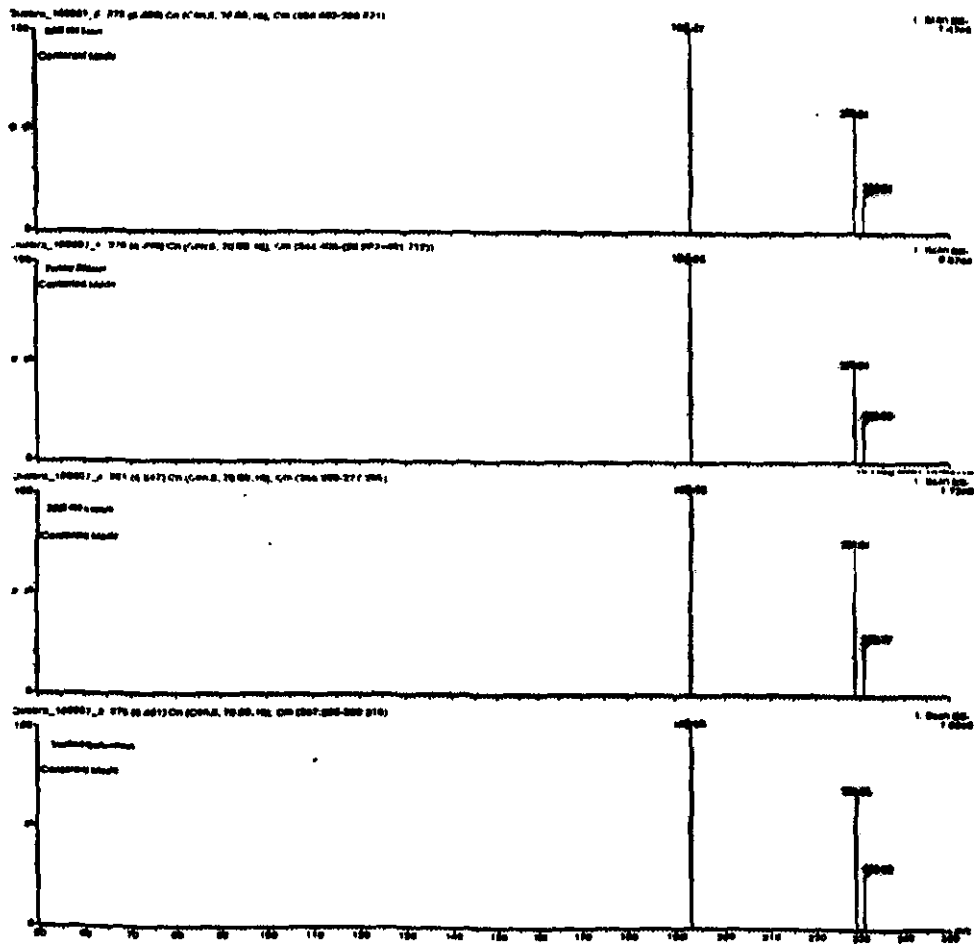


Fig. 3.14. LC/MS/ESI spectrum of L-quebrachitol in centroid mode (a) standard, samples isolated from (b) *Hevea* clone RRII 430 A-serum, (c), factory effluent, (d) RRII 105 latex

Methods of serum extraction were found to have direct effect on the yield of quebrachitol from the latex sera. Analyses indicated that a higher

extraction rate (percentage of recovery) of quebrachitol was obtained from the C-serum (1.41% w/w) followed by A-serum (0.48% w/w) (Table 3.3). The average rate of extraction of quebrachitol was 1.29% (w/w) using serum obtained by centrifugation, while the remaining methods yielded 0.22% to 0.27%. The clone RRII 430 was superior to other clones and gave the highest recovery rate (1.41%) by centrifugation method. The alcoholic extraction yielded the same percent (0.15%) as reported earlier for fresh latex (Yaojun *et al.*, 2004).

Table.3.3. Clonal variation in the recovery of L-quebrachitol under different extraction methods

Clone	Percentage of quebrachitol				
	Acid coagulation (A- serum)	Alcoholic extraction	Centrifugation (C-serum)	Cold treatment of latex	Factory effluent
RRII 105	0.14	0.15	1.25	0.12	
RRIM 600	0.12	0.13	1.26	0.12	
RRII 414	0.27	0.28	1.25	0.22	
RRII 430	0.49	0.50	1.41	0.43	
Average	0.26	0.27	1.29	0.22	0.005

The amount of quebrachitol recovery from factory effluent was very low (0.005%). It may be due to high amount of preservatives (ammonia) added for the prolonged storage of latex and dilution of serum resulted by the water used in NR processing. C-serum and A-serum obtained from RRII 430

showed higher amount of extractable quebrachitol. In general RRII 400 series clones recorded a relatively higher percent of quebrachitol than other *Hevea* clones studied.

3.5. Discussion

Hevea latex contains a variety of organic and inorganic non-rubber components in addition to rubber. They can be readily harvested through the tapping of latex from the tree. Among the non-rubber components, inositols form an important organic group. L-quebrachitol is the most abundant inositol in latex and is indirectly involved in latex production. It is an optically active inositol of potential utility to mankind. Quebrachitol is a raw material for inositol derivatives. Inositol derivatives have crucial role in cell signalling mechanisms; hence it is used in pharmacological and medical research field (Kiddle, 1995; De Almedia, 2001). By suitable chemical modifications other special inositol derivatives of potential application can be synthesized from this compound *e.g.* Valienamine, Cyclophilitol, Conduritol, Aminocyclitols, Isoavenaciolide, Oudemansin X, Bengamide E, *etc.* (Lau, 1993: Kiddle, 1995; Sakdapipanich, 2005).

Isolation of quebrachitol from natural rubber serum has been reported from 1932 onwards (Woodman and Rhodes, 1932; Rhodes and Wiltshire, 1932; Yaojun and Donghua 1999). However, so far it was practically not viable, in which latex serum from smoked rubber factories was used for

extraction and no much details have been reported about the purity of the isolated compound. A few earlier reports were available for the extraction of quebrachitol from natural rubber serum and synthesis of medicines (Lau, 1993; Yaojun *et al.*, 2004). But in these reports, the extraction and isolation procedures were not clearly mentioned.

Recently there were some patent applications (CN 103058834A; CN 20131075778; CN 103724459A; CN 102516041A) submitted for methods of extracting quebrachitol from rubber waste water and skim serum. All of them include heating the waste water and removal of floccules, vaccum distillation, utilization of full fllm separation technology, decolouration, extraction with ethyl acetate or ether to remove lipids and repeated crystallisation using ethanol. The application CN 102557892A relates to extraction of quebrachitol from natural rubber latex. The process involves centrifugation, solidification, concentration, precipitation, filtration and decolourisation using activated carbon column. There after concentration of the eluate, silica gel treatment, dispersion, drying and refluxing with methanol for 3-5 times and each time 1-2 hours. The combined extracts were concentrated under pressure at 60^0C. Further crystal precipitation by cooling and recrystallisation. There was wastage of resources, low product quality and requirement of high energy by these methods. Meng *et al.,* (2014) discloses a method, which involves concentration of rubber from rubber waste water by

adding acetic acid, solidification, filtration and sterilization to get concentrated whey. Thereafter column chromatography has been devised using silica gel for purification. During this process whey concentrate was sonicated with methanol and mixed with silica gel. The solvent after evaporation has to be passed through silica column of 200-300 mesh and has to be eluted using chloroform as elutent followed by a mixture of chloroform, methanol and acetic acid. The sub fractions after thin layer chromatography has to be combined and dried under reduced pressure to get quebrachitol crystals. These processes require column chromatography purification which involves toxic solvents as elutent in purification steps and the energy consumption is also very high.

There is wide variety and molecular ranges for carbohydrate compounds including inositols. Most suitable method for their separation and detection has to be considered during analysis. Alcholic extraction of carbohydrate compounds including polyols was found suitable for identification and quantification by many studies (Yaojun *et al.*, 2004; Duguesnoy *et al.*, 2008; Sanseera *et al.*, 2012). Cyclitols from Carob pod extract were isolated using Dowex 1-X8 anion exchange resin (100-200 mesh) column and purified by passing through a column of Dowex 50w-XA cation exchange resin (50-100 mesh) in lithium form and eluted with 87 % ethanol (Stephanie, 1986). A similar trend with modification was followed

for the separation of pinitol, myo-inositol and chiro-inositol (Streeter, 1998; 2001a). Methanolic extract of the root of *Elegnus formosana* Nakai yielded L-quebrachitol (Huang and Luo, 1994). L-quebrachitol was obtained from macerated twig of *Allophyllus edulis* through alcoholic extraction (Daiz *et al.*, 2008). Myo-inositol and methyl-inositols (L-quebrachitol) were isolated by alcoholic extraction from CO_2 extraction residue of sea buckthorn berries (Yang *et al.*, 2009; 2011). L-quebrachitol, was extracted from *Mitrephora vulpina* (Moharam *et al.*, 2010). Aerial part of *Artemisia sodiroi* Hurm, was extracted and fractionated using hexane, ethyl acetate and methanol. It was found that the methanolic fraction contains L-quebrachitol (Briceño *et al.*, 2011). Alcoholic extraction (ethyl alcohol) of latex samples was carried out for isolation of L-quebrachitol from *Hevea* latex and found appropriate owing to the elimination of toxic solvents (Fig.3.3). Isolation of L-quebrachitol from *Hevea* plants was more advantageous because of its relatively large presence in latex and collection along with rubber by tapping compared to the destructive collection of plant materials in other plant groups.

In the present study to accomplish the objectives of standardization of an appropriate method for the isolation, purification and quantification of L-quebrachitol from latex serum, first of all, the complete resolution of all the inositol components was carried out. Here the serum samples, the lyophilised C and A-sera of both the *Hevea* clones (RRII 105 and RRIM 600) revealed

the presence of L-quebrachitol and glucose in the HPLC chromatogram (Fig.3.1). Concentration of samples through lyophilisation was tried as a purification step in this study. A fairly large amount of L-quebrachitol, sucrose and glucose were found in these samples. There may be chances of a small quantity of impurities after partial purification using an aminopropyl column (Waters Spherisorb NH_2 column). Further, separation and identification of individual carbohydrates in C-serum and A-serum was accomplished (Fig.3.1).

Liquid chromatography is commonly used for the separation, identification and quantification of complex mixtures of compounds based on their polarities and interaction with a stationary phase and gradient change between aqueous and organic mobile phase with time (Gajewski *et al.*, 2009; Koyuncu and Dilmacünal, 2010). Chromatographic methods are the most powerful analytical technique for the analysis of different types and concentration of sugars and related compounds because of its rapid, specific, sensitive and precise measurements. These compounds are separated based on their partition coefficients, polarity, size and depending on the type of the column used. Therefore in the present study liquid chromatography was carried out for separation, identification and quantification.

The column composition in liquid chromatography is very important for the development of methods for the analysis of different metabolite

classes depending on their nature. The majority of compounds are separated using a reverse-phase (RP) C_{18} column where, hydrophobic compounds are eluted at the end using a predominantly organic phase (acetonitrile/methanol) (Kuhlmann *et al.*, 1995). Polar compounds are not readily retained on such stationary phases. The reverse phase (RP) or non-polar stationary phase are packed with porous silica particles coated with non-polar material (styrene-divinylbenzene copolymers) (Bakry *et al.*, 2006). Relatively very polar molecules like sugars and amino acids can be separated using a column packed with porous graphite carbon or other hydrophilic interaction chemistries. Thus, it is not possible to separate the whole metabolite profile on a single LC-chromatographic system/column. Consequently, to get the most out of metabolite profiling, a combination of methods is needed to identify different classes of metabolites (Garcia-Plazaola and Becerril, 1999; De Vos *et al.*, 2007; Pan *et al.*, 2008; Giavalisco *et al.*, 2009). Moreover the accurate quantification of metabolic compounds is very much dependent on the resolving capabilities of the column used and sample processing. Separation of carbohydrates in dairy products was achieved with Aminex HPX-87 carbohydrate column (Richmond *et al.*, 1982). Separation of all inositol isomers using calcium (Ca^{2+}) form cation exchange resin as stationary phase and water as mobile phase by liquid chromatography was found efficient (Sasaki *et al.*, 1988). Resolution of myo-inositol and related substances from biological samples was achieved with Aminex HPX-87 carbohydrate column

using milliQ water as the mobile phase (Perello *et al.,* 2004). In the present study the identification and quantification L-quebrachitol was conducted using Shodex sugar SC1011 column with water as mobile phase and sample processing as per the methods developed previously (Gopalakrishnan *et al.*, 2010). Shodex sugar SC1011 column was found more appropriate for the separation of L-quebrachitol in the present study.

The complexity of the latex serum was evident from the HPLC chromatogram (Fig.3.2) developed without purification steps and it necessitates an appropriate purification method. HPLC profile (Fig.3.3) of samples processed by adopting the purification steps developed (Table 3.1) revealed only a single peak, indicating that the isolated compound is devoid of any components or contaminants present in the serum. The isolated compound was identified as L-quebrachitol by comparing its retention time with that of standard. The retention time of standard L-quebrachitol (Sigma) was 32.021 minutes and that of the sample L-quebrachitol obtained from natural rubber latex serum was found to have a retention time of 31.896 minutes (Fig.3.3).

Two methods were followed and found that both the methods were suitable for purification of the compound from the serum. Between these methods, the second method was found better than the first one due to reduction in steps involved in purification (Table 3.1). Generally crystallization

is a part of purification so the isolated compound was crystallized for obtaining pure solid form. The crystallization process from a solution involves the initial nucleation of the material to be crystallized, its growth to the final size or crystal size distribution and recovery from solution. Crystallizing inositol from a solution is a straight forward procedure of concentration of inositol in water at an elevated temperature, *via* evaporation, or some other means of water removal, followed by temperature reduction to initiate nucleation. Crystallization of L-quebrachitol from *Hevea* serum showed same trend and was found more appropriate. Complete evaporation of eluate at 80^0C followed by cold shock was found suitable for crystallization of the isolates.

The identity and purity of the isolated compound was established through FT-IR spectrum. The spectrum (Fig.3.4 a and b) showed strong wide peak at 3331 cm^{-1} representing the overlap of stretching vibration absorption peaks of five hydroxyl groups and the inter and intra molecular hydrogen bonding effects. The multiple absorption peaks at 2939 cm^{-1}, 2928 cm^{-1}, 2901 cm^{-1}, 2882 cm^{-1}, 2835 cm^{-1} were denoted by the six C-H bonds and one methyl group. Absorption peak at 1139, 1102, 1086,1063,1048,1015 cm^{-1} corresponds to the strong stretching vibrations of five C-OH, C-OCH and C-O bonds. The IR spectrum of the isolated compound was found super identical with that of the standard L-quebrachitol. The complex lower region of the spectrum is referred as finger print region. It was called so because

almost every organic compound produces unique pattern in this region. Therefore the identity can often be confirmed by comparison of this region to a known spectrum. The characteristic and distinct finger prints for L-quebrachitol exist in the spectrum of isolated compound from *Hevea* latex. This confirms the identity of the isolated compound in absolutely pure form as both the sample and standard spectra had exactly identical dips at particular wave numbers. The finding was in accordance with earlier reports (Huang and Luo, 1994; Briceño, 2011; Jiang *et al.*, 2014)

High Performance Liquid Chromatography (HPLC) coupled to mass spectrometry (MS) is very sensitive and an obvious approach for the analysis of targeted and untargeted metabolites. It is a rapid and robust technology. Mass spectrometry is an analytical technique which in combination with GC or LC is used for measuring a multitude of molecules in biological and non-biological samples. The advantage of LC-MS over other spectroscopic techniques (such as NMR) is that it putatively annotates the spectral components of a compound based on their mass and retention time. The molecular mass of the lower molecular weight organic compounds can be measured within an accuracy of 5 ppm or less which is sufficient for the characterisation and putative identification of a compound (de Hoffmann and Stroobant, 2007).

T206

Different methods are available for the ionization of the compound to be analysed. The selection of the ionization technique depends on the nature of the analysis. The most commonly used ionization techniques for biological mass spectrometry are atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) (de Hoffmann and Stroobant, 2007). These ionization methods work in both positive and negative mode, depending on the chemical nature of the compounds under investigation. ESI is described as a soft ionisation technique, as it produces ions with very low internal energy and therefore minimal chances of analyte fragmentation. The fragments of desired ions are separated based on their mass to charge ratio (*m/z*). The separated ions are detected as an electronic signal converted into a digital output in the format of *m/z* and abundance. These are presented as mass spectra. ESI method of ionisation is good for polar, charged molecules and ions. In the present study ESI technique was adapted for ionization of the compound isolated form latex serum.

The isolated compound (L-quebrachitol) was found to have a mass of 193.10 Dalton which was eluted at about 7.82 minutes and that of standard 7.75 minutes (Fig.3.5). The molecular weight of the compound is 194.13. In the present study mass detection with ESI in negative mode was adopted. Thus the LC/MS/ESI analysis (Fig.3.7) revealed that the molecular weight of

the isolated compound was 193.10 and matching with that of the standard L-quebrachitol (193.13).

The ^{1}H-NMR reveals that both the sample and standard spectrum had identical chemical shifts. The ^{1}H-NMR spectra of the isolated compound coincided with those of authentic L-quebrachitol (Fig.3.8). Therefore, the isolated compound was identified as L-chiro methyl inosoitol (L-quebrachitol). ^{1}H-NMR profiles is a good strategy to assign the molecular structure of the sample in the experimental measurements based on the comparison of ^{1}H-NMR chemical shift data (De Almeida *et al.*, 2012). The ^{13}C-NMR spectrum of the isolated compound showed the presence of 7 well resolved signals (Fig.3.9). δ 56.71 denote one C-O primary carbon and six anomeric carbons represented by δ 68.09, δ 70.77, δ 72.19, δ 72.35, δ 73.38 and δ 81.21. The compound confirmation as L-quebrachitol was done in comparison with standard and earlier reports (Diaz *et al.*, 2008; Sanseera *et al.*, 2012; De Almeida *et al.*, 2010; 2012; Jiang *et al.*, 2014).

Various extraction procedures were adopted by several workers for the isolation of quebrachitol. Studies on recovery rate of quebrachitol from different *Hevea* clones and varying extraction methods of latex serum revealed that the protocol developed was suitable for the isolation of quebrachtiol from the sera obtained from any type of latex extraction methods (Figs.3.10 and 3.11). Purity and identity of the isolated compound

was further confirmed by standard techniques like FT-IR (Fig.3.11), LC/MS/ESI (Fig.3.12, 3.13 and 3.14). It was in conformity with earlier results (Diaz *et al.*, 2008; Sanseera *et al.*, 2012; De Almeida *et al.*, 2012; Jiang *et al.*, 2014).

Isolation of a substance from its sources is one of the uses of extraction to obtain the compound for some end use or as a preliminary step for further analytical procedures. In the present study, methods of serum extraction were found to have direct effect on the final yield of quebrachitol from the latex sera. A higher extraction rate (percentage of recovery) of quebrachitol was obtained from the C-serum (1.41% w/w) followed by A-serum (0.48% w/w). The average rate of extraction of quebrachitol was 1.29% (w/w) using serum obtained by centrifugation, while the remaining methods yielded 0.22% to 0.27%. A very low rate of quebrachitol recovery from factory effluent was recorded (0.005%). It might be due to high amount of preservatives and prolonged storage of latex and very high serum dilution by processing activities. It was found that C-serum and A-serum obtained from RRII 430 showed higher amount of extractable quebrachitol. In general RRII 400 series clones had a relatively higher percent of quebrachitol than other *Hevea* clones studied. The popular clone RRII 105 is cultivated in more than 80% of the traditional rubber growing region whereas RRIM 600 is mainly cultivated in the non-traditional region. Therefore, a comparative

account of recovery rate of quebrachitol in these clones was worked out. The recovery rate of quebrachitol was almost similar when the latex was processed through alcoholic coagulation or through cold treatment of latex in clones RRII 105 and RRIM 600. The average recovery of quebrachitol from the latex of RRII 105 and RRIM 600 by alcoholic extraction was 0.15 and 0.13% (w/v), respectively whereas it was 0.12% for both clones by cold treatment and comparable with acid coagulation method (Table.3.3).

The added latex preservatives have generally affected the extraction rate of quebrachitol (Bennett, 1969). Comparatively a smaller recovery of quebrachitol was observed in the factory effluent sample. Preservatives such as ammonia could have changed the non-rubber constituents in the latex, affecting the colloidal stability and thereby hindering the release of some non-rubber constituents such as quebrachitol. Thus the very low rate of quebrachitol recovery from factory effluent may be due to serum dilution during NR processing, high amount of preservatives and prolonged storage of latex. The addition of chemical preservatives and duration of preservation were reported to have a profound effect on the extractable amount of quebrachitol from latex. The ammonia content of the preservatives is the key factor determining the preserving effect on latex. Latex can be preserved effectively only if the ammonia content of the latex was maintained more than 0.01% (Yaojun *et al.*, 2004).

Inositols/polyols are cyclohexane compound existing as nine stereoisomers. Among these isomers myo-inositol is the precursor of other inositols, *O*-methyl inositols and inositol derivatives. The most studied of all inositols is myo-inositol because of its wide availability (Clement and Darnell, 1980; Loewus *et al.*, 1984; Streeter *et al.*, 2001a; 2001b; Chiera *et al.*, 2006; Manchanda and Garg, 2008). Commonly found *O*-methyl inositol forms are ononitol and pinitol. Ononitol is the precursor of pinitol (Loewus and Murthy, 2000; Chiera *et al.*, 2006). Different plants produce varying proportions of different types of inositols.

Among the non-rubber components (water, carbohydrates, proteins, inorganic acids, lipids, *etc.*) of latex of *Hevea* (Smith, 1954; Lowe, 1961), inositols form a rich group of carbohydrates, especially quebrachitol (Bealing, 1981). Considerable quantities of inositols are available in the latex of *Hevea* which are now go completely unutilized and discarded as waste material after taking the rubber from latex. Potential availability of the raw material for large scale production of inositol (quebrachitol) from latex has been worked out and found encouraging. The present research work provides a suitable method for the isolation of L-quebrachitol in a pure form from *Hevea brasiliensis* latex serum. A process patent (IP: 238511 dt. 09/02/2010) was obtained for the methodology of L-quebrachitol isolation from latex serum. There exists a vast potential for exploiting this commercially high

value secondary metabolite in large scale. An efficient and quick extraction method and identification of appropriate serum source would give opportunities to use natural rubber latex as a source for the valuable pharmaceutical compounds like quebrachitol, which would fetch additional income to the stakeholders apart from rubber. The potential utility of L-quebrachitol as a chiral compound in asymmetrical organic synthesis was well established (Akiyama *et al.*, 1992; Kiddle, 1995; Akiyama, 1996). But the importance of L-quebrachitol in various physiological functions is not yet clearly studied. In this respect, the mechanism and factors influencing the action of the compound to control diseases/amelioration of stresses are a new research area, which needs indepth studies.

Chapter 4

Water relations of *Hevea* latex in relation to the content of inositols and other osmolytes

4.1 Introduction

Hevea brasiliensis, the major source of natural rubber accounts for about 99% in the World. There is an increasing demand for natural rubber. The traditional belt of India faces the problem of land constraints for the extension of rubber cultivation. The only scope of extension of cultivation is the non-traditional regions *i.e.* North-eastern states, Konkan and some pockets of Central India. These areas are prone to adverse environmental conditions such as drought, cold and high light intensities.

The merits and demerits of extension of rubber cultivation to marginally suitable regions are already reported (Pushparajah, 1983). Environmental conditions like soil moisture deficit coupled with high temperature, high light intensities and low relative humidity are known to inhibit growth and productivity of rubber plants (Sethuraj *et al.*, 1989 Chandrashekar *et al.*, 1990; Ouseph *et al.*, 1990;; Baskar *et al.*, 1991; Mohanakrishna *et al.*, 1991; Rao *et al.*, 1998). The drought free traditional region of India experiences a water deficit of 450 mm. During summer period the girth increment was minimum in North Konkan parts (Chandrashekar *et al.*, 1996). Trunk shrinkage was observed in rubber trees under rain fed condition at North Konkan as a result of the negative turgor pressure experienced by the xylem vessels during peak summer (Chandrashekar *et al.*, 1996; 1998; Devakumar *et al.*, 1998). There were

clonal differences existing in the degree of drought tolerance capacity and irrigation responses in *Hevea* plants (Chandrashekar, 1997). Plant water status and maximum sap flux density was decreased under soil drought and tapping during dry season (Kunjet *et al.,* 2013; Annamalainathan *et al.*, 2013). Under low temperature conditions in non-traditional regions the girth increment of *Hevea* trees was minimum (Chandrashekar *et al.*, 1998; Annamalainathan *et al.*, 1998; Devakumar *et al.*, 1999).

Harvesting of latex from *Hevea* trees is carried through tapping the tree bark. When *Hevea* tree is tapped, the latex vessels are opened up and the latex exudes from the vessels by hydrostatic pressure. Eventually there is pressure drop in the laticifers and thereby water from the surrounding cells enters into the laticifers and results in dilution of the latex and continuous flow of latex. This leads to osmotic imbalance in the laticifer tissue and bursting of lutoid particles present in the latex which accelerates coagulation of latex. Latex flow from the tree is controlled by plant water status (Buttery and Boatman, 1976). With respect to xylem sap flow rate and gradual recovery of turgor after tapping, diurnal variation of phloem turgor pressure was observed (An *et al.*, 2014a; 2014b). The latex flow was inhibited by water stress and water vapour deficit of the atmosphere (Paaradekooper and Sookmook, 1969; Buttery and Boatman, 1976; Sethuraj *et al.*, 1984;

Paikanathan *et al.*, 1989; Rao *et al.*, 1990, Vijayakumar *et al.*, 1988; 1991; Dey *et al.*, 1999).

Latex flow during tapping results in movement of solutes and water from the surrounding tissues into the laticifers. The water transfer within laticifers indicates that the cytoplasmic medium maintains a sufficiently negative osmotic potential. It was reported that turgor pressure of laticifers and surroundings cells are maintained by sugars, polyols and potassium (Buttery and Boatman, 1966). According to d'Auzac and Jacob (1989) the osmoticum of *Hevea* latex is maintained mainly by carbohydrate components. The daily growth maintenance and dehydration avoidance was associated with the loading of osmolytes at whole plant level, expression of aquaporins and regulation of transpiration (Junjittakarn *et al.*, 2012). During summer season there was significant reduction in latex yield but it was more in rain fed trees than irrigated trees (Vijayakumar *et al.*, 1998). The yield of latex during rainy and summer season is influenced by osmotic potential of B and C-sera of latex (Satheesan, *et al.*, 1982). It was reported that high yielding clones that had high rubber yield during summer maintained a high osmotic concentration in the C-serum of latex. The capacity of the tree to overcome the fluctuation in osmotic environments in latex influences the yield of latex from the tree (Raghavendra *et al.*, 1984).

Contribution of changes in osmotic potential towards drought and salt tolerance in *Eucalyptus* species have been studied by a number of workers (Ladiges, 1975; Clayton-Greene, 1983; Myers and Neales, 1986; Lemcoff *et al.*, 1994; Stoneman *et al.*, 1994; White *et al.*, 2000). Changes in the osmotic potential may be due to active accumulation of solutes *i.e.* osmotic adjustment (Turner and Jones, 1980; Chaves *et al.*, 2002) or through reduction of cellular water leading to increase in the concentration of constitutive solutes. Primary metabolites (sugars *i.e.* sucrose, glucose or fructose) contributes substantially to the osmoticum. Major inorganic ions contributing to osmotic potential are K^+, Na^+, Ca^{2+}, and Mg^{2+}. Cyclitols such as quercitol (Paul and Cockburn, 1989), pinitol (Nguyen and Lamant, 1988) and quebrachitol (Popp *et al.*, 1997) are important groups of compatible solutes wide spread in woody plants.

Hevea latex analyses revealed that quebrachitol concentration is about 1.2% (w/v) while the sucrose concentration is 0.4% (w/v) (Bealing, 1969; 1981). Osmolytes other than the organic solutes present in the latex are the mineral components. They also act as activators/ cofactors of enzymes in the rubber biosynthetic pathways. Many of the key latex biosynthetic enzymes require magnesium (Mg^{2+}) as cofactor (d'Auzac, 1965). Magnesium is therefore considered as one of the physiological factors related to rubber yield (d'Auzac and Jacob, 1989). The high level of magnesium and

Mg/Pi ratio was reported to be associated with early coagulation of latex at the tapping panel (Beaufils, 1957). Potassium (K^+) is the major component of mineral elements in the latex. The role of phosphorus and potassium in latex stability has been reported by Philpot and Garth (1953). Potassium is involved in the activation of tonoplast pyro phosphatase and pyruvate kinase and is a major mineral element in the latex (Jacob *et al.,* 1989). Yield and rate of latex flow were found to be enhanced by K^+ application (Watson, 1989). Moreover, K^+ is one of the major osmolyte of latex and it is reported as one of the factors for the changes in osmotic potential under water stress in *Hevea* (Karyudi, 2004). The present study was focused on quantification of total inositol contents in the latex of *Hevea* clones to understand the clonal/ seasonal variation, if any in the levels of inositols and effect on latex water relations through their involvement in osmotic potential. The study was also aimed at understanding the seasonal status of other osmolytes including the inorganic ions such as magnesium (Mg^{2+}), calcium (Ca^{2+}), potassium (K^+) and phosphorus (P) in the latex and their contribution to the water relations leading to latex yield in different *Hevea* clones.

4.2 Experimental details

4.2.1 Clonal and seasonal variation in total inositol content

Fresh latex samples were collected from thirteen *Hevea* clones *viz.* RRII 105, RRII 208, RRII 118, RRII 5, RRII 300, RRII 308, RRIM 600,

RRIM 703, SCATC 93/114, SCATC 88/13, PR255, PR 261 and HN1 planted in the field of RRII. The trees were 8 years old and the latex was harvested through tapping under S/2 d3 6d/7 systems without stimulation. Latex samples were collected from five replications with three trees per clone.

4.2.1.1 Estimation of total inositol content

Approximately, 1g of latex sample was mixed with 1 ml of 80% alcohol (1:1 w/v) and the serum was separated after removing the rubber particles through filtration from the coagulated latex. The residue was re-extracted with 80% alcohol after boiling for 30 minutes. The ethanol extracts were pooled together, made up to 10 ml with 80% alcohol in stoppered measuring cylinder and used for the estimation of total inositol.

The total inositol content in latex was measured according to the method of Bernad *et al.*, (1958) as modified by Low (1978). About 0.1 ml of ethanol extract was evaporated to dryness and made up to 0.1 ml with distilled water. To this 2.5 ml of 1 M sodium acetate buffer was added followed by 0.3 ml of 0.01 M sodium metaperiodate and the absorbance was read at 260 nm using a spectrophotometer (Shimadzu, Japan). This reaction mixture was then heated at 65^0C for 2 hours and absorbance was read again at 260 nm after cooling the sample to room temperature. The difference in optical densities before and after heating the reaction mixture was due to the oxidation of inositol to formaldehyde. A standard curve was made using pure

myo-inositol as standard. The inositol content was calculated in each sample and expressed as mg/tree/tap (mg/t/t). The analysis of inositol content in the latex samples was carried out at specific intervals representing three seasons, *viz.* June to September (monsoon- S1), October to January (post-monsoon- S2) and February to May (summer- S3).

4.2.2 Latex osmolytes and water relations of latex

Hevea clones (RRII 43, RRII 118, PB 311, RRII 105, GT 1, RRII 308, RRIM 600 and GL1) which were classified as relatively drought tolerant and susceptible and in the 8th year of tapping at Rubber Research Institute of India (RRII), Kottayam were used for the study. The experiment was conducted in randomized block design (RBD) of five replications with three trees per clone. The observations were made during the periods of peak yielding (October to December) and stress season (summer months- February to May). All the trees were tapped under S/2 d2 6d/7 system. The sucrose, reducing sugars, free amino acids and inorganic ions such as potassium (K^+), magnesium (Mg^{2+}), phosphorus (P), and calcium (Ca^{2+}) were estimated. Total inositol content was also estimated from the latex of these *Hevea* clones as mentioned in section 4.2.1.

4.2.2.1 Estimation of sucrose content

Approximately 1.0g of latex was in taken a vial and 1 ml 2.5% trichloroacetic acid (TCA) was added. Latex was coagulated immediately

and the coagulum was squeezed with a glass rod. The extract was transferred into a 10 ml stoppered measuring cylinder. The extraction was repeated thrice with small volumes of TCA and transferred to the cylinder and made up to 10 ml with TCA. Shaken well and filtered through whatman No.1 filter paper. The filtrate was used for estimation of sucrose. Sucrose content was estimated according to the method of Scott and Melvin (1953).

An aliquot of the extract (0.1 ml) was pipetted in to each tubes and made upto 0.5 ml with 2.5%TCA. The tubes were cooled on ice bath for some time. Then 3 ml anthrone reagent was added to all the tubes. Mixed well and after thorough cooling all the tubes were heated in a boiling water bath for ten minutes and after cooling the absorbance was measured at 620 nm. Sugar content was calculated using standard graph plotted against sucrose and expressed as mg/100g latex.

4.2.2.2 Estimation of reducing sugars

Latex samples, approximately 1.0g were extracted with 1 ml of 80% alcohol (1:1 w/v) and heated on a water bath at 80^0C for 30 minutes. Extraction was repeated once with the rubber coagulum and the extract was collected in 10 ml stoppered measuring cylinder. The extract collected was made up to 10 ml with 80% alcohol and used for the estimation of reducing sugars according to standard method (Nelson, 1944). An aliquot of 0.5 ml was pipetted out into test tubes and completely evaporated on a water bath at

80^0C. After cooling 0.5 ml distilled water and 1 ml freshly prepared copper reagent were added and mixed thoroughly. The assay mixture was heated on a water bath for 20 minutes. After cooling 1 ml of arsenomolybdate solution was added and the reaction mixture was shaken well. It was made up to 25 ml after 15 minutes and the absorbance was read at 520 nm. Reducing sugar content was calculated using standard graph plotted against glucose and expressed as mg/100g latex.

4.2.2.3 Estimation of total amino acids

Total free amino acids were extracted and estimated by following the method of Moore and Stein (1948). One gram of fresh latex was extracted as mentioned in section 4.2.2.2. In a 25 ml test tube, 0.5 ml ethanol extract was taken and evaporated to dryness, cooled and 0.5 ml distilled water was added. 1 ml ninhydrin reagent was added into this and the assay mixture was boiled in a boiling water bath. After cooling 5 ml of diluent (water: propanol, 1:1 V/V) was added to each tube and the content was mixed thoroughly. The absorbance was read at 570 nm.

4.2.2.4 Estimation of inorganic ions

Latex samples from individual trees were collected in ice. 5g of the latex was taken in a vial and extracted with 5 ml 2.5% TCA. The extract was transferred into a 25 ml stoppered measuring cylinder. The extraction was repeated twice or thrice and the volume was made up to 25 ml. Appropriate

amount of aliquots were used for the estimation of inorganic ions. Magnesium (Mg^{2+}) and Calcium (Ca^{2+}) were estimated using an Atomic absorption spectrometer (Avanta-GBC Scientific equipment Company Ltd., Australia). Potassium (K^{+}) and phosphorus (P) were estimated using Auto analyzer (Auto analyzer 3 - Bran Luebbe, Germany).

4.2.3. Water relation parameters of latex

4.2.3.1 Estimation of osmotic potential

C-serum was collected through centrifugation of latex samples at 23,000 rpm for 45 minutes at 4^0C. Osmotic potential of the latex serum was determined using PSΨPRO Water potential System (Wescor, USA). Osmotic potential recorded in MPa were converted to bars (-bars) and then to osmotic concentration expressed as mOsmol/Kg (Kanta and Varshney, 2006) by using the following relationship.

-1MPa = -10 bar

-1 bar = 40 mOsmol/Kg

4.2.3.2 Contribution of solutes to osmotic potential

Osmotic contribution of organic solutes to osmotic potential was calculated by Van't Hoff's equation. The contribution of inorganic ions to osmotic potential was calculated based on Taiz and Zeiger, (1991). Accordingly the concentration of cations was converted into moles and from this osmolality was worked out by multiplying with factor 1.84. The osmotic

potential was then calculated by multiplying the osmolality with 2.48 (Kramer and Boyer, 1995). Latex yield from all the clones was recorded during peak yielding and stress season.

4.2.4 Latex yield

Latex yield was determined by measuring the whole latex collected from the tree by tapping and after cessation of the flow, expressed as volume of latex in ml/tree/tap.

4.2.5 Statistical analysis

The data were analysed by analysis of variance (ANOVA) and treatment means were compared by DMRT. The package used for analysis was SPSS version 10.0 for windows.

4.3 Results

4.3.1 Clonal and seasonal variation in total inositol content

In the present study inositol content in the latex of different *Hevea* clones showed significant clonal variations. The high inositol content was found in the latex of the *Hevea* clones such as RRII 105, RRII 5 and RRIM 703 followed by SCATC 88/13, RRII 208 and RRIM 600 and the low levels in HN1, RRII 300 and SCATC 93/114 clones (Fig.4.1). The metabolically superior and high active clones generally showed higher production of inositols. It may be noteworthy that metabolically active clones are generally high yielders of latex as well (Fig.4.2).

Apart from the clonal difference, there was seasonal variation in the levels of inositols in the latex. The inositol content was the highest in RRII 105 followed by RRII 703, SCATC 88/13 and the lowest in HN1 and SCATC 93/114 during monsoon season. The maximum inositol content was observed during post-monsoon in RRII 5, RRII 105 and minimum in SCATC 93/114. During summer season a higher content was noticed in PR 255, RRIl 105 and the lowest in SCATC 93/114 (Table 4.1). RRII 118 and SCATC 93/114 recorded a stable level and ranking with respect to the content of inositol in latex.

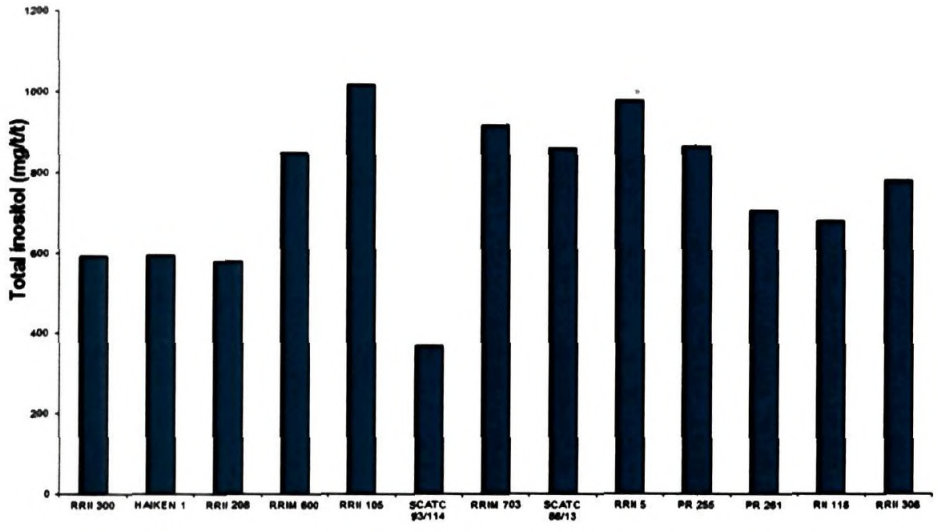


Fig. 4.1. Total inositol content in different *Hevea* clones

Table. 4.1. Clonal and seasonal variation in inositol content of different *Hevea* clones (mg/t/t) where S1, S2, S3 denotes the monsoon season, post monsoon and summer (stress) season

Clones	Seasons		
	S1	S2	S3
RRII 300	867.79	446.38	405.88
HN 1	693.38	583.58	419.04
RRII 208	1310.57	706.15	497.06
RRIM 600	1211.16	688.77	599.49
SCATC 93/114	569.20	227.6	271.53
RRII 105	1496.18	783.92	615.61
RRIM 703	1384.51	740.01	549.86
SCATC 88/13	1333.88	658.15	574.52
RRII 5	1311.65	1024.04	476.69
PR 255	1163.17	678.96	705.14
PR 261	1135.35	487.17	452.57
RRII 118	975.28	554.77	448.82
RRII 308	1103.7	626.9	583.21
F	4.65**	5.79**	2.05*
CD ($P \leq 0.01$)	363.1	222.9	223.9

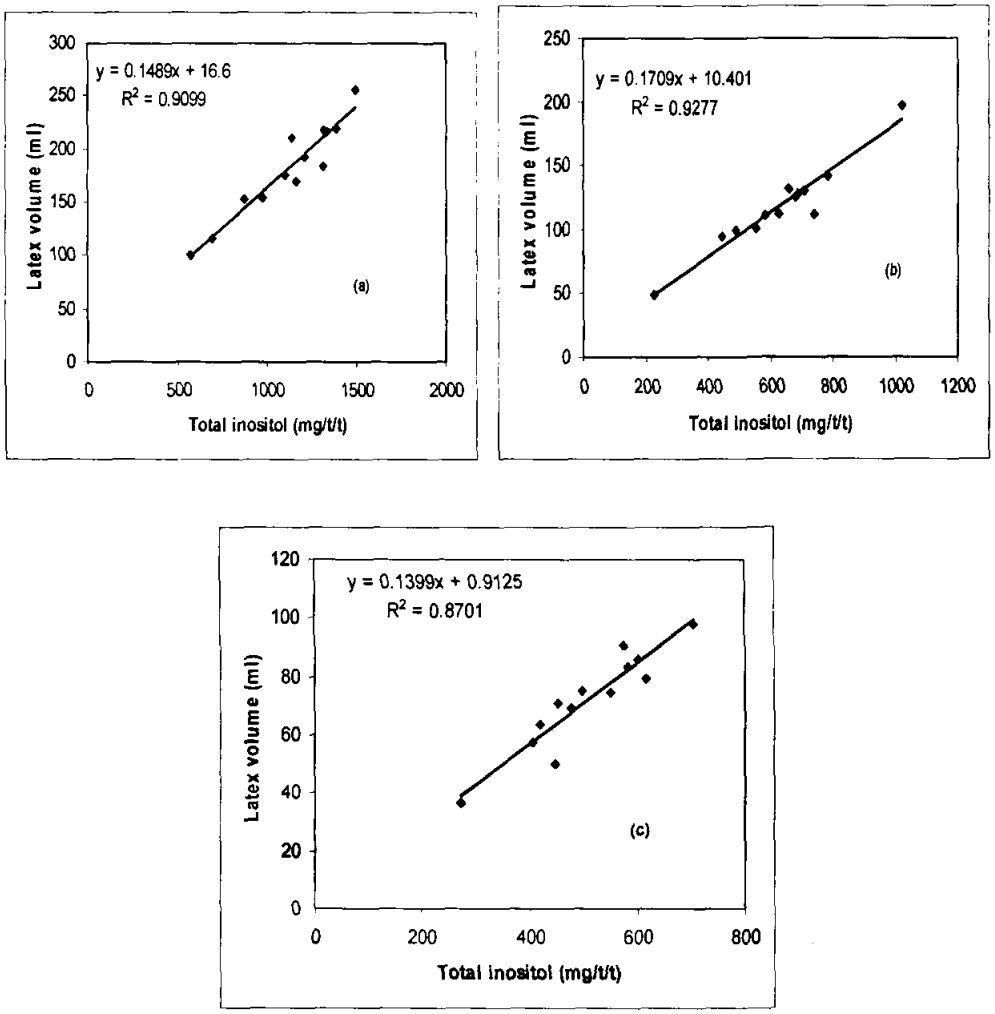


Fig. 4.2. Correlation of total inositol content with latex yield during different seasons (a) monsoon (b) post-monsoon (c) summer

4.3.2 Latex osmolytes and water relations of latex

The drought-induced changes in sugar concentrations of latex varied among *Hevea* clones. Concentrations of sucrose showed significant clonal, seasonal and clone x season interaction effect. The *Hevea* clone RRII 118 recorded relatively higher sucrose concentration during stress season. GL 1, RRII 308 and GT1 had high sucrose concentration than other clones (Fig.4.3a). Level of reducing sugar declined significantly from peak yielding to stress in clone RRII 308 whereas PB 311 showed a slight increase in the level during stress season (Fig.4.3b). There was considerable accumulation of total amino acids during stress season in all the *Hevea* clones and significantly higher level was observed in PB 311 clones from peak yielding to stress season (Fig.4.3c). Total inositol content was increased significantly from peak yielding to stress season (Fig.4.3d). *Hevea* clones RRII 105 and GT1 recorded high level of inositol content followed by RRIM 600 under stress season and clones RRII 118 and PB 311 were on par with each other for its concentration in latex. Inositol/cyclitol concentration in RRII 105 and GT1 was found to be the highest soluble solute fraction of latex high under stress season (Fig. 4.3d).

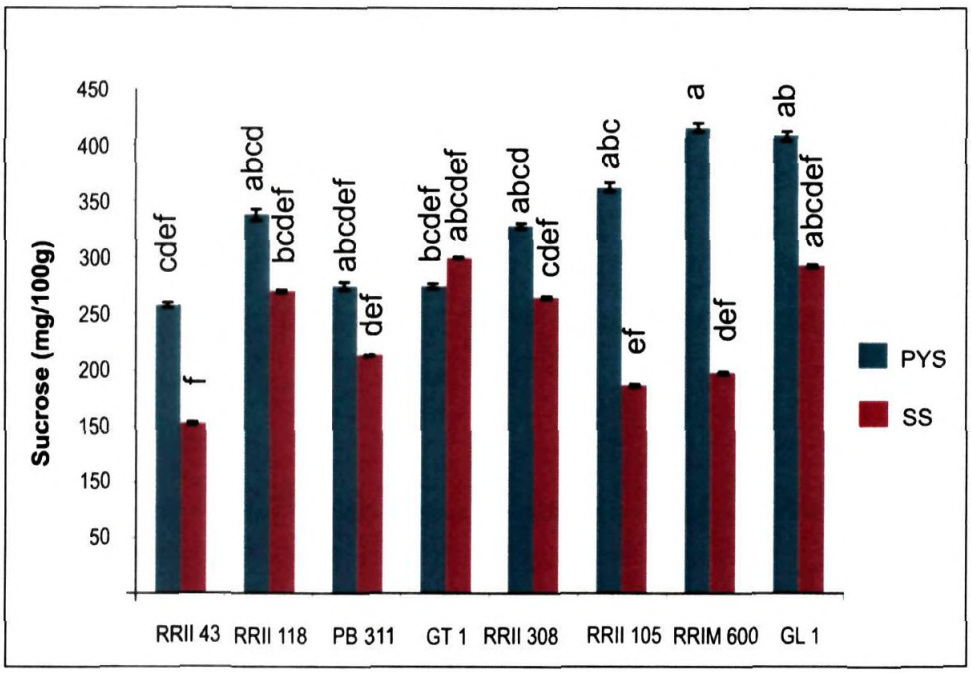
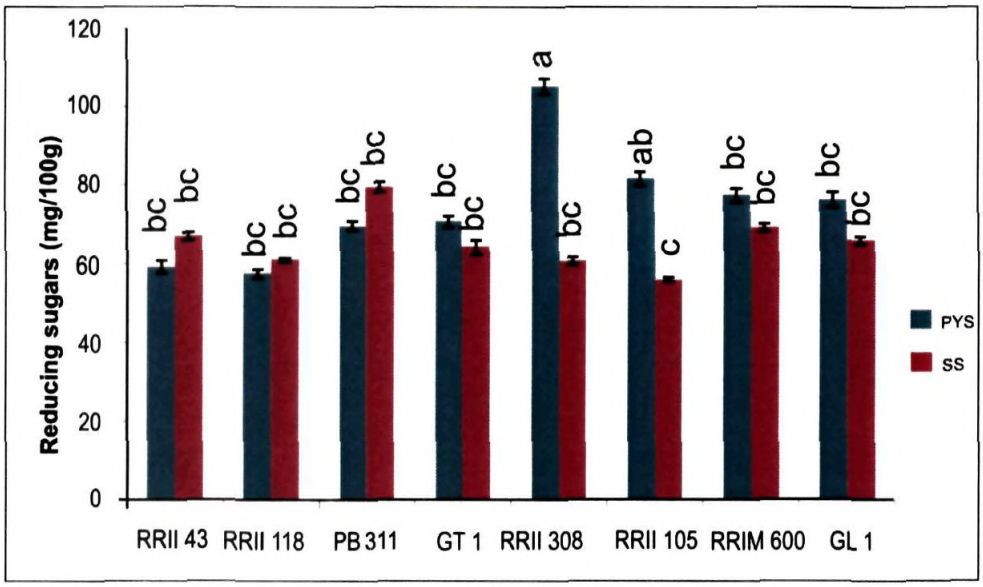
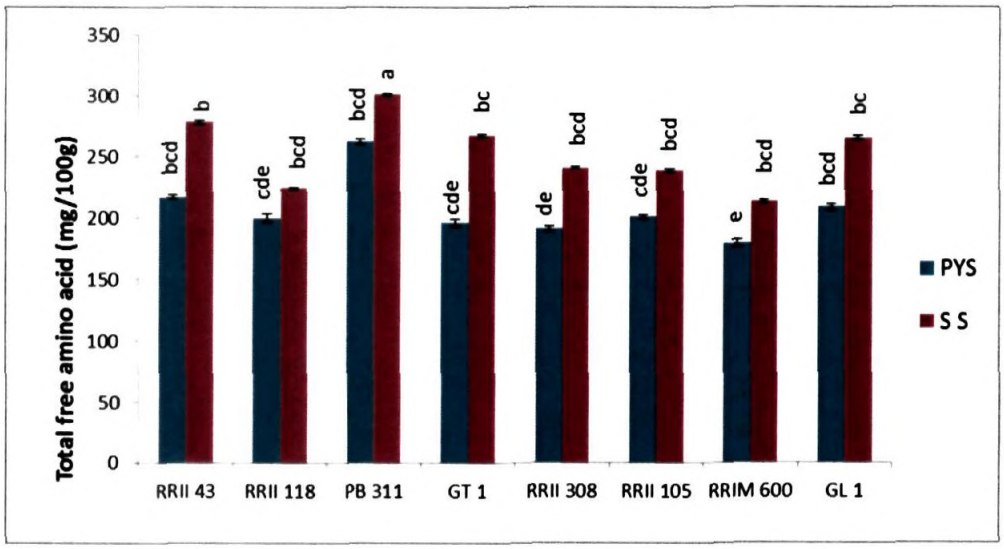


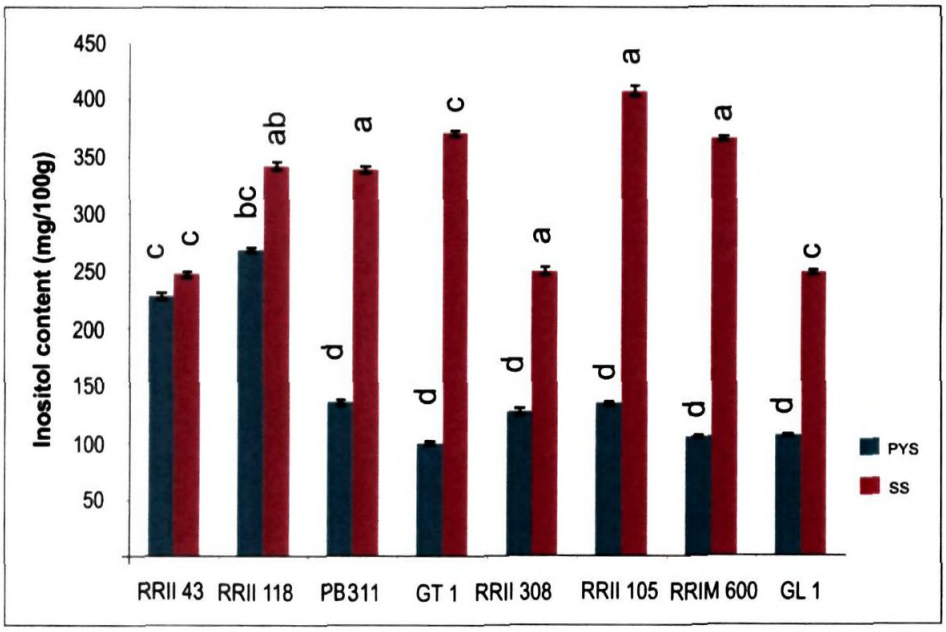
Fig.4.3 (a) sucrose



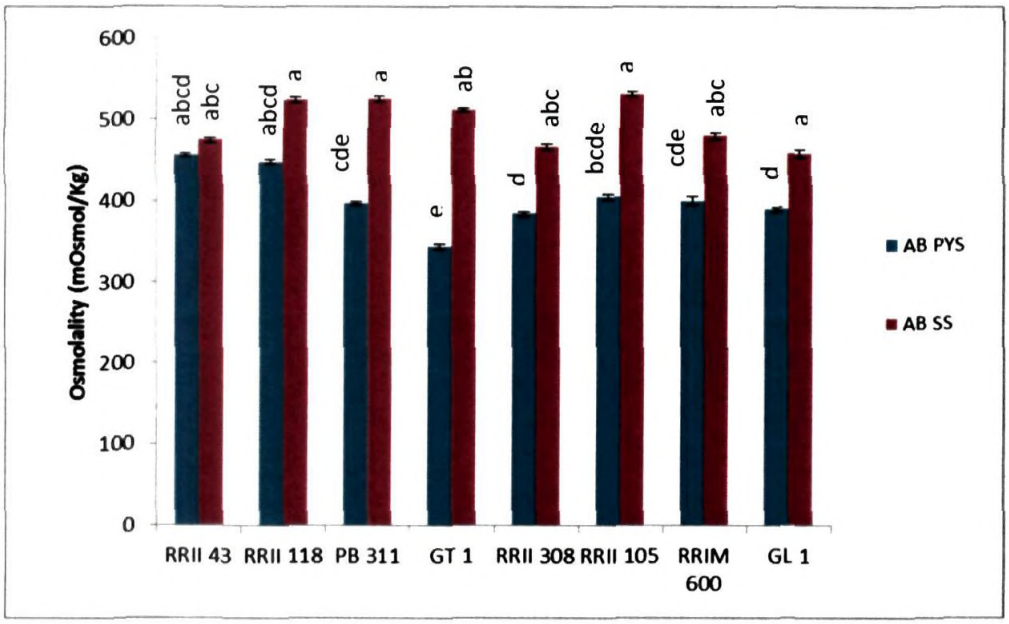
(b). Reducing sugars



(c). Total free amino acid



(d). Total inositol content



(e). Total osmolality

Fig. 4.3 Osmolyte concentration and total osmolality in latex of different *Hevea* clones during peak yielding (PYS) and stress seasons (SS) (a) Sucrose, (b) Reducing sugar, (c) Total free amino acid, (d) Total Inositol, (e) Total osmolality

DMRT was performed using SPSS for comparing clonal and seasonal variations. Significant at $P < 0.05$ level.

Latex ionic contents showed significant clonal variation (Table 4.2). A higher concentration was observed for inorganic ions such as $K,^{+}$ $Mg,^{2+}$ P during the peak yielding season where as Ca^{2+} showed high magnitude under summer months (stress season). Significant clonal variation and clone x season interaction was observed for the latex Mg^{2+} content. Ca^{2+} concentration was found low in latex compared to other cations studied.

the *Hevea* clones studied (Table 4.2). The latex calcium (Ca^{2+}) contents showed both clonal and seasonal variations. Significant clonal and sesonal variation and clone x season interactions were observed for latex P. The K^+ content, a major ionic component in the latex was relatively abundant in the latex of all the clones during peak yielding season. Latex K^+ content showed significant seasonal and clone x season interaction effect (Table 4.2). The latex K^+ concentration showed limited range of variation in GT 1 and GL 1 with respect to the season.

Table. 4.2 Latex ionic compositions during peak yielding and stress seasons in different *Hevea* clones

Clone	Peak yielding season				Stress season			
	K^+ (mg/100g)	Ca^{2+} (µg/100g)	Mg^{2+} (mg/100g)	P (mg/100g)	K^+ (mg/100g)	Ca^{2+} (µg/100g)	Mg^{2+} (mg/100g)	P (mg/100g)
RRII 43	212.43	68.75	56.39	21.455	161.98	117.17	29.36	16.622
RRII 118	208.11	91.48	32.5	27.626	211.56	100.12	37.43	18.835
PB 311	239.77	79.00	60.2	42.291	259.21	96.77	73.58	19.648
GT 1	257.68	55.38	45.44	34.189	257.13	67.77	38.68	39.277
RRII 308	268.05	59.03	60.89	34.434	254.94	80.36	68.01	29.948
RRII 105	240.56	62.05	76.73	39.359	231.67	96.05	57.71	29.046
RRIM 600	270.05	71.36	78.27	26.887	197.38	90.83	78.09	17.246
GL 1	254.69	36.66	59.38	20.378	251.47	66.76	67.86	10.343

K^+ CD (clone x season) at $P< 0.05 = 37.57$

Ca^{2+} CD (clone) at $P< 0.05 = 14.67$ CD (season) at $P< 0.05 = 7.34$

Mg^{2+} CD (clone x season) at $P< 0.05 = 17.8$

P CD (clone x season) at $P< 0.05 = 4.00$

4.3.2.1 Latex osmotic potential

Latex osmolality increased significantly in all the *Hevea* clones from peak yielding to stress season (Fig.4.3e). Osmotic concentration was found high during stress season than peak yielding season in all clones. The magnitude of reduction in osmotic potential was more in relatively drought tolerant clones under stress condition.

Table. 4.3 Osmotic potential, osmotic concentration, total latex yield and osmoregulation in different *Hevea* clones

Clone	Peak yielding season			Stress season			
	Osmotic potential (-bars)	Osmotic concentration (mOsmol/kg)	Latex yield (ml)	Osmotic potential (-bars)	Osmotic concentration (mOsmol/kg)	Latex yield (ml)	Osmoregulation (bars)
RRII 43	11.4 ± 0.37	456.1	61.8	11.88± 0.43	475.11	28.1	-0.48
RRII 118	11.21 ± 0.37	448.32	158.7	13.11± 0.75	524.32	70.6	-1.90
PB 311	9.93 ± 0.32	397.09	181.0	13.13± 0.7	525.36	131.7	-3.2
GT 1	8.61 ± 0.66	344.58	116.9	12.8 ±0.33	511.8	47.3	-4.19
RRII 308	9.62 ± 0.38	384.85	130.4	11.67 ±0.38	466.71	90.8	-2.05
RRII 105	10.13 ± 0.5	405.36	149.1	13.28 ±0.31	531.18	91.0	-3.15
RRIM 600	10.02 ± 0.97	400.86	89.5	12.00 ±0.73	480.23	40.7	-1.98
GL 1	9.75 ± 0.49	390.1	30.0	11.47 ±1.2	458.72	16.5	-1.72
CD at P < 0.05			49.46			36.31	

Osmotic concentration

CD (clone x season) at P< 0.05 = 67.43

Osmotic concentration of latex varied among the clones which had significant seasonal and clone x season effects (Table 4.3). The clones, RRII 43 and RRII 118 had higher osmolality than other clones during peak yielding season. Clones such as RRII 105, PB 311 and RRII 118 had the highest osmolality in stress season (summer) (Fig.4.3e). These clones were also shown to have increased tissue osmolality than other clones.

4.3.2.2 Contribution of solutes (osmolytes) to osmotic potential and osmotic adjustment

Osmolality means the measure of the number of particles present in solution and is independent of the size or weight of the particles and it is expressed as mOsmol/kg. The distribution of water among the different fluid compartments, particularly between the extracellular and intracellular fluids was determined by osmolality. It effects the distribution of water through the generation of osmotic pressure. *Hevea* latex contains number of soluble organic components which influences the latex osmolality. There was an observed reduction in the contribution of sugar molecules to the total latex osmolality in *Hevea* during summer season. Significant clonal, seasonal and clone x season interaction was observed to its contribution to total measured osmotic potential (Fig.4.4). High level of sucrose content was observed in RRIM 600 and GL 1 followed by RRII 105. The contribution to total osmotic potential by sucrose, reducing sugars and the content of inositol ranged from −0.35 to −0.64 and −0.48 to −0.72 MPa in peak yielding and stress seasons,

respectively (Fig.4.5). The percentage of contribution of sucrose ranged from 16% to 27.5% under peak yielding season and from 9% to 16.5% under stress season (Table 4.4). The magnitude of reduction of sucrose contribution to total latex osmolality was high in RRII 43.

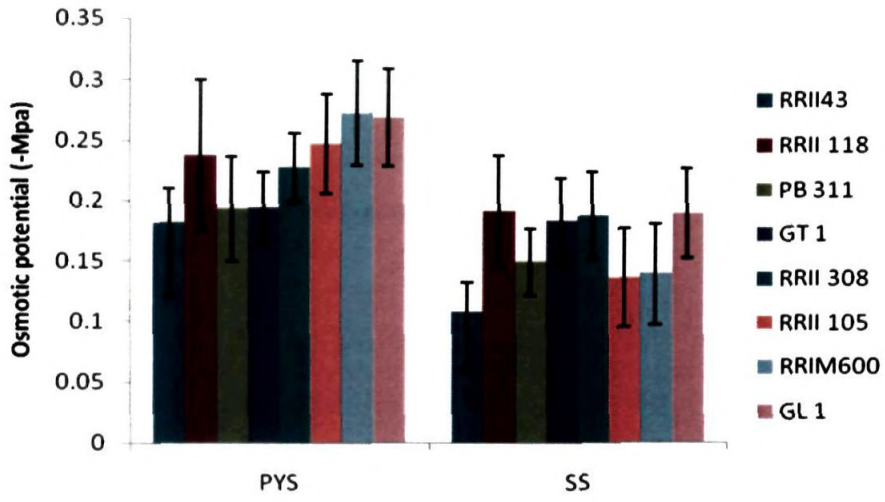


Fig. 4.4 Contribution of sucrose to the total osmotic potential during peak yielding (PYS) and stress seasons (SS)

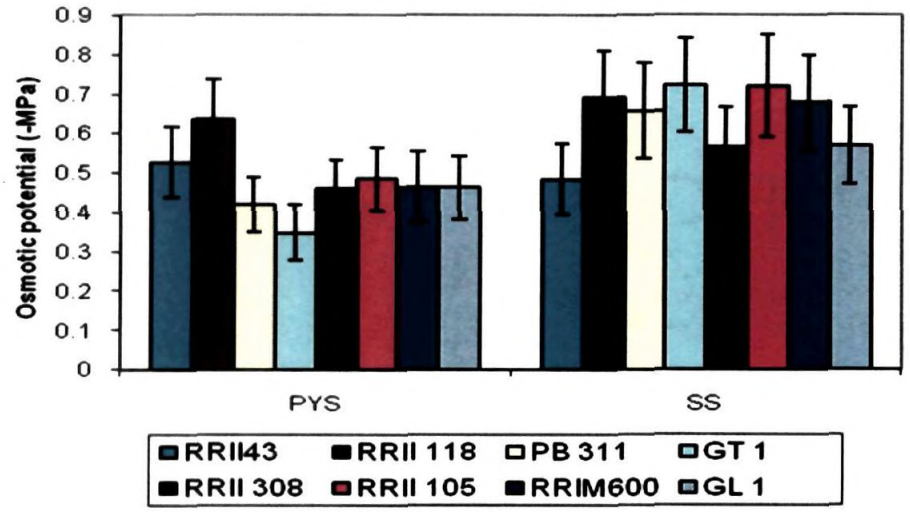


Fig. 4.5 Contribution of total sugars (sum of sucrose, reducing sugar and inositols) to the osmotic potential during peak yielding (PYS) and stress seasons (SS)

Concentration of reducing sugars was generally low and contribution was about 3.6 to 6.7% and 3 to 4.7% during peak yielding and stress seasons of the total osmolality Drought-induced inositol accumulation was significantly higher in clone RRII 105 (Fig.4.3d). The contribution of inositols to the measured osmotic potential ranged from −0.11 to −0.36 and −0.33 to −0.544 MPa during peak yielding and stress seasons (Fig.4.6). Inositols alone accounted around 27.8% to 41% of the osmotic components during stress season (Table 4.4). High rate of contribution was found in RRII 105, RRIM 600 and GT1. The clones RRII 118 and PB 311 were on par with each other in respect to the contribution of inositols to osmotic adjustment. Sugars and inositols together contributed to more than 50% of the total osmolality of the latex in some of the clones studied during stress season (Fig.4.7). The concentration of amino acids and its contribution to the solute pool increased with water scarcity. Contribution of total free amino acids to the measured osmotic potential of latex was higher under stress season in all the clones. It was around 6% to 7.8 % during peak yielding season and 5.6 % to 12.7% during stress season (Table 4.4).

Table 4.4 Contribution of osmolytes (-bars) to latex osmotic potential of different *Hevea* during peak yielding (PYS) and stress seasons (SS)

		Clones							
Solute		RRII 43	RRII 118	PB 311	GT1	RRII 308	RRII 105	RRIM 600	GL1
Sucrose	PYS	1.81 (16)	2.37 (21)	1.93 (19)	1.94 (23.5)	2.23 (23.2)	2.47 (24.4)	2.72 (27.1)	2.68 (27.5)
	SS	1.07 (9)	1.91 (15)	1.49 (11.3)	1.83 (14.3)	1.87 (16)	1.36 (10.2)	1.39 (11.6)	1.897 (16.5)
Reducing sugar	PYS	0.407 (3.6)	0.407 (3.6)	0.49 (4.9)	0.492 (5.7)	0.643 (6.7)	0.585 (5.8)	0.544 (5.4)	0.535 (5.5)
	SS	0.472 (4)	0.424 (3.2)	0.555 (4.2)	0.441 (3.5)	0.426 (3.7)	0.395 (3.0)	0.488 (4.1)	0.463 (4.7)
Inositol	PYS	3.054 (26.8)	3.584 (32.0)	1.815 (18.3)	1.059 (12.3)	1.701 (17.7)	1.80 (17.8)	1.407 (14.0)	1.418 (14.5)
	SS	3.306 (27.8)	4.563 (34.8)	4.519 (34.4)	4.946 (38.6)	3.332 (28.6)	5.44 (41.0)	4.882 (41.0)	3.318 (28.9)
Free aminoacid	PYS	0.748 (6.6)	0.874 (7.8)	0.673 (6.8)	0.582 (6.8)	0.665 (6.9)	0.688 (6.8)	0.642 (6.4)	0.682 (7.0)
	SS	1.110 (9.3)	0.746 (6.0)	1.662 (12.7)	1.122 (8.8)	1.012 (8.7)	1.006 (7.6)	0.889 (7.4)	1.101 (9.6)
K^+	PYS	2.483 (21.8)	2.429 (21.7)	2.765 (27.8)	3.008 (35)	3.135 (32.6)	2.919 (28.8)	3.099 (31)	2.973 (30.5)
	SS	2.429 (20.4)	2.429 (18.5)	3.025 (23)	3.001 (23.4)	2.903 (25)	2.713 (23.2)	2.304 (19.2)	2.982 (26)
Mg^{2+}	PYS	1.059 (9.3)	0.611 (5.5)	1.110 (11.2)	0.711 (8.3)	0.628 (6.5)	0.994 (8.8)	1.337 (13.3)	0.975 (10)
	SS	0.551 (4.6)	0.703 (5.4)	1.381 (10.5)	0.726 (5.7)	1.283 (11)	1.084 (8.2)	1.466 (12.2)	1.274 (11.1)
Ca^{2+}	PYS	0.0021 (0.002)	0.00032 (0.003)	0.0002 (0.002)	0.0002 (0.002)	0.0003 (0.003)	0.00003 (0.0003)	0.00005 (0.005)	0.0001 (0.001)
	SS	0.00003 (0.0003.)	0.00047 (0.003)	0.0003 (0.0002)	0.00002 (0.02)	0.002 (0.002)	0.00022 (0.002)	0.00003 (0.0003)	0.00004() (0.0003)
P	PYS	1.039 (9.1)	0.691 (6.2)	1.059 (10.7)	0.751 (8.7)	0.531 (5.5)	0.699 (6.9)	0.612 (6.1)	0.449 (4.6)
	SS	0.415 (3.6)	0.446 (3.4)	0.491 (3.7)	0.536 (4.2)	0.749 (6.4)	0.726 (5.5)	0.431 (3.6)	0.258 (2.2)

Values in parenthesis denotes the percent contribution of osmolyte to total osmotic potential

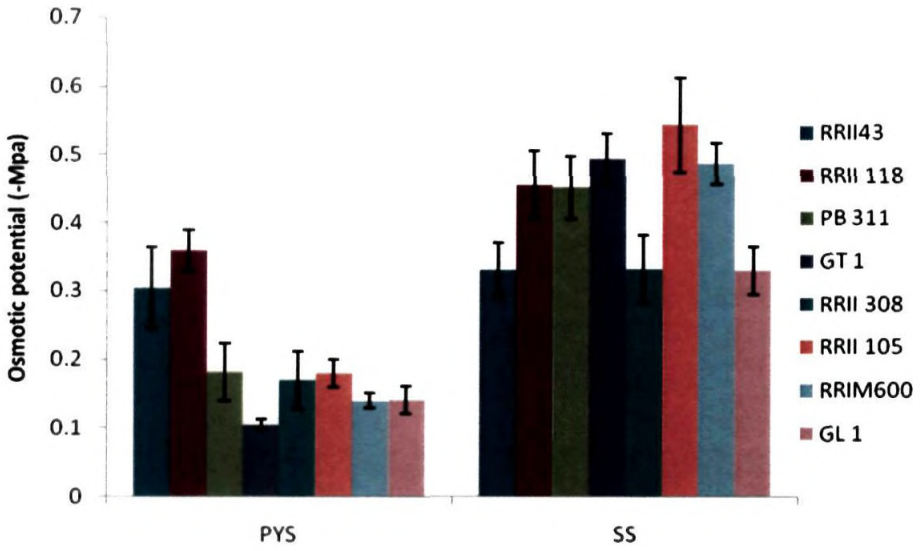


Fig. 4.6 Contribution of inositols to the total osmotic potential during peak yielding (PYS) and stress seasons (SS)

Contribution of inorganic ions to osmolality and osmotic potential was worked out from its concentrations in the latex. The contribution of latex Ca^{2+} content towards the water relation components, *i.e.* osmotic potential was very low (Table 4.4). The contribution of Mg^{2+} to the osmolality was also found low in all the clones studied. During peak yielding season the contribution of to osmotic adjustment was 5% to 13% and under stress season it was about 5% to 12 % (Table 4.4). Phosphorus level and contribution to osmotic adjustment was relatively higher under peak yielding season (Table 4.4). There was significant clonal and clone x season effects with respect to the contribution of K^+ to osmolality and osmotic adjustment in the latex. The percentage of K^+ contribution to osmotic potential ranged between 22% to 35% and 19% to 26% during peak yielding and stress seasons, respectively. Among the inorganic ions analyzed, potassium has a

major role in latex osmotic potential. Contribution of all the estimated inorganic ions to the total osmolality ranged from 29 to 42% during stress season (Fig.4.8). The differences in contribution of total sugars and inorganic ions to osmotic potential showed significant clonal and seasonal variation. Differences between the contribution of soluble carbohydrates and inorganic ions indicated that osmotic adjustment is mainly contributed by soluble carbohydrates *i.e.* soluble sugars and inositols during stress season. It was high in RRII 118 and GT1 where as it was on par in clones RRII 105 and RRIM 600 (Fig.4.9). The total latex volume produced during peak yielding season was significantly high in all the clones (Table 4.3). Total latex volume was significantly high in PB 311 followed by RRII 118 and RRII 105 under peak yielding season. Even though a marked reduction was noticed in total latex volume, PB 311 maintained the highest latex yield followed by RRII 105 and RRII 308 during stress season (Table 4.3). Irrespective of seasons the latex yield was higher in PB 311. The clones such as RRII 105, RRII 308 and RRII 118 recorded relatively little reduction in yield compared to other clones. Among the *Hevea* clones GT 1 showed better osmoregulation which may be due to relatively high contribution of inositols and stable K^+ to osmotic adjustment and low osmotic potential during peak yielding season. Followed that RRII 105, PB 311 and RRII 308 were the other clones having stable higher osmoregulatory potential (Table 4.4).

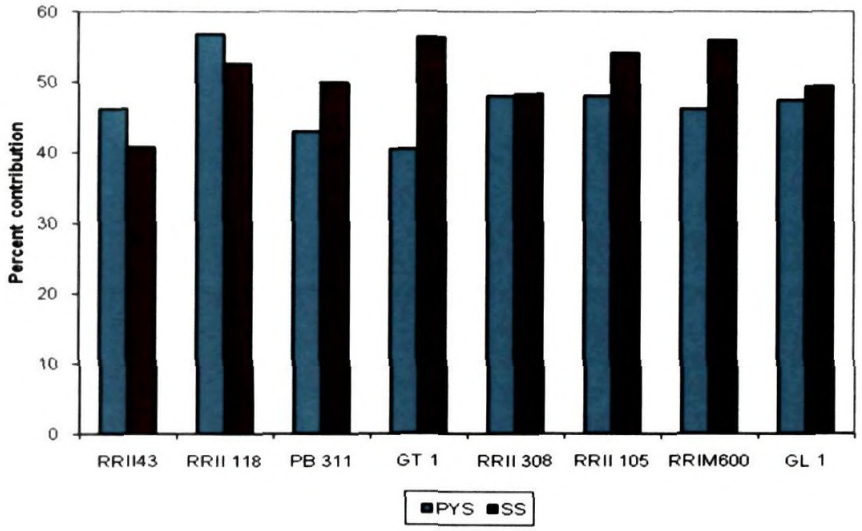


Fig 4.7. Percent contribution of total sugars (sum of sucrose, reducing sugar and inositols) to the osmotic potential during peak yielding (PYS) and stress seasons (SS)

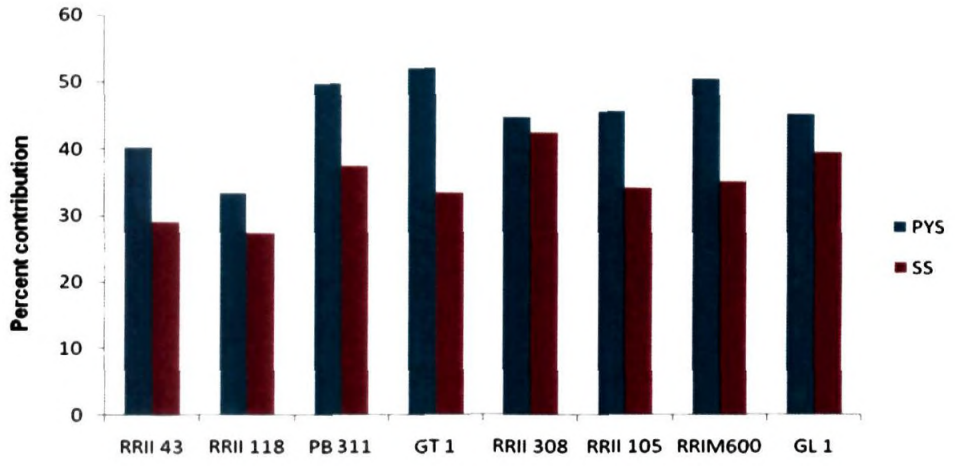


Fig 4.8 Percent contribution of total inorganic ions to the osmotic potential during peak yielding (PYS) and stress seasons (SS)

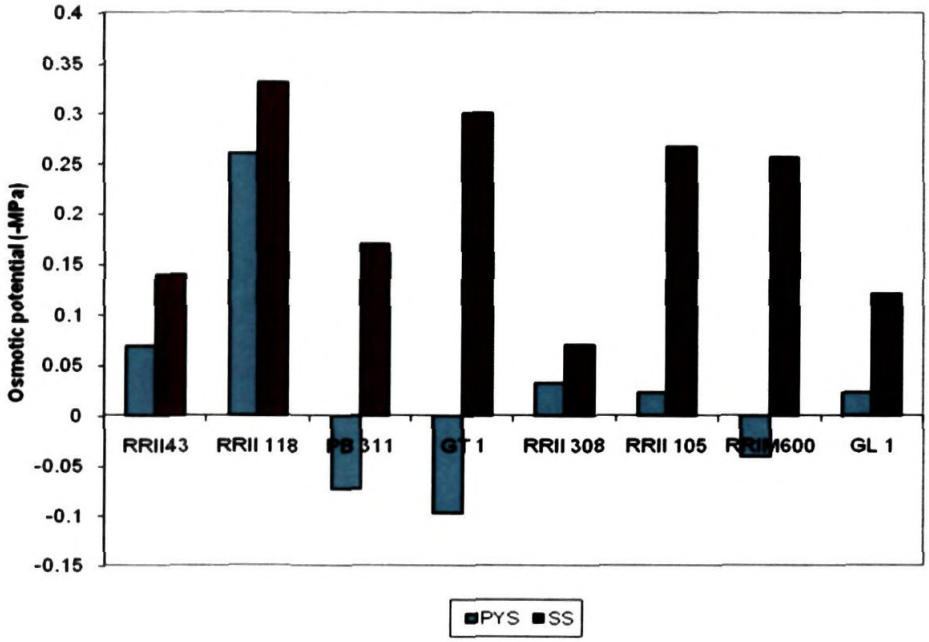


Fig. 4.9 Differences between the contribution of sugars (including inositols) and inorganic ions to osmotic potential in the latex of *Hevea* clones during peak yielding (PYS) and stress seasons (SS)

4.4 Discussion

Adverse environmental conditions such as drought, cold or high soil salinity obstruct plant growth and plants require specific adaptation strategy to tolerate these conditions. In response to environmental stresses, a number of low molecular weight compounds can accumulate in plants such as protective amino acids, sugar alcohols, sugars and betaine-type quaternary amines. The function of these compounds includes the stabilization of cellular structures, photosynthetic complexes, cell membranes, specific enzymes and other macromolecules, the scavenging of reactive oxygen species or acting as metabolic signals in stress conditions. The significant

role of osmoprotective compounds towards development of adaptative features under adverse environmental conditions is supported by numerous studies obtained with natural variants, mutants or transgenic plants with different capabilities to accumulate these metabolites (Bohernt and Jensen, 1996. Shen *et al.*, 1997; Sheveleva *et al.*, 1998; Merchant *et al.*, 2010)

In many plants, carbohydrates and polyols are the major soluble constituents and are often linked with osmoregulation (Koppenaal *et al.*, 1991; McManus *et al.*, 2000; Rajam *et al.*, 1998; Sacher *et al.*, 1985; Wingler, 2002). In the present study, clonal variations in inositol content in the latex of *Hevea* clones were evident (Fig.4.1). Metabolically more active *Hevea* clones generally showed increased production of inositols. It may be noted that metabolically active clones are generally high rubber yielders also (Fig.4.2). Total inositol content showed significant variation with respect to seasons. In many clones, monsoon season recorded the highest inositol content in the latex. The level decreased during post-monsoon season and the lowest level was noticed during summer season (Table 4.1). This seasonal variations noticed in the total inositol content of latex is closely related to the total latex volume produced by the tree during respective seasons (Fig.4.2). Generally in all the clones, the maximum quantity of latex is produced during the monsoon and post-monsoon seasons and the yield decline was noticed during summer season. Thus the total available quantity of inositols

from the latex will be high from June onwards till January period in the traditional rubber growing belt.

The concentration of osmotically active substances was calculated on latex weight basis and found to be greater in the summer months than the peak yielding season (Fig.4.3). It has been reported that phloem solute concentrations significantly increased across a range of plant genera in response to water deficit (Pate and Arthur, 1998; Pate *et al.*, 1998; Gessler *et al.*, 2008; Merchant *et al.*, 2010b; 2010c). The seasonal differences in osmolyte concentrations indicated that there was a change in tissue water status with lower tissue water content in the summer months resulting in greater tissue osmolality. The seasonal changes in osmotically active substances in tissues are species specific. Concentrations of osmotically active substances in *Eucalyptus* species did not vary seasonally and showed the same pattern on a dry weight or plant water basis except in leaf tissues, which had significantly greater concentrations of osmotically active substances in summer compared to winter (Merchant *et al.*, 2009).

The role of carbohydrates and inorganic ions as active osmolytes has been studied in many plant species with contrasting results depending on genotype, severity and duration of the stress, or tissue studied (Munns, 2002). In the present study, the contribution of different organic and inorganic solutes to osmotic potential of latex differed among *Hevea* clones

(Table 4.4). Fluctuations in the sugar content depended on varietal and water status of the tissue under stress season. Accumulation of sugars during water deficit condition was well documented (Mostajeran and Rahimi-Eichi, 2009). Soluble sugars act as osmoprotectant, stabilizing cellular membranes and maintaining turgor pressure and key substrates in biosynthetic processes. During the stress season, the concentration of sucrose and their contribution to osmotic potential in *Hevea* showed significant clonal, seasonal and clone x season interaction effect (Figs.4.3a, 4.4). The contribution of sugar to osmotic potential was reduced during stress season in all the clones studied. However, the decline in contribution of sugar to osmotic potential was low in clone GT1. In Chickpea, the sugar pool accounted for about 25% of total osmotic adjustment and 55% of total sugars were contributed by sucrose (Singh *et al.,* 1990). Munns and Weir (1981) have reported a 70-90 % contribution of sugars to the osmotic adjustment in drought-stressed wheat plants. The percent contribution of free amino acids to osmotic adjustment of the latex in *Hevea* was greater under stress season and ranged from 6% to 13% (Table 4.4). Free amino acids from the phloem sap of *Eucalyptus* were found large enough to have apparent influence on osmotic relations (Pritchard, 2007). Nair, (1992) reported a high level of free amino acid content in high yielding clones of *Hevea.* Sugars and total inorganic content constitutes the main osmolytes during peak yielding season in *Hevea* latex (Table 4.4). The possible reason for the decrease of sugars might be the

conversion of sugars to inositols. Keller and Ludlow (1993) reported an increase in sugar alcohol content with a decrease in sugar concentration. However, in stressed chickpea plants inositol was found accumulated without affecting the sugar level (Munns, 2002). In the present study it was observed that there was an increase in inositol content at the expense of sugar content. Inositol content in the latex was significantly higher under deficit water regime (Fig.4.3e).

The percent contribution of soluble sugar fraction including the inositols to the total measured osmotic potential of *Hevea* latex ranged from 40.5% to 56.8 % during stress season (Fig.4.7). Relatively higher level was observed in clone GT1, RRIM 600, RRII 105 and RRII 118. Clone PB 311 and RRII 308 were on par with each other in respect to the contribution of carbohydrates to total osmolality. On an average, inositols contributed for about 12% to 32% and 28% to 41% to the total osmotic potential during peak yielding and stress seasons respectively in *Hevea* (Fig.6). Among sugars, a greater percent contribution was observed for inositol to the total osmolality and found higher in RRII 105. Inositol content was significantly increased under stress season (Fig.4.3d). The concentration of quercitol, a cyclitol, did not change in response to water deficit in field-grown chestnut oak (*Quercus prinus*) (Gebre and Tschaplinski, 2002). In woodland or plantation trees of *Eucalyptus* species, there was no substantial change in quercitol concentrations

between seasons in tissues except the leaves of plantation trees, where the summer concentration of quercitol (270 mmol kg^{-1} DW) was double than that in winter (140 mmol kg^{-1} DW) (Merchant *et al.,* 2006a; 2006b; Merchant, 2009) as well as in oak species (Popp *et al.,* 1997; Passarinho *et al.,* 2006).

Composition of *Hevea* latex showed that it contains 1.2% w/v of quebrachitol (mono-methyl inositol) in comparison with 0.4% of w/v sucrose (Bealing, 1969; 1981). Clonal variation was observed for its concentration and ranged from 1-3% of the whole latex (Gopalakrishnan *et al.*, 2008). Under drought stress conditions inositols, mainly pinitol was found to be the major contributor of osmoticum in legumes (Ford, 1984). In agreement with this result, there was a highly significant accumulation of inositols in *Hevea* plants, which accounted for about 28% to 41% of the osmotic pool during stress season. It is notable that inositol was found in all *Hevea* clones. Significant clonal and seasonal variations were observed for inositol contribution to total measured osmotic potential (Fig.4.6). Quercitol, a polyol accumulated in *Eucalyptus* under drought condition and differences in species was most probably not due to the presence or absence of any specific biosynthetic pathway, but possibly due to different regulation or expression of these pathways (Merchant *et al.,* 2007a; 2007b).

The ionic contribution to total osmolality ranged from 29% to 42% during stress season (Fig.4.8). During peak yielding season, GT1 showed

higher level followed by PB 311 and RRIM 600 and clones RRII 105 and GL1 were on par with respect to the ionic contribution to total osmotic potential. Significant clonal and clone x season interaction effect was observed for Mg^{2+} concentration and its contribution to osmotic potential of latex (Table 4.2 and 4.4). Mg^{2+} concentration in the latex was reported to be associated with premature coagulation of latex at the tapping panel (Beaufils, 1957). Thus, it might influence the premature coagulation of latex in the tapping panel during different seasons. Amount of Ca^{2+} in latex was low compared to other cations (Table 4.2). Contribution of Ca^{2+} to osmolality was very low compared to other inorganic ions and varied in all the *Hevea* clones. The Ca^{2+} influx up regulate different calcium dependent protein kinases (CDPK) and mitogen activated protein kinases (MAPK). These were involved in the cascade of cytosolutes and antioxidants synthesis. Solutes help to maintain the cellular water status and free radical were scavenged by antioxidants (Maestri *et al.*, 2002). This might be a reason for low contribution of Ca^{2+} to osmolality in *Hevea* latex. The total concentration of Ca^{2+} and the level of contribution to osmotic potential increased with progressive soil water deficit. Concentration of phosphorus was higher under peak yielding season. Phosphorus showed significant clonal and clone x season with respect to its contribution to osmotic adjustment.

Potassium was the major solute in latex of all species, which contributed to about 35% of the osmolality under peak yielding season (Table 4.4). It is required for both stabilizing cytoplasmic pH and decreasing the osmotic potential in vacuoles. K^+ was the principal solute species and its contribution to the osmolality of the cell sap increased from 33% to 48% in faba bean in response to drought stress (Amede and Schubert, 2003). There was an accumulation of K^+, Mg^{2+} and Cl^- in the cell sap under stress in legumes (Amede and Schubert, 2003). The available K^+ from phloem sap has significant influence to osmotic relations owing to its concentration and low molecular weight (Pate *et al.*, 1974; Pritchard, 2007). Ca^{2+} contributed considerably to osmotic adjustment in chickpea and maintains the homeostasis by balancing negative charges of accumulated organic compounds (Ford, 1984). Salinity treatment in salt-tolerant and salt-sensitive *Phaseolus* species showed differential accumulation of Na^+, Cl^- and K^+ in the plant and significantly contributed to the measured osmotic potential. There were ample evidences that glycophytes adjust to high salt concentrations by lowering tissue osmotic potentials with an increase of inorganic ions and/or compatible solutes (Munns, 2002). This would also help to maintain root water absorption and its flux to the shoot under salt stress conditions.

The major environmental factor limiting crop productivity world-wide is drought (Chimenti *et al.*, 2006) and crops with increased resistance to

this stress appear to be crucial for keeping yield in areas where dry seasons are common. Relative water content (RWC) as well as water potential (ψ_w) and its components, turgor potential (ψ_p) and osmotic potential (ψ_π), are the parameters most commonly used to assess the plant water status (Kiane *et al.*, 2007). In the present study, C-serum osmotic concentration of latex varied among the clones which had significant seasonal and clone x season effects (Table 4.3). Osmotic concentration was found high during the stress season in all the *Hevea* clones (Fig.4.3.e). Correlation between osmotic adjustment and drought stress has been found in several tree species, including *Ziziphus rotundifolia* Lamk. (Arndt *et al.*, 2001), *Vitis vinifera* L. (Patakas *et al.*, 2002), *Eucalyptus* (Ngugi *et al.*, 2003), *Populus tremula* L. and *Tilia cordata* Mill. (Aasamaa *et al.*, 2004). It was reported that osmotic concentration influences the latex flow in *Hevea* trees (Satheesan *et al.*, 1982; Raghavendra *et al.*, 1984). Latex yield in both the season was higher in PB 311, RRII 105, RRII 308 and RRII 118 showed relatively little reduction in yield compared to other clones. Substantial osmotic adjustment was observed in PB 311 by accumulating soluble sugars, inositol content, free amino acids, K^+ and Mg^{2+} to readjust the internal osmotic pressure and to improve its water status. Accumulation of soluble sugars, inositols, K^+ and free amino acids at higher concentration often assist in turgor maintenance and help to enhance drought tolerance. Clones such as RRII 105, RRII 308 and RRII 118 were on par with PB 311 with respect to osmotic adjustment

and is mainly contributed by inositol and soluble sugars. Clone GT1 showed high accumulation of solutes such as soluble sugars and inorganic ions during stress season. Moreover at peak yielding season the osmotic potential was very low compared to other clones and might be the reason for its higher osmotic adjustment.

Under temporary or prolonged periods of water shortage, osmotic adjustment is one of the crucial processes involved in plant adaptation to drought (Chaves *et al.*, 2003). Plants subjected to water deficits may synthesize and accumulate amino acids (*e.g.* proline and aspartic acid), proteins, sugars (*e.g.* sucrose, glucose and mannitol), methylated quaternary ammonium compounds (*e.g.* glycine betaine and alanine betaine) and organic acids (Ingram and Bartels, 1996). Compatible solutes in high concentrations contribute to the lowering of osmotic potential ($\Psi\pi$) and allow influx of water into the cells, thereby maintaining the turgor potential (Ψp) eventually tolerance to low soil water potentials (Tyree and Jarvis, 1982; Bray 1993).

The effective OA in *Jatropha curcas* plants was attributed by inorganic solutes such as K^+, Na^+ and Cl^-, in the leaves and roots (Ianucci *et al.*, 2002). The relative contribution of K^+ to the osmotic potential of *Jatropha curcas* plants under water stress was greater compared to other inorganic ions. K^+ ion is known to be highly soluble and play key osmoregulatory role in the stomatal guard cells and turgor maintenance (Taiz

and Zeiger, 1991). Organic solutes, mostly soluble sugars, total free amino acids (TFAA) and glycinebetaine effectively participate in osmotic adjustment of leaf mesophyll tissue and in roots of *J. curcas* plants. The increase in the concentration of leaf TFAA was more under severe drought conditions (Ianucci *et al.*, 2002).

Significant differences in cyclitol and carbohydrate concentrations between winter and summer seasons were reported in *Eucalyptus* species (Merchant *et al.*, 2010a). The present study was mainly focused to evaluate the organic and inorganic solute accumulation and their role in osmotic adjustment (OA) under stress season (summer months) in *Hevea* plants. Our findings suggest that osmotic adjustment of latex of *Hevea* plants to drought is mainly by high accumulation and percent contribution of inositol content to total carbohydrate pool. Inositol makes up a 40% of contribution to the total osmotic adjustment. Osmolality of latex was significantly increased during stress period compared to peak yielding season. There were significant changes in sucrose, inositol and other estimated ionic and amino acid concentration.

There are many adaptive mechanisms found in plants in response to environmental changes such as drought, high temperature and high light stresses. These mechanisms include changes in biochemical, morphological and physiological processes which naturally occur in plants. Accumulation

of compatible solutes is considered as an important metabolic response to environmental changes which leads to metabolic adjustment. Role of freezing tolerance through quercitol accumulation in *Quercus suber* leaves were reported in the winter months (Passarinho *et al.,* 2006). In *Hevea* high concentration of cyclitols (inositols), total sugar and free amino acids was reported in high yielding clones (Nair, 1992). Latex osmotic potential under drought was related to latex yield (Vijayakumar *et al.*, 1998). Organic and inorganic solutes of latex contributed to the osmotic potential. Modification of internal osmotic potential in response to the change in water availability is commonly observed in all terrestrial and marine plants. A limited range of solutes are suitable as cellular osmotica, as their physiological properties help to maintain the physiological function at low water potential. Among many functional adaptations that enable growth and productivity of trees against a wide range of environment, is their capacity to regulate osmotic potential.

Compatible solutes such as proline, soluble sugars *etc* maintain the cellular osmotic potential/ osmotic adjustment and can be used as a metabolic trait in relation to drought stress (Thapa *et al.*, 2011). Phloem sap osmotic potential considered as surrogate measure of $\delta^{13}C$ and physiological status of the plant (Tausz *et al.*, 2008; Merchant *et al.*, 2011; 2012). Significant changes in phloem sugar composition reported to have significant

influence on osmotic potential (Merchant *et al.*, 2010b, 2010c, 2011). In the present study, total inositol content in the latex of different *Hevea* clones showed significant clonal variation. The high yielding clones generally showed increased production of inositols. Osmotic contribution of solutes of latex showed the positive role of inositols and other solutes to total latex osmolality. Osmotic adjustment of latex during stress season in *Hevea* is mainly achieved by the inositol content. Accordingly under stress conditions clones such as RRII 105, PB 311, RRII 118 and GT 1 maintained latex osmotic potential and had better physiological status.

Chapter 5

General Conclusion

Summary and Conclusion

Secondary metabolites are specialized compounds of diverse chemical structures, which are synthesised from primary metabolites. Chemically these compounds are highly diverse and their metabolic pathways are complex. The biosynthesis and accumulation of secondary metabolites are usually organ, tissue and developmental stage specific. Plant secondary metabolites have broad application potential in industry, agriculture, medicine and food science. They play an important role in the interaction of plants with their biotic and abiotic environment. Inositol or polyol is one such compound of vast application and physiological function. Different types of inositols of varying proportions are produced by many plants. The most common form of inositol is myo-inositol. A group of inositol derivatives ranging from simple inositol phosphates to complex membrane-associated ones related to important cellular functions are synthesised from myo-inositol. A variety of species-specific epimers and methyl ethers are formed from myo-inositol by epimerization and methylation. These compounds accumulated under environmental stresses like water deficit, salinity and extreme temperatures in plants and are recognized as osmoprotectant metabolites. L-quebrachitol, a methylated form of inositol is an alternative raw material for the synthesis of a number of inositol derivatives and biologically active compounds related to pharmaceutical and medical research field. The peculiarity of *Hevea* latex is

that, even though the predominant sugar form present is sucrose, the total cyclitol (inositol) concentration is higher than sugars. A higher part of the non rubber components is mainly comprised of inositols, particularly L-quebrachitol. In this context, the objectives of the present study were isolation, quantification and characterization of L-quebrachitol from *Hevea* latex. Further goals of the study were to analyze different serum extraction methods from the latex of different *Hevea* clones, and evaluate the effect of inositols and other osmolytes in the context of latex water relations and osmoregulation in different *Hevea* clones.

To achieve the development of an appropriate method for the isolation, purification and quantification of L- quebrachitol from latex serum, at first, separation and identification of serum components was attempted. C- and A- sera of two clones (RRII 105 and RRIM 600) were prepared and analysed by HPLC method. The solvent system, acetonitrile: water (85:15 v/v) and flow rate 0.5 ml/min. were optimized for the separation of compounds from crude extracts. Serum samples of both the clones showed the presence of L-quebrachitol, sucrose and glucose. A fairly large amount of L-quebrachitol, sucrose and glucose were found in these samples. As the latex serum is complex in nature, appropriate methods were developed for the isolation of L-quebrachitol from latex serum. In this perspective two methods were tried for the purification of the latex serum. Both were found

appropriate for purification of the compound. However, the second method was found better owing to the reduction in number of steps involved in purification. The isolated compound was crystallized for obtaining pure solid form. Crystallizing inositol from a solution is a straight forward procedure of concentration of inositol in water at an elevated temperature, *via* evaporation, or some other means of water removal, followed by temperature reduction to initiate nucleation. The purified extract (latex serum) was analysed using high performance liquid chromatography (HPLC) and found only a single peak at the retention time 31.896 minutes and identified in comparison with the retention time (32.021min.) of the standard (Sigma). Further, the isolated compound was characterized by standard techniques such as FT-IR, LC/MS/ESI, ^{1}H-NMR and ^{13}C NMR.

The FT-IR spectrum of the isolated compound was found similar to that of the standard L-quebrachitol. The strong wide absorption band at 3331 cm^{-1} represents the characteristic -OH stretching of the compound. The multiple absorption bands at 2939 cm^{-1}, 2928 cm^{-1}, 2901 cm^{-1}, 2882 cm^{-1}, 2835 cm^{-1} represents the six C-H bonds and one methyl group. The finger print region of the isolated compound from *Hevea* latex was also matching with that of the standard. The LC/MS/ESI in negative mode confirmed the molecular weight of the isolated compound to be 193.10. It was in conformity with the molecular weight of the standard L-quebrachitol

(193.13). The ^{1}H-NMR studies indicated that the compound isolated from *Hevea* latex and standard was identical. The spectrum showed presence of three protons at *δ 4.35 – 4.33, δ 3.86* – 3.85 and δ 3.67 – 3.66 (doublet), δ 3.1 – 3.06 (double doublet), δ 4.51- 4.47, δ 4.70 – 4.67 (multiple shifts). The presence of one methoxy group indicated by the singlet signal at δ 3.3 (Fig.3.8). The ^{13}C-NMR spectrum of the isolated compound showed the presence of 7 well resolved signals at δ 81.21, *δ* 73.38, *δ 72.35, δ 72.19, δ 70.77, δ 68.09 and δ* 56.71. The present research work leads to the development of a suitable method for the isolation of L-quebrachitol in a pure form from *Hevea brasiliensis* latex serum. Further the methodology of L-quebrachitol isolation from latex serum was patented (IP: 238511 dt. 09/02/2010).

Isolation of L-quebrachtiol by adopting the method developed was studied in the serum obtained from different latex extraction methods in different *Hevea* clones. The results indicated that the protocol for the isolation of L-quebrachtiol from *Hevea* latex sera developed was suitable for serum obtained from different latex extraction methods and clones. The identity and purity of the isolated compound was confirmed through standard techniques such as FT-IR, LC/MS/ESI and NMR. The yield of quebrachitol from the latex serum was directly affected by the methods of serum extraction. Clonal variation was also very evident. Higher amount of

extractable quebrachitol was found from the C-serum and A-serum of RRII 430. In general RRII 400 series clones recorded a relatively higher percent of quebrachitol than other *Hevea* clones studied. Higher extraction rate (percentage of recovery) of quebrachitol was obtained from the C-serum (1.41% w/w) followed by A- serum (0.48% w/w) and lowest from the factory effluent. The average rate of extraction of quebrachitol from alcoholic extraction was 0.27% (w/w) of latex serum and 0.22% (w/w) from latex serum obtained through cold treatment.

In order to assess the relationship of inositols and other solutes to water relation of latex (osmotic potential) and their role in drought responses, the content of total inositols, sugars, free amino acids, reducing sugars and different ions such as potassium (K^+), magnesium (Mg^{2+}), phosphorus (P), and Calcium (Ca^{2+}) were studied in the latex of different *Hevea* clones during different seasons. Contribution of all these solutes to osmotic potential (ψ_π) and osmotic regulation was attempted. Inositol content showed significant clonal and seasonal variation. These variations noticed in the total inositol content in the latex are closely related to the total latex produced by the tree during different seasons. Concentrations of sugars showed significant clonal, seasonal and clone x season interaction effects. Reducing sugar level was significantly reduced in all the clones from peak yielding to stress in all the clones except PB 311. Free aminoacids were higher in

summer (stress season) in all the clones and showed significant seasonal variations. Total inositol content showed significant seasonal difference and was higher during stress season. A high level of inositol content was recorded in *Hevea* clones RRII 105 and GT1 followed by RRIM 600. Most of the soluble solute fraction of latex of the clones RRII 105 and GT1 under stress season was accounted by inositol/cyclitol concentration. Latex ionic contents showed significant clonal variation. The latex calcium (Ca^{2+}) contents showed both clonal and seasonal variations. Significant clonal variation and clone x season interaction was observed for the latex Mg^{2+} content. Latex P level showed significant clonal and seasonal variations and clone x season interaction effects. The K^{+} content, the major ionic component in the latex has significant seasonal and clone x season interaction effects.

With respect to the studies of water relation component of latex, osmotic potential, a significant increase in osmolality was found in all *Hevea* clones between peak yielding and stress season. There was significant seasonal and clone x season interaction effect for osmotic concentration of latex. *Hevea* clones such as RRII 105, PB 311, RRII 118 and GT 1 had high osmolality in stress season (summer). The contribution of different organic and inorganic solutes to osmotic potential differed among *Hevea* clones. Inositol content accounted for about 28% to 41% to the total osmotic

potential during stress season in *Hevea.* Significant clonal and seasonal variations were found in the magnitude of contribution of total sugars and inorganic ions to osmotic potential. Differences between the contribution of soluble carbohydrates (including inositols) and inorganic ions indicate that osmotic adjustment is mainly contributed by soluble sugars and sugar alcohols *i.e.* soluble sugars and inositols during stress season. Among the sugars, the contribution of inositol to osmotic potential was significantly high. Clones such as PB 311, RRII 105, RRII 308 and RRII 118 showed better latex yield and little reduction in latex volume under stress compared to other clones. The clone PB 311 showed substantial osmotic adjustment and was dependent on organic solutes (soluble sugars, inositol content and free amino acids) and inorganic ions (K^+ and Mg^{2+}) to modify the osmotic pressure and to improve its water status. Clones such as RRII 105, RRII 308 and RRII 118 were on par with PB 311 with respect to osmotic adjustment and is mainly contributed by inositol and soluble sugars. The reason for the high osmotic adjustment in clone GT1 might be due to the increased accumulation of sugars and inorganic ions during stress season and very low osmotic potential during peak yielding season. Significant changes in the phloem sap composition has specific role on osmotic potential. Under stress condition (summer months) clones such as RRII 105, PB 311, RRII 118 and GT 1 maintained latex osmotic potential and healthy physiological status.

In conclusion, the study successfully accomplished the isolation of L-quebrachitol from *Hevea* latex serum (IP. No. 238511). The amount of L-quebrachitol extracted from natural rubber latex varied with respect to clone and the source of serum. Total inositol content in the latex of different *Hevea* clones showed significant clonal variation. Studies on latex osmolytes and water relations of latex showed positive role of inositols and other solutes in osmoregulation of latex. As better osmoregulation is a desired trait in drought tolerance, inositol content can be incorporated in crop improvement programmes for screening drought resistant genotypes. The mechanism of long distance transport, carbon partitioning, defense and response to environmental fluctuations were related to the differences in phloem pressure. Therefore studies in the direction of carbon and oxygen isotope discrimination with respect to phloem loading and water relations under changing environments would give a comprehensive association of growth and yield with physiological performance of the plant. As an important prominent plantation crop, rubber tree has considerable significance to Indian economy. The study revealed that bioresources such as raw latex, effluents from latex centrifugation factory or coagulated latex can be used for the production of secondary metabolite (L-quebrachitol) of commercial importance. Considerable quantities of inositols particularly L-quebrachitol available in the latex, now remains unutilized and discarded as waste material after processing the rubber from latex. The present quick

extraction technique from appropriate serum sources would give great opportunities to natural rubber latex as a source of L-quebrachitol which would fetch additional income to the rubber industry. Further studies for the refinement of the method for pilot plant/commercialisation are underway.

References

References

Aasamaa, K., Sõber, A., Hartung, W. and Niinemets, Ü. (2004). Drought acclimation of two deciduous tree species of different layers in a temperate forest canopy. *Trees*, 18:93-101.

Abbas, A.A., Anees, A.A., Irshad, M., Zafaryab, M., Rizvi, M.M.A.and Ahmad, A. (2014). Rice bran phytic acid induced apoptosis through regulation of Bcl-2/Bax and p53 genes in HepG2 human hepatocellular carcinoma cells. *Asian Pacific Journal of Cancer Prevention*, 15: 3731-3736.

Abid, G., Silue, S., Muhovski, Y., Jacquemin, J.M., Toussaint, A. and Baudoin, J.P. (2009). Role of myo-inositol phosphate synthase and sucrose synthase genes in plant seed development. *Gene*, 439: 1-10.

Abreu, E.F.M. and Araga F.J.L. (2007). Isolation and characterization of a myo-inositol-1-phosphate synthase gene from yellow passion fruit (*Passiflora edulis f. flavicarpa*) expressed during seed development and environmental stress. *Annals of Botany*, 99: 285-292

Adams, M.A., Richter, A., Hill, A.K. and Colmer, T.D. (2005). Salt tolerance in *Eucalyptus sp.*: Identity and response of putative osmolytes. *Plant Cell and Environment*. 28: 772-787.

Adams, P., Thomas, J.C., Vernon, D.M., Bohnert, H.J. and Jensen, R.G. (1992). Distinct cellular and organismic responses to salt stress. *Plant Cell and Physiology*, 33: 1215-1223.

Adiwilaga, K. and Kush, A. (1996). Cloning and characterization of cDNA encoding farnesyl diphosphate synthase from rubber tree *(Hevea brasiliensis)*. *Plant Molecular Biology,* 30: 935-946.

Ahn, C., Park, U. and Park, P.B. (2011). Increased salt and drought tolerance by d-ononitol production in transgenic *Arabidopsis thaliana. Biochemical and Biophysical Research Communications* 415: 669-74.

Ajithkumar, P. and Panneerselvam, R. (2013). Osmolyte accumulation, photosynthetic pigment and growth of *Setaria italica* (L.) P. Beauv. under drought stress. *Asian Pacific Journal of Reproduction,* 2(3): 220-224.

Akiyama, S.K. (1996). L-quebrachitol as a new chiral bulding block. *Journal of Synthetic Organic Chemistry Japan*, 54(2): 84-93.

Akiyama, T., Takechi, N. and Ozaki, S. (1992). *Bulletin of Chemical Society of Japan*, 65: 316-372.

Amalou, Z., Bangratz, J. and Chrestin, H. (1992). Ethrel (ethylene releaser)-induced increases in the adenylate pool and transtonoplast ΔpH within *Hevea* latex cells. *Plant Physiology,* 98: 1270-1276.

Amede, T. and Schubert, S. (2003). Mechanisms of drought resistance in grain legumes I: Osmotic adjustment. *Sinet: Ethiopia Journal of Science*, 26(1):37- 46.

An, F., Cahill, D., Rookes, J., Lin, W. and Kong, L. (2014). Real-time measurement of phloem turgor pressure in *Hevea brasiliensis* with a modified cell pressure probe. *Botanical Studies,* 55 (19): 1-11.

An, F., Lina, W., Cahill, D., Rookes, J. and Kong, L. (2014). Variation of phloem turgor pressure in *Hevea brasiliensis*: An implication for latex yield and tapping system optimization. *Industrial Crops and Products*, 58: 182-187.

Anderson, L. (1972). The cyclitols. In: *Chemistry and Biochemistry,* Vol. Ia (Eds. Pigman, W. and Horton, D). Academic Press, New York, pp.519 - 559.

Andersen, H.D., Wang, C.H., Arleth, L., Peters, G.H. and Westh, P. (2011). Reconciliation of opposing views on membrane–sugar interactions. *Proceedings of the National Academy of Sciences of the United States of America,* 108: 1874-1878.

Angyal, S.J., Greeves, D. and Mills, J.A. (1974). Complexes of carbohydrates with metal cations. II. Confirmation of alditols in aqueous solution. *Australian Jouranl of Chemistry.* 27: 1447-1456.

Angyal, S.J. and Hoskinson, R.M. (1963). *Methods in Carbohydrate Chemistry*, 2: 87.

Annamalainathan, K., Aunmod, M.A. Krishnakumar, R. Sreelatha, S. and James Jacob. (2008). Tapping induced changes in respiration rate, biochemical status, and ATP content of the soft tissue of natural rubber plants. *Journal of Plantation Crops*, 36(3): 245-258.

Annamalainathan, K., Joseh, J., Alarm, B., Sathessh, P.R. and Jacob, J. (2013). Seasonal changes in xylem sap flow rate in mature rubber plants. *Journal of Plantation Crops*, 41(3): 343-349.

Annamalainathan, K., Prakash, G.P., Vijayakumar, K. R., Jacob, J. and Sethuraj, M. R. (1998). Growth and yield of natural rubber under rainfed and irrigated conditions in the drought prone North Konkan region of Maharashtra. *National symposium on Current Trends in Plant Physiology and Plant Biochemistry,* 1998, University of Hyderabad, India, pp.153.

Archer, B. L., Audley, B.G., Mc Sweeney, G.P. and Hong, T.C. (1969). Studies on the composition of latex serum and bottom fraction particles. *Journal of Rubber Research Institute of Malaya,* 21(4): 506-509.

Archer, B.L., Bamard, D., Cockbain, E.G.C., Dickenson, P.B. and Mc Mullen, A. (1963). Structure, composition and biochemistry of *Hevea* latex. 11. In: *Chemistry and physics of rubber like substances,* (Eds. Bateman, L. Mc Laren and Sons), London. p43.

Arndt, S.K., Clifford, S.C., Wanek, K. Jones, H.G. and Popp. M. (2001). Physiological and morphological adaptations of the fruit tree *Ziziphus rotundifolia* in response to progressive drought stress. *Tree Physiology,* 21:705-715.

Ashraf, M. and Harris, P.J.C. (2004). Potential biochemical indicators of salinity tolerance in plants. *Plant Science,* 166: 3-16.

Aswatreratanakul, K., Zhang, Y.W., Wititsunnakul, D., Wititsunnakul, R., Takahash, S., Rattanapittayaporn, A. and Koyama, T. (2003). Molecular cloning, expression and characterization of cDNA encoding cis-prenyl transferases *Hevea brasiliensis*: A key factor participating in natural rubber biosynthesis. *European Journal of Biochemistry.* 270: 4671-4680.

Babita, M., Maheswari, M., Rao, L.M., Shankerb, A.K., and Rao, G.D. (2010). Osmotic adjustment, drought tolerance and yield in castor (*Ricinus communis* L.) hybrids. *Environmental and Experimental Botany,* 69(3): 243-249.

Bakry, R., Stoggl, W.M., Hochleitner, E.O., Stecher, G., Huck, C.W. and Bonn, K.K. (2006). Silica paricle encapsulated poly (styrene-divinylbenzene) monolithic stationary phase for micro high performance liquid chromatography. *Journal of Chromatography A,* 1132: 183-189.

Bartels, D. and Nelson, D. (1994). Approaches to improve stress tolerance using molecular genetics. *Plant Cell and Environment,* 17: 659-667.

Bartels, D. and Sunkar, R. (2005). Drought and salt tolerance in plants. *Critical Reviews in Plant Science,* 24: 23-58.

Baskar, C.V.S., Mohankrishna, T., Chandrashekar, T.R., Rao, P.S., Vijayakumar, K. R. and Sethuraj, M. R. (1991). Effect of irrigation on growth and other physiological parameters of *Hevea brasiliensis* in the Konkan region of India. Physiology and exploitation of *Hevea brasiliensis*. *Proceedings of IRRDB Symposium*, 1990, Kunmin, China, PP. 130-135.

Bealing, F. J. (1969). Carbohydrate metabolism in *Hevea* latex: Availability and utilization of substrates. *Journal of Rubber Research Institute of Malaya,* 21(4): 445- 455.

Bealing, F. J. (1981). Quebrachitol synthesis in *Hevea brasiliensis*. *Journal of Rubber Research Institute of Malaya,* 29(20): 111-122.

Bealing, F.J. and Chua, S.E. (1972). Output, composition and metabolic activity of *Hevea* latex in relation to tapping intensity and the onset of brown blast. *Journal of Rubber Research institute of Malaya,* 23(3): 204-231.

Beaufils, E.R. (1957). Research on rational exploitation of *Hevea* using a physiological diagnosis based on mineral analysis of various parts of the plant. *Fertilite*, 3: 27-37.

Benjamin, J., Levine, J., Fux, M., Aviv, A., Levy, D. and Belmaker, R.H. (1995) Double-blind, placebo-controlled, crossover trial of inositol treatment for panic disorder. *American Journal of Psychiatry,* 152: 1084-1086.

Bennett, R. C. (1969). *Chemical Engineering Progress Symposium Series*, 65 (95): 34.

Bernad, W. A., Roy, M. B., and Roscoe, O. R. (1958). The enzymatic synthesis of inositol phosphatide. *Journal of Biological Chemistry,* 233(5): 1077.

Bhatnagar-Mathur, P., Vadez, V. and Sharma, K.K. (2008). Transgenic approaches for abiotic stress tolerance in plants, retrospect and prospects. *Plant Cell Report,* 27: 411- 424.

Bieleski, R.L. (1982). Sugar alcohols. In: *Plant carbohydrates*, (Eds. Loewus, F. A. and Tanner, W.), Berlin Heidelbery New York, Springer-Verlag, pp. 158-192.

Bieleski, R.L. and Briggs, B.G. (2005). Taxonomic patterns in the distribution of polyols with in the Proteaceae. *Australian Journal of Botany,* 53: 205-217.

Blum, A. (1997). Crop responses to drought and the interpretation of adaptation in drought tolerance in higher plants. In: *Genetical Physiological and Molecular Biology Analysis*, (Ed. Belhassen, E.) Kluwer Academic Publishers, Dordrecht, The Netherland. pp. 57-70.

Bohernt, H.J. and Jensen. R.G. (1996). Strategies for engineering water stress tolerance in plants. *Trends in Biotechnology,* 14: 89-97.

Bohnert, H.J. Nelson, D.E. and Jensen, R.G. (1995). Adaptation to environmental stresses. *Plant Cell,* 7: 1099-1111.

Boominathan, P., Shukla, R., Kumar, A., Manna, D., Negi, D., Verma, P.K. and Chattopadhyay, D. (2004). Long term transcript accumulation during the development of dehydration adaptation in *Cicer arietinum. Plant Physiology*, 135: 1608-1620.

Bouteau, F., Dellis, O., Bousquet, U. and Rona, J.P. (1999). Evidence of multiple sugar uptake across the plasma membrane of laticifer protoplasts from *Hevea. Bioelectrochemistry and Bioenergetics,* 48: 135–139.

Bray, E. (1993). Molecular response to water deficit. *Plant Physiology.* 103:1035-1040.

Breda, N., Huc, R., Granie, R.A. and Dreyer, E. (2006). Temperate forest trees and stands under severe drought: A review of ecophysiological responses, adaptation processes and long-term consequences. *Annals of Forest Science*, 63: 1-20.

Briceño, T., Compadre, C.M., Ordóñez, P. Malagón, O. (2011). Isolation and identification of secondary metabolites from *Artemisia sodiroi* Hieron. *Avances,* 3(2): A46-A49.

Briens, M. and Larher, F. (1983). Sorbitol accumulation in Plantaginaceae: Further evidence for a function in stress tolerance. *Z. Pflanzenphysiology,* 110: 447-458.

Buttery, B.R. and Boatman, S.G. (1966). Manometric measurement of turgor pressures in laticiferous phloem tissues. *Journal of Experimental Botany*, 17(51): 283-296.

Buttery, B.R. and Boatman, S.G. (1976). Water deficit and flow of latex. In: *Water deficits and Plant Growth* (Ed. Kozlowski, T. T.), Academic Press, New York, pp. 233-289.

Carr, M.K.V. (2012). The water relations of rubber (*Hevea brasiliensis*): A review. *Experimental Agriculture*, 48(2): 176-193.

Chandrashekar, T.R. (1997). Stomatal response of *Hevea* to atmospheric and soil moisture stress under dry subhumid climatic condition. *Journal of Plantation crops,* 25: 146-151.

Chandrashekar, T.R., Jana, M.K., Thomas, J., Vijayakumar, K.R., and Sethuraj, M.R. (1990). Seasonal changes in physiological characteristics and yield in newly opened trees of *Hevea brasiliensis* in North Konkan. *Indian Journal of Natural Rubber Research,* 3: 88-97.

Chandrashekar, T.R., Marattukulam, J.G. and Nazeer, M.A. (1996). Growth reaction of *Hevea brasiliensis* to heat and drought stress under dry subhumid climatic conditions. *Indian Journal of Natural Rubber Research,* 9(1): 1-5.

Chandrashekar, T.R., Nazeer, M.A., Marattukulam, J.G., Prakash, G.P., Anamalainathan, K. and Thomas, J. (1998). An analysis of growth and drought tolerance in rubber during the immature phase in dry subhumid climate. *Experimental Agriculture,* 34: 287-300.

Chaves, M.M., Maroco, J.P. and Pereira. J.S. (2003). Understanding plant response to drought from gene to whole plant. *Functional Plant Biology,* 30: 239-264.

Chaves, M.M., Pereira. J.S., Maroco, J.P., Rodrigues, M.L., Ricerdo, C.P.P., Osórino, M.L., Carvalho, I., Faria, T. and Pinheiro, C. (2002). How plants cope with water stress in the field: Photosynthesis and growth. *Annals of Botany*, 89:907-916.

Chen, H, and Jiang, J. (2010). Osmotic adjustment and plant adaptation to environmental changes related to drought and salinity. *Environment Review.* 18: 309-319.

Chen, X.M., Hu, L., Lu, H., Liu, Q.L. and Jiang, X.N. (2005). Overexpression of *mtlD* gene in transgenic *Populus tomentosa* improves salt tolerance through accumulation of mannitol. *Tree Physiology*, 25: 1273-1281.

Chen, T.H.H. and Murata, N. (2011). Glycinebetaine protects plants against abiotic stress: mechanisms and biotechnological applications. *Plant Cell and Environment*, 34: 1-12.

Chida, N., Suzuki, M., Suwama, M. and Ogawa, S.J. (1989). *Journal Carbohydrate Chemistry*, 8: 319.

Chien, C.T., Lin, T.P., Juo, C.G. and Her, G.R. (1996). Occurrence of a novel galactopinitol and its changes with other non-reducing sugars during development *of Leucaena leucocephala* seeds. *Plant Cell Physiology,* 37: 539-544.

Chiera, J.M., Streeter, J.G. and Finer, J.J. (2006). Ononitol and pinitol production in transgenic soybean containing the inositol methyl transferase gene from *Mesembryanthemum crystallinum. Plant Science.* 171: 647-654.

Chimenti, C.A., Marcantonio, M. and Hall, A.J. (2006). Divergent selection for osmotic adjustment results in improved drought tolerance in maize (*Zea mays* L.) in both early growth and flowering phases. *Field Crop Research.* 95, 305-315.

Chinnusamy, V., Zhu, J. and Zhu, J.K. (2007). Cold stress regulation of gene expression in plants. *Trends in Plant Science,* 12: 444-451.

Chong, F.C. (1981).The role of carbohydrates in the exploitation and latex flow of *Hevea,* Thesis. Ghent University, Belgium.

Chow, K.S., Wan, K.L., Isa, M.N.M., Bahari, A., Tan, S.H., Harikrishna, K. and Yeang, H.Y. (2007). Insights into rubber biosynthesis from transcriptome analysis of *Hevea brasiliensis* latex. *Journal Experimental Botany,* 58: 2429-2440.

Claramma, N.M., Varghese, L. and Mathew, N.M. (1995). Influence of storage on properties of natural rubber latex concentrate and valcanizates. *Indian Jouranl of Natural Rubber Research,* 8(1): 1-7.

Clayton-Greene, K.A. (1983). The tissue water relationships of *Callitris columellaris, Eucalyptus melliodora* and *Eucalyptus microcarpa*

investigated using the pressure-volume technique. *Oecologia,* 57: 368-373.

Clements, R.S.J. and Darnell, B. (1980). Myo-inositol content of common foods: Development of a high-*myo*-inositol diet. *American Journal of Clinical Nutrition*, 33: 1954-1967.

Clément-Demange, A., Legnate, H., Seguin, M., Carron, M.P., Le Guen, V., Chapuset, T. and Nicolas, D. (2000). Rubber Tree. In: *Tropical Plant breeding*, (Ed. Charrier, A., Jacquot, M., Hamon, S. and Nicolas, D.), Collection Repères, Montpellier, France, pp. 455-480.

CN Patent Application No. 102557892A. (2012). (in Chinese).

CN Patent Application No. 201310007459. (2013). (in Chinese).

CN Patent Application No. 103058834A. (2013). (in Chinese).

CN Patent Application No. 103724459A. (2014)..(in Chinese).

CN Patent Application No. 102516041A. (2012). (in Chinese)

Cochard, H., Coll, L., Roux, X.L. and Ameglio. T. (2002). Unraveling the effects of plant hydraulics on stomatal closure during water stress in walnut. *Plant Physiology*, 128:282-290.

Cohen, J.D. and Bandurski, R.S. (1982). Chemistry and physiology of the bound auxins. *Annual Review of Plant Physiology*, 33: 403-430.

Conde, A., Chaves, M.M. and Gerós, H. (2011a). Membrane transport, sensing and signalling in plant adaptation to environmental stress. *Plant and Cell Physiology,* 52: 1583-1602.

Conde, A., Regalado, A., Rodrigues, D., Miguel Costa J, Blumwald, E., Chaves, M.M. and Gerós, H. (2014). Polyols in grape berry: Transport and metabolic adjustments as a physiological strategy for water-deficit stress tolerance in grapevine. *Journal of Experimental Botany,* 28: 3-18.

Conde, A., Silva, P., Agasse, A., Conde, C. and Gerós, H. (2011b). Mannitol transport and mannitol dehydrogenase activities are coordinated in *Olea europaea* under salt and osmotic stresses. *Plant and Cell Physiology,* 52: 1766-1775.

Cornish, K., Siler, D.J., Grosjean, O. and Goodman, N. (1993). Fundamental similarities in rubber particle architecture and function in the three

evolutionarily divergent plant species. *Journal of Natural Rubber Research,* 8: 275-285.

Cote, G.G. and Crain, R.C. (1993). Biochemistry of phosphoinositides. *Annual Review of Plant Physiology and Plant Molecular Biology*, 44: 333-56.

Dall'Antonia, A.C., Martins, M.A., Moreno, R.M.B., Mattoso, L.H.C., Job, A.E., Ferreira, F.C. and Gonçalves, P.S. (2006). Evaluation of raw natural rubber clones using standard tests and dynamic mechanical properties. *Polímeros,* 16: 239-245.

d'Auzac, J. and Jacob, L.J. (1989). The composition of latex from *Hevea brasiliensis* as a laticiferous cytoplasm. In: *Physiology of rubber tree latex.* (Eds. d' Auzac, J., Jacob, J. L. and Christen, H.), CRC Press, Boca Raton, Florida, pp. 60-68.

d'Auzac, J. and Rabaillier, D. (1969). L' ethylene, nouvel agent stimulant de la production de latex chez *l' Hevea brasiliensis. Revue Generale des Caoutchoucs et Plastiques*, 46 (7&8): 857-858.

d'Auzac, J., Jacob, L.J., Prevot, J.C., Clement, A., Gallois, R., Crestin, H., Lacote, R., Pujade-Renaud, V. and Gohet, E. (1997). The regulation of cis-polyisoprene production (natural rubber) from *Hevea brasiliensis. Recent Research Development in Plant Physiology,* 1: 273-381.

De Almeida, M.V., Courl, M.R.C., De Assis, J.V. and Anconi, C.P.A. (2012). ^{1}H -NMR analysis of *O*-methyl-inositol isomers: A joint experimental and theoretical study. *Magnetic Resonance in Chemistry,* 50(9): 608-614.

De Almeida, M.V., De Assis, J.V., Courl, M.R.C., Anconi, C.P.A., Guerreiro, M.C., Dos Santos, H.F. and De Almeida, W.B. (2010). Experimental and theoretical investigation of epoxide quebrachitol derivatives through spectroscopic analysis. *Organic Letters*, 12(23): 5458-5461.

De Almeida, M.V., Figueiredo, R.M., Dos Santos, H.F., Da Silva, A.D. and De Almedia, W.B. (2001). Synthesis and theoretical study of azido and amino inositol derivatives from L-quebrachitol. *Tetraheron Letters*, 42: 2767-2769.

De Almeida, M.V., Silva, A.D., Souza, M.V.N and Benicio, A.A. (2003). A cascata dos fosfoinositideos. *Quimica Nova*, 26: 105-111.

de Fay, E. and Jacob, J.L. (1989). Anatomical organisation of the laticiferous system in the bark. In: *Physiology of rubber tree latex* (Eds. d' Auzac, J., Jacob, J. L. and Christen, H.) CRC Press, Florida, pp. 4-14.

de Faÿ, E., Moraes, L.A.C. and Moraes, V.H.F. (2010). Cyanogenesis and the onset of tapping panel dryness in rubber tree. *Pesquisa Agropecuária Brasileira*, 45: 1372-1380.

de Hoffmann, E. and Stroobant, V. (2007). Mass spectrometry- principles and application (3rd edition) Wiley, Great Briatin.

Deng, Y.J. and Deng, D.Y. (2000). Study on extraction of quebrachitol from natural rubber. *Natural Product Research and Development*, 12(6): 61-65. (in Chinese)

Del Vecchio, P., Esposito, D., Eicchi, L. and Barone, G. (1999). The effects of polyols on the thermal stability of calf thymus DNA. *International Journal of Biological Marcomolecules*, 24: 361-369.

Devakumar, A.S., Rao, G.G., Rajagopal, R., Rao, P.S., George, M.J., Vijayakumar, K.R. and Sethuraj, M.R. (1988). Studies on soil-plant atmosphere system in *Hevea* II. Seasonal effects on water relations and yield. *Indian Journal of Natural Rubber Research*, 1(2): 45-60.

Devakumar, A.S., Prakash, G.P., Sathik, M.B.M. and James, J. (1999). Drought alters the canopy architecture and microclimate of *Hevea brasiliensis* trees. *Trees,* 13: 161-167.

De Vos, R., Moco, C.S., Lommen, A., Keurentjes, J. J., Bino, R. J. and Hall, R. D. (2007). Untargeted large scale plant metabolomics using liquid chromatography coupled mass spectrometry. *Nature Protocol*, 2: 778-791.

Dey, S.K., Vijayakumar, K.R., Nair, D.B. and Subramanian, P. (1999). Effect of temperature and vapour pressure on major yield components of rubber in humid and sub-humid climatic regions, *Indian Journal of Natural Rubber Research*, 12(1&2): 69-76.

Dhir, B., Nasim, S.A., Samantary, S. and Srivastava, S. (2012). Assessment of osmolyte accumulation in heavy metal exposed *Salvinia natans*. *International Journal of Botany,* 8: 153-158.

Diamant, S., Eliahu, N., Rosenthal, D. and Goloubinoff, P. (2001). Chemical chaperons regulate molecular chaperones in vitro and in cells under combined salt and heat stress. *Journal of Biological Chemistry,* 276: 39586-39591.

Diamantoglou, S. (1974). Über das physiologische Verhalten von Cycliten in vegetativen Teilen höherer Pflanzen. *Biochemie und Physiologie der Pflanzen,* 166: 511-523.

Diaz, M., Gonzalez, A., Castro-Gamboa, I., Gonzalez, D. and Rossini, C. (2008). First record of L-quebrachitol in *Allophylus edulis* (Sapindaceae). *Carbohydrate Research*, 343(15): 2699-2700.

Dickenson, P.B. (1964). The ultrastructure of latex vessels of *Hevea brasiliensis. Proceedings of the Natural Rubber Producers' Research Association Jubilee Conference,* 1964, Cambridge, London, pp. 54-66.

Dickenson, P. B. (1969). Electron microscopical studies of latex vessel system of *Hevea brasiliensis. Journal of Rubber Research Institute of Malaya*, 21(4): 543-559.

Donahue, J.L., Alford, S.R., Torabinejad, A.J, Torabinejad, J., Kerwin, R.E., Nourbakhsh, A., Ray, W.K., Hernick, M., Huang, X., Lyons, B.M., Hein, P.P. and Gillaspy, G.E. (2010). The *Arabidopsis thaliana* myo-inositol-1-phosphate synthase1 gene is required for *myo*-inositol synthesis and suppression of cell death. *The Plant Cell*, 22: 999-903.

Drew, E.A. (1984). Physiology and metabolism of cyclitols. In: *Storage carbohydrates in plants*, (Ed. Lewis, D.H.), Cambridge University Press, Cambridge, U. K.

Duquesnoy, E., Castrola, V. and Casnova, A. (2008). Identification and quantitative determination of carbohydrates in ethanolic extract of two conifers using ^{13}C-NMR spectroscopy. *Carbohydtrate research*, 343(5): 813-902.

Dusotoit-Coucaud, A., Brunel, N., Kongsawadworakul, P., Viboonjun, U., Lacointe, A., Julien, J.L., Chrestin, H. and Sakr, S. (2009). Sucrose importation into laticifers of *Hevea brasiliensis*, in relation to ethylene stimulation of latex production. *Annals of Botany,* 104: 635-647.

Dusotoit-Coucaud, A., Kongsawadworakul P, Maurousset L, Viboonjun U, Brunel, N., Pujade-Renaud, V., Chrestin, H. and Sakr, S. (2010). Ethylene stimulation of latex yield depends on the expression of a sucrose transporter (*HbSUT1B*) in rubber tree (*Hevea brasiliensis*). *Tree Physiology*, 30: 1586-1598.

Ebi, G.C. and Kolawole, E.G. (1992). Some aspects of stabilization and degradation of Nigerian natural rubber. (11): The environmental degradation of DBHBT – Modified unprocessed, unvulcanized Nigerian natural rubber. *Journal of Applied Polymer Science,* 10(12): 2239-2244.

Einat, H., Clenet, F., Shaldubina, A., Belmaker, R.H. and Bourin, M. (2001). The antidepressant activity of inositol in the forced swim test involves 5-HT2 receptors. *Behavioural Brain Research,* 118: 77-83.

Ericsson, A., Hansen, J. and Dalgaard, L. (1978). A routine method for quantitative determination of soluble carbohydrates in small samples of plant material with gas-liquid chromatography. *Analytical Biochemistry*, 86: 552-560.

Eschbach, J.M., Tupy, J. and Lacrotte, R. (1986). Photosynthate allocation and productivity of latex vessels in *Hevea brasiliensis. Biologia Plantarum,* 28: 321-328.

Evans, R.D., Black, R. A., Loescher, W.H. and Fellow, R.J. (1992). Osmotic relation of the drought tolerant shrub *Artemesia tridentate* in response to water stress. *Plant cell and Environment,* 15: 49-59.

Farouk, S. and Abdul Qados, A.M.S. (2013). Osmotic adjustment and yield of cowpea in response to drought stress and chitosan. *Indian Journal of Applied Research*, 3(10): 1-6.

Ford, C.W. (1982). Accumulation of *O*-methyl inositol in water stressed *Vigna species. Phytochemistry,* 21(5): 1149-1151.

Ford, C.W. (1984). Accumulation of low molecular weight solutes in water stressed tropical legumes. *Phytochemistry*, 23: 1007-1015

Ford, C.S., Jones, N.B. and Vans Staden, J. (2000). Cryopreservation and plant regeneration from somatic embryo of *Pinus patula. Plant Cell Reports*, 25: 610-615.

Fux, M., Levine, J., Aviv, A. and Belmaker, R.H. (1996). Inositol treatment of obsessive compulsive disorder. *American Journal of Psychiatry*, 153: 1219-1221.

Gajewski, M., Katarzyna, K. and Bajer, M. (2009). The influence of post harvest storage on quality characteristics of fruit of egg plant cultivars. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 37: 200-205.

Galinski, E.A. (1993). Compatible solutes of halophytic bacteria: Molecular principles, water-soluble interaction, stress-protection. *Experimentia,* 49: 487- 496.

García-Plazaola, J.I. and Becerril, J.M. (1999). A rapid HPLC method to measure lipophilic antioxidants in stressed plants: Simultaneous determination of carotenoids and tocopherols. *Phytochemical Analysis,* 10: 307-313.

Gebre, G.M. and Tschaplinski, T.J. (2002). Solute accumulation of chestnut oak and dogwood leaves in response to through fall manipulation of an upland oak forest. *Tree Physiology,* 22: 251-260.

Gelling, I.R. and Porter, M. (1988). Chemical modification of natural rubber. *Nature,* 249: 29-30.

Gerli, S., Mignosa, M. and Di Renzo, G.C. (2003). Effects of inositol on ovarian function and metabolic factors in women with PCOS: A randomized double blind placebo-controlled trial. *European Review for Medical and Pharmacological Sciences,* 7: 151-159.

Gerli, S., Papaleo, E., Ferrari, A. and Di Renzo, G.C. (2007). Randomized, double blind placebo-controlled trial: Effects of Myo-inositol on ovarian function and metabolic factors in women with PCOS. *European Review for Medical and Pharmacological Sciences*, 11(5): 347-354.

George, E.S., Punnoose, K.I., Prasannakumari, P. and Nair, N.U. (2006). Clonal variation in latex nutrient content and nutrient drainage from some of the promising *Hevea* clones through latex. *Proceedings of International Rubber Conference*, 13-14 November 2006, Ho Chi Minh City, Vietnam, pp. 259-265.

Gessler, A., Tcherkez, G., Peuke, A.D., Ghashghaie, J. and Farquhar, G.D. (2008). Experimental evidence for diel variations of the carbon isotope composition in leaf, stem and phloem sap organic matter in *Ricinus communis. Plant Cell and Environment*, 31:941–953.

Giavalisco, P., Kohl, K., Hummel, J., Seiwert, B. and Willmitzer, L. (2009). ^{13}C isotope-labeled metabolomes allowing for improved compound annotation and relative quantification in liquid chromatography-mass spectrometry-based metabolomic research. *Analytical Chemistry*, 81(15): 6546-51.

Gillaspy, G.E., Keddie, J.S., Oda, K. and Gruissem, W. (1995). Plant inositol monophosphatase is a lithium-sensitive enzyme encoded by multigene family. *Plant Cell,* 7: 2175-2185.

Gohet, E., Chantuma, P., Lacote, R., Obouayeba, S., Dian, K., Clément-Demange, A., Kurnia, D. and Eschbach, J.M. (2005). Latex clonal typology of *Hevea brasiliensis*: Physiological modelling of yield potential and clonal response to ethephon stimulation. In: *Proceedings of the IRRDB Workshop on Exploitation Physiology*, Kottayam, India. pp. 199-217.

Gomez, J.B. (1983). Physiology of latex (Rubber) production. *Monograph,* No. 8. Rubber Research Development Board, Kula Lumpur.

Gomez, J.B. and Moir, G.F.J. (1979). The ultra cytology of latex vessels in *Hevea brasiliensis*. Monograph No. 4. *Malaysian Rubber Research Development Board,* Kuala Lumpur.

Gopalakrishnan, J., Thomas, M. and Jacob, J. (2008). Inositols: A commercially important sugar alcohol from natural rubber latex. *Natural Rubber Research,* 21 (1&2): 130-133.

Gopalakrishnan, J., Thomas, M and Jacob, J. (2010). A process for obtaining pure L- quebrachitol from rubber latex serum. *Indian patent No.238511.*

Gopalakrishnan, J., Thomas, M., Philip, A., Krishnakumar, R. and Jacob, J. (2010). Contribution of latex cations to the water relations and latex yield in *Hevea brasiliensis. Natural Rubber Research,* 23(1&2): 93-97.

Gopalakrishnan, J., Thomas, M., Preenu, A.A. Annamalainathan, K., Krishnakumar, R. and Jacob. J. (2011). Recovery of L-quebrachitol from different sources latex serum of *Hevea brasiliensis. Natural Rubber Research*, 24 (2): 253- 258.

Gorham, J. and Jones, R.G.W. (1981). Low molecular weight carbrohydrates in some salt stresses plants. *Plant Physiology*, 53: 27-33.

Gregersen, G., Bersting, H., Theil, P. and Servo, C. (1978). Myoinositol and function of peripheral nerves in human diabetes. *Acta Neurologica Scandinavica,* 58: 241-248.

Gregersen, G., Bartelsen, B. and Harbo, H. (1983). Oral supplementation of myo-Inositol: Effects of peripheral nerves in human diabetics and on

the concentration in plasma, erythrocytes, urine, and muscle tissue in human diabetics and normals. *Acta Neurologica Scandinavica,* 67:164-172.

Guo, C. and Oosterhius, D.M. (1997). Effect of water deficit stress and genotypes on pinitol. *Environmental and Experimentanal Botany*, 37: 147-152.

Gupta, A.K. and Kaur, N. (2005). Sugar signalling and gene expression in relation to carbohydrate metabolism under abiotic stresses in plants. *Journal of Bioscience,* 30(5): 101-115.

Halford, N.G. and Paul, M.J. (2003). Carbon metabolite sensing and signalling. *Plant Biotechnology Journal*, 1: 381-398.

Halford, N.G., Curtis, T.Y., Muttucumaru, N., Postles, J. and Mottran, D.S. (2010). Sugars in crop plants. *Annals of Applied Biology*, 158: 1-25.

Hao, B. and Wu, J. (2000). Laticifer differentiation in *Hevea brasiliensis*: Induction by exogenous jasmonic acid and linolenic acid. *Annals of Botany (Lond),* 85: 37- 43.

Hare, P.D., Cress, W.A. and Van Staden, J. (1998). Dissecting the roles of osmolyte accumulation during stress. *Plant Cell and Environment,* 21: 535– 553.

Hart, W.J. (1945). Method for recovering quebrachitol from rubber latex serum. *US Patent No. 2378141.*

Hasegawa, P.M., Bressan, R.A., Zhu, J.K. and Bohernt, H.J. (2000). Plant celluar and molecular responses to high salinity. *Annual Review of Plant Physiology and Molecular Biology,* 51: 463-499.

Hauser, M.T., Aufsatz, W., Jonak, C. and Luschnig, C. (2011). Transgenerational epigenetic inheritance in plants. *Biochimica et Biophysica Acta*, 1809: 459-468.

Hincha, D.K. and Hagemann, M. (2004). Stabilization of model membranes during drying by compatible solutes involved in the stress tolerance of plants and microorganisms. *Biochemical Journal*, 383: 277-283.

Ho, L.C. (1988). Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. *Annual Review of Plant Physiology and Plant Molecular Biology*, 39: 355-378.

Hoekstra, F.A., Golovina, E.A. and Buitinik, J. (2001). Mechanisms of plant desiccation tolerance. *Trends in Plant Sciences,* 6: 431- 438.

Hoffman-Ostenhof, O. and Pittner, F. (1982). The biosynthesis of inositols and its isomers. *Canadian Journal of Chemistry*, 60: 1863-1871.

Huang, K.F. and Luo, W. (1994). Crystal structure of L-quebrachitol. *Journal of Chinese Chemical Society*, 41: 115-117.

Huang, Z.D. and Pan, Y.Q. (1992). Rubber cultivation under climatic stresses in China. In: *Natural rubber: biology, cultivation and technology* (Eds. Sethuraj, M.R. and Mathew, N.M.), Amsterdam, The Netherlands: Elsevier Science Publishers BV. pp 220-238.

Hübel, F. and Beck, E. (1996). Maize root phytase: Purification, characterization, and localization of enzyme activity and its putative substrate. *Plant Physiology*. 112: 1429-1436.

Iannucci, A., Russo, M., Arena, L., Di Fonzo, N. and Martiniello, M. (2002). Water deficit effects on osmotic adjustment and solute accumulation in leaves of annual clovers. *European Journal of Agronomy*. 16: 111-122.

Ingram, J. and Bartels. D. (1996). The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 47: 377- 403.

Isarangkool, N.A.S., Do, F.C., Pannengpetch, K., Junjittakarn, J., Maeght, J.L., Rocheteau, A. and Cochard, H. (2010). Transient thermal dissipation method of xylem sap flow measurement: Multi-species calibration and field evaluation. *Tree Physiology*, 30:139-148

Isarangkool, N.A.S., Do, F.C., Pannengpetch, K., Junjittakarn, J., Maeght, J.L., Rocheteau, A. and Cochard, H. (2011). Water loss regulation in mature *Hevea brasiliensis*: Effects of intermittent drought in the rainy season and hydraulic regulation. *Tree Physiology*, 31(7): 751-762.

Ishitani, M., Majumder, A.L., Bornhouser, A., Michalowski, C., Jensen, R.G., and Bohnert, H.J. (1996). Coordinate transcriptional induction of myo-inositol metabolism during environmental stress. *Plant Journal*, 9: 537-548.

Jacob, J.L., d' Auzac, J. and Prevot, J.C. (1993). The composition of natural rubber from *Hevea brasiliensis. Clinical review in Allergy*, 11: 325-337.

Jacob, J.L., Prevot, J.C., Roussel, D., Lacrotte, R., Serres, E., d' Auzac, J., Eschbach, J.M. and Omont, H. (1989). Yield limting factors latex

physiological parameters, latex diagnosis and clonal typology. In: *Physiology of rubber tree latex.* (Eds. d' Auzac, J., Jacob, J.L. and Chrestin, H.), Boca Raton, CRCpress, pp. 346-382.

Jacob, J.L., and Sontag, N. (1974). Purification et etude de la phosphatase wide lutoidique du latex *d'Hevea brasiliensis. Biochemie,* 56*:* 13 -15.

Jaindl, M. and Popp, M. (2006). Cyclitols protects glutamine synthetase and malate dehydrogenase against heat induced deactivation and thermal denaturation. *Biochemical and Biophysical Research Communications*, 345: 761-765.

Janska, A., Marsik, P., Zelenkova, S. and Ovesna, J. (2010). Cold stress and acclimation: What is important for metabolic adjustment? *Plant Biology,* 12: 395-405.

Jan van Alphen (1951). Quebrachitol: Cyclic polyalcohol from natural rubber latex. *Industrial and Engineering Chemistry*, 43: 141-145.

Jayasekera, N.E.M. and Karunasekera, K.B. (1984). Effect of environment on clonal performance with respect to early vigour and yield in *Hevea brasiliensis*, *Proceedings of IRRDB Meeting on Hevea Physiology, Exploitation and Breeding*, Montpellier, France, pp. 250-255.

Jenning, J.D., Ehrenshaft, M., Pharr, D.M. and Williamson, J. D. (1998). Role of mannitol and mannitol dehydrogenase in active oxygen mediated plant defense. *Proceedings of National Acadamey of Science of United States of America,* **95**: 15129-15133.

Jiang, A. (1988). Climate and natural production of rubber (*Hevea brasiliensis*) in Xishuangbanna, southern part of Yunnan province, China. *International Journal of Biometeorology*, 32(4): 280-282.

Jiang, S.K., Zhang, G.M., Wu,Y., Meng, Z.H. and Xue, M. (2014). Isolation and characterisation of L-quebrachitol from rubber factory waste water. *Journal of Rubber Research,* 17(1): 23-33.

Jones, W.R.G., Storey, R., Leigh, R.A., Ahmad, N. and Pollard, A. (1977). A hypothesis on cytoplasmic osmoregulation. In: *Regulation of Cell Membrane Activities in Plants* (Eds. Marre, E. and Cifferi, O.), Elsevier, Amsterdam, pp. 121-136.

Junjittakarn, J., Limpinuntana, V., Pannengpetch, K., Isarangkool, N.A.S., Rocheteau, A., Cochard, H. and Frederic, C. (2012). Short term

effects of latex tapping on micro-changes of trunk girth in *Hevea brasiliensis*. *Australian Journal of Crop Science,* 6(1): 65-72.

Kallio, H., Lassila, M., Jarvenpaa, E., Haraldsson, G.G., Jonsdottir, S. and Yang, B. (2009). Inositols and methyl inositols in sea buckthorn berries. *Journal of Chromatography B,* 877: 1426-1432.

Kanta, R. and Varshney, U.K. (2006). Effects of osmotic and ionic stress on water relations of Isabgol (*Plantago ovata* Forsk.) seedlings. *Journal of Plant Biology*, 23(3): 237-240.

Kaplan, F. and Guy, C.L. (2004). Beta-Amylase induction and the protective role of maltose during temperature shock. *Plant Physiology*, 135: 1674-1684.

Karyudi, N. (2004). Varation of osmoregulation in *Hevea* rubber clones in response to water stress. *Proceedings of IRRDB conference*, 7-8 September 2004, Kunming, Yunnan, China, pp. 317-323.

Kaushik, J.K. and Bhat, R. (1998).Thermal stability of proteins in aqueous polyol solutions: Role of the surface tension of the water in stabilizing effect of polyols. *Journal of Physical Chemistry*, 102: 7058-7066.

Kelavkar, U.P. and Chhatpar, H.S. (1993). Polyol concentrations in *Aspergillus repens* grown under salt stress. *World Journal of Microbiology and Biotechnology,* 9: 579-582.

Keller, F. and Ludlow, M.M. (1993). Carbohydrate metabolism in drought stressed leaves of pigeon pea (*Cajanus cajan*). *Journal of Experimental Botany,* 44: 1351-1359.

Khraiwesh, B., Zhu, J.K. and Zhu, J. (2012). Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochimica et Biophysica Acta*, 1819(2): 137-148.

Khurana, N., Chauhan, H. and Khurana, P. (2012). Expression analysis of a heat-inducible, Myo-inositol-1-phosphate synthase (MIPS) gene from wheat and the alternatively spliced variants of rice and Arabidopsis. *Plant Cell Reports,* 31: 237-251.

Kiane, S.P., Tália, P., Maury, P., Grieu, P., Heinz, R., Perrault, A., Nishinakamasu, V., Hopp, E., Gentzbittel, L., Paniego, N. and Sarrafi, A. (2007). Genetic analysis of plant water status and osmotic

adjustment in recombinant inbred lines of sunflower under two water treatments. *Plant Science*. 172: 773-787.

Kiddle, J.J. (1995). Quebrachitol: A versatile building block in the construction of naturally occurring bioactive materials. *Chemical Review*, 95(6): 2189-2202.

Klages, K. Donnison, H. and Boldingh, M.R.E. (1998). Myo-inositol is the major sugar in *Actinida arguta* during early fruit development. *Australian Journal of Plant Physiology,* 25: 61-67.

Klig, L.S. and Henry, S.A. (1984). Isolation of the yeast INO1 gene: Located on an autonomously replicating plasmid, the gene is fully regulated. *Proceedings of National Acadamey of Science of United States of America*, 81: 3816-3820.

Klink, V.P., Hosseini, P., Matsye, P., Alkharouf, N.W. and Matthews, B.F. (2009). A gene expression analysis of synctia laser microdissected from the roots of the *Glycine max* (soyabean) genotype PI 548402 (Peking) undergoing a resistant reaction after infection by *Heterodera glycines* (soybean cyst nematode). *Plant Molecular Biology,* 71: 525-567.

Koch, K. (2004). Sucrose metabolism: Regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Current Opinion in Plant Biology*, 7: 235-246.

Kongsawadworakul, P. and Chrestin, H. (2003). Laser diffraction: A new tool for identification and studies of physiological effectors involved in aggregation-coagulation of the rubber particles from *Hevea* latex. *Plant Cell Physiology,* 44: 707-717.

Kongsawadworakul, P., Viboonjun, U., Romruensukharom, P., Chantuma, P., Ruderman, S. and Chrestin, H. (2009). The leaf, inner bark and latex cyanide potential of *Hevea brasiliensis*: Evidence for involvement of cyanogenic glucosides in rubber yield. *Phytochemistry,* 70: 730-739.

Koppenaal, R.S., Tschaplinski, T.J. and Colombo, S.J. (1991). Carbohydrate accumulation and turgor maintenance in seedling shoots and roots of two boreal conifers subjected to water stress. *Canadian Journal of Botany*, 69: 2522-2528.

Koyuncu, M.A. and Dilmaçünal, T. (2010). Determination of vitamin C and organic acid changes in strawberry by HPLC during cold storage. *Notulae Botanicae Horti Agrobotanici Cluj-Napoca*, 38: 95-98.

Kozikowski, A.P., Ognyanov, V.I., Fanq, A.H., Nahorski, S.R. and Wilcox, R.A. (1993). Synthesis of 1D-3-deoxy-,1D-2,3-dideoxy-, and 1D-2,3,6-trideoxy-myo-inositol-1,4,5-trisphosphate from quebrachitol, their binding affinities, and calcium release activity. *Journal of American Chemical Society*, 115: 4429-4434.

Kozikowski, A.P., Fanq, A.H. and Rusnak, J.M. (1989). A simplified route to the phosphatidyl inositol cascade inhibitor- (-) - 1L-1- Deoxy-1-Fluro-myo-inositol. *Tetrahedron Letters*, 30 (26): 3365-3368.

Kramer, P.J. and Boyer, J.S. (1995). Water relations of plants and soils. Academic Press, San Diego, pp.480.

Krapp, A. and Stitt, M. (1995). An evaluation of direct and indirect mechanisms for the sink-regulation of photosynthesis in spinach: Changes in gas exchange, carbohydrates, metabolites, enzyme activities and steady state transcript levels after cold-girdling source leaves. *Planta,* 195: 313-323.

Kuhlmann, F.E., Apffel, A., Fischer, S.M., Goldberg, G. and Goodley, P.C. (1995). Signal enhancement for gradient reverse-phase high-performance liquid chromatography-electrospray ionization mass spectrometry analysis with trifluoroacetic and other strong acid modifiers by postcolumn addition of propionic acid and isopropanol. *Journal of the American Society for Mass Spectrometry,* 6(12): 1221-1225.

Kuhn, R. and Klesse, P. (1958), *Chemische Berichte*, 91: 1989-1991.

Kunjet, S., Thaler, P., Gay, F., Chuntuma, P., Sangkhasila, K. and Kasemsap, P. (2013). Effects of drought and tapping for latex production on water relations of *Hevea brasiliensis* trees. *Kasetsart Journal (Natural Science),* 47: 506-515.

Kuo, T. M., Lowell, C. A. and Nelsen, T. C. (1997). Occurrence of pinitol in developing soybean seed tissues. *Phytochemistry,* 45(1): 29-35.

Ladiges, P.Y. (1975). Some aspects of tissue water relations in three populations of *Eucalyptus viminalis* Labill. *New Phytologist.* 75: 53-62.

Lahuta, L.B., Pluskota, W.E., Stelmazewska, J. and Szablińka, J. (2014). Dehydration induces expression of galactinol synthase and raffinose synthase in seedlings of pea (*Pisum sativum* L.). *Journal of Plant Physiology*, 171(14): 1306-1314.

Larcher, W. (2003). *Physiological plant ecology*. 4th Edn. Springer.

Lau, C.M. (1993). New materials from natural rubber serum. In: *Proceedings of International Rubber Research and Development Board Symposium*, 1993, Hortford, England, pp. 70-79.

Lau, C.M. (1996). Quebrachitol – an additional role for *Hevea* latex. *Rubber Developments*. 49 (1/2): 11-13.

Lemcoff, J.H., Guarnaschelli, A.B., Garau, A.M., Basciauli, M.E. and Ghersa, C.M. (1994). Osmotic adjustment and its use as a selection criterion in *Eucalyptus* seedlings. *Canadian Journal of Forest Research*. 24: 2404-2408.

Lemos, T.L., Machado, L.L., Souza, J.S.N., Fonseca, A.M., Maia, J.L. and Pessoa, D.L.P. (2006). Antioxidant, icthyotoxicity and brine shrimp lethality tests of *Magonia glabrata* (Sapindaceae). *Fitoterapia,* 77: 443-445.

Le Rudulier, D., Strom, A.R., Dandeker, A.H., Smith, L.T. and Valentine, R.C. (1984). Molecular biology of osmoregulation. *Science,* 224: 1064-1068.

Levine, J., Barak, Y., Gonzalves, M., Szor, H., Elizur, A., Kofman, O. and Belmaker, R.H. (1995). Double-blind, controlled trial of inositol treatment of depression. *American Journal of Psychiatry*, 152: 792-794.

Lewis, D.H. (1984). Physiology and metabolism of alditols. In: *Storage Carbohydrates in Vascular Plants*. (Ed. Lewis, D.H.), Cambridge: Cambridge Univ. Press, pp. 157-179.

Li, D., Deng, Z., Chen, C., Xia, Z., Wu, M., He, P. and Chen, S. (2010). Identification and characterization of genes associated with tapping panel dryness from *Hevea brasiliensis* latex using suppression subtractive hybridization. *BMC Plant Biology,* 10: 140-151.

Licy, J., Thomas, M., Panickar, A.O.N., Abraham, J., Saraswathyamma, C.K. and Sethuraj, M.R. (1993). Genetic parameters and heterosis in rubber (*Hevea brasiliensis* Muell. Arg.): 2. Association of yield and

certain yield attributes. PLACROSYM X, 1992, Kasaragod, India, *Journal of Plantation Crops,* 21 Supplement: 264-267.

Licy, J., Panickar, A.O.N., Premakumari, D., Nazeer, M.A., Varghese, Y.A., Saraswathyamma, C.K. and Sethuraj, M. R. (1993). Genetic parameters and heterosis in rubber (*Hevea brasiliensis* Muell. Arg.): 3. Variability and hybrid vigour for yield and certain yield attributes. *Golden Jubliee Symposium, Horticultural Research: Changing Scenario*, 24-28 May 1993, Bangalore, India.

Liu, X. and Grieve, C. (2009). Accumulation of chiro-inositol and other non-structural carbohydrates in *Limonium* species in response to saline irrigation waters. *Journal of American Society of Horticultural Science*, 134(3): 329-336.

Lo Bianco, R., Rieger, M. and Sung, S.S. (2000). Effect of drought on sorbitol and sucrose metabolism in sinks and sources of peach. *Physiologia Plantarum*, 108: 71-78.

Loescher, W.H. (1987). Physiology and metabolism of sugar alcohols in higher plants. *Physiologia Plantarum*, 70: 553-557.

Loewus, F.A. (1990). Structure and occurrence of inositols in plants. In: *Inositol metabolism in plants.* (Eds. Morre, D.J., Boss, W.F. and Loewus, F.A.). New York, WileyLiss, pp. 1-11.

Loewus, F.A. and Loewus, M.W. (1983). Myo-ionositol, its biosynthesis and metabolism. *Annual Review of Plant Physiology,* 34: 137-161.

Loewus, M.W., Bedgar, D.L., and Loewus, F.A. (1984). 1L-myoinositol 1-phosphate synthase from pollen of *Lilium longiflorum.* an ordered sequential mechanism. *Journal of Biological Chemistry*, 259: 7644-7647.

Loewus, F.A. and Loewus, M.W. (1987). Biosynthesis and metabolism of ascorbic acid in plants. *CRC Critical Review in Plant Science*, 5: 101-119.

Loewus, F.A. and Murthy, P.P.N. (2000). Myo-inositol metabolism in plants. *Plant Science,* 150: 1-19.

Loewus F.A., Tanner, W., Loewus F.A. and Dickinson, M.W. (*1982*). Cyclitols. In: *Encyclopedia of Plant Physiology, New Series, Vol. 13A, Plant Carbohydrates*, (Eds. Loewus, F.A. and Tanner, W.), Berlin: Springer-Verlag, pp. 194-216.

Loewus, M.W., Tupy, J. and Loewus, F.A. (1986). 1L-myoinositol-1-phosphate synthase in serum from *Hevea* latex. *Plant Science.* **44**: 29-32.

Low, F.C. (1978). Distribution and concentration of major soluble carbohydrates in *Hevea* latex, the effects of ethephon stimulation and possible role of these carbohydrates in latex flow. *Journal of Rubber Research Institute of Malaya,* 26(1): 21-32.

Low, F.C. and Gomesz, J. (1982). Carbohydrate status of exploited *Hevea* I. The effect of different exploitation system on the concentration of major soluble carbohydrates in latex. *Journal Rubber Research Institute of Malaya,* 30(1): 1-18.

Low, F.C., Gomez, J. and Hashim, J.B. (1983). Carbohydrate status of exploited *Hevea* II. Effect of micro trapping on the carbohydrate content of latex. *Journal of Rubber Research Institute of Malaya,* 31(1): 27-34.

Lowe, J.S. (1961). The substrate for VFA formation in natural rubber latex. *Proceedings of Natural Rubber Research Conference,* 1960, Kuala Lumpur, pp. 822.

Lozano, P., Combes, D. and Iborra, J.I. (1994). Effect of polyols on α-chymotrypsin thermostability: A mechanistic analysis of the enzyme stabilization. *Journal of Biotechnology,* 35: 9-18.

Ludlow, M.M. and Muchow. R.C. (1990). A critical evaluation of traits for improving crop yields in water-limited environment. *Advances in Agronomy*, 43: 107-153.

Luo, Y., Qin, G., Zhang, J., Liang, Y., Song, Y., Zhao, M., Tsuge, T., Aoyama, T., Liu, J., Gu, H. and Qu, L.J. (2011). D-myo-inositol-3-phosphate affects phosphatidyl inositol-mediated endomembrane function in *Arabidopsis* and is essential for auxin-regulated embryogenesis. *Plant Cell,* 23(4): 1352-1372.

Madden, T.D., Bally, M.B., Hope, M.J., Cullis, P.R., Schieren, H.P. and Janoff, A.S. (1985). Protection of large unilamellar vesicles by trehalose during dehydration: Retention of vesicle contents. *Biochimica et Biophysica Acta,* 817: 67-74.

Maestri, E., Klueva, N., Perrotta, C., Gulli, M., Nguyen, H.T. and Marmiroli, N. (2002). Molecular genetics of heat tolerance and heat shock proteins in cereals. *Plant Molecular Biology*, 48: 667-681.

Maheswari, M., Varalekshmi, Y., Vijayalakshmi, A., Yadav, S.K., Sharmila, P., Venkateswarlu, B., Vanaja, M. and Parathasaradhi, P. (2010). Metabolic engineering using *mtlD* gene enhances tolerance to water deficiet and salinity in *Sorghum*. *Biologia Planatrum*, 54: 647-652.

Majumder, A.L., Dattagupta, S., Goldwasser, P., Donahue, T.F. and Henry, S.A. (1981). The mechanism of interallelic complementation at the *ino-l* locus in yeast: immunological analysis of mutants. *Molecular Genetics and Genomics*, 184: 347-354.

Majumder, A.L., Johnson, M.D. and Henry, S.A. (1997). 1L-myo-inositol-1-phosphate synthase. *Biochimica et Biophysica Acta,* 1348: 245-256.

Manchanda, G. and Garg, N. (2008). Salinity and its effect on the functional biology of legume. *Acta Physiologia Plantarum*, 30: 595-618.

Mathews, C.K. and Van Holde, K.E. (1990). *Biochemistry*. Redwood City, CA: Benjamin/Cummings Publishing Co.

Mcgacack, J. and Binmore, G.B. (1930). Method for recovering quebrachitol from rubber latex serum. *US Patent No. 1758616.*

McManus, M.T., Bieleski, R.L., Caradus, J.R. and Barker, D.J. (2000). Pinitol accumulation in mature leaves of white clover in response to a water deficit. *Environmental and Experimental Botany*, 43: 11-18.

Meng, Z., Chen, J., Xue, M. and Lu, Z. (2014). Method for extracting quebrachitol from rubber waste water. *CN 102721270B.*

Merchant, A. and Adams, M.A. (2005). Stable osmotica in *Eucalyptus spathulata*- responses to salt and water deficit stress. *Functional Plant Boiology*. 32: 797- 805.

Merchant, A., Richter, A., Popp, M. and Adams, M.A. (2006a). Targeted metabolite profiling provides a functional link among eucalypt taxonomy, physiology and evolution. *Phytochemistry*. 67(4): 402-408.

Merchant, A., Callister, A., Arndt, S.K., Tausz, M., Adams, M.A. (2007a). Contrasting physiological responses to water deficit in six *Eucalyptus* species. *Annals of Botany*, 100: 1507-1515.

Merchant A., Ladiges, P.Y., and Adams M.A., (2007b). Quercitol links the physiology, taxonomy and evolution of 279 *Eucalyptus* species. *Global Ecology and Biogeography*, 16: 810-819.

Merchant, A., Tausa, M., Arndt, S.K. and Adams, M.A. (2006b). Cyclitols and carbophydrates in leaves and roots of 13 *Eucalyptus* species suggest contrasting physiological tresponses to water deficit. *Plant Cell and Environment,* 29: 2017-2029.

Merchant, A., Arndt, S.K., Rowell, D.M., Posch, S., Callister, A., Tausz, M. and Adams, M.A. (2010a). Seasonal changes in carbohydrates, cyclitols, and water relations of 3 field grown *Eucalyptus* species from contrasting taxonomy on a common site. *Annals of Forest Science*, 67(1): 104p1-104p7.

Merchant, A., Peuke, A.D., Keitel, C., Macfarlane, C., Warren, C.R. and Adams, M.A. (2010b). Phloem sap and leaf $\delta^{13}C$, carbohydrates and amino acid concentrations in *Eucalyptus lobulus* change systematically according to flooding and water deficit treatment. *Journal of Experimental Botany*, 61:1785-1793.

Merchant, A., Tausz, M., Keitel, C. and Adams, M.A. (2010c). Relations of sugar composition and $\delta^{13}C$ in phloem sap to growth and physiological performance of *Eucalyptus globulus* (Labill). *Plant Cell and Environment,* 33:1361-1368.

Merchant, A., Wild, B., Richter, A., Bellot, S., Adams, M.A. and Dreyer, E. (2011). Compound- specific differences in ^{13}C of soluble carbohydrates in leaves and phloem of 6-month-old *Eucalyptus globulus* (Labill). *Plant Cell and Environment*, 34:1599-1608.

Merchant, A. (2012). Developing Phloem $\delta^{13}C$ and sugar composition as indicators of water deficit in *Lupinus angustifolius*. *Horticultural Science*, 47(6): 691-696.

Merchant, A. and Richter, A. (2011). Polyols as biomarkers and bioindicators for 21st century plant breeding. *Functional Plant Biology,* 38: 934-940.

Merchant, A., Arndt, S.K., Callister, A., Adams, M. A. (2009). Quercitol plays a key role in stress tolerance of *Eucalyptus leptophylla* (F. Muell) in naturally occurring saline conditions. *Environmental and Experimental Botany*, 65(2-3): 296-303.

Michell, R. H. (2007). Evolution of the diverse biological roles of inositols. *Biochemical Society Symposia*, 74: 223–246.

Michell, R.H. (2008). Inositol derivatives: Evolution and functions. *Nature Reviews Molecular Cell Biology*, 9(2): 151-61.

Mitsuhashi, N., Kondo, M., Nakaune, S., Ohnishi, M., Hayashi, M., Nishimura, H.I., Richardson, A., Fukaki, H., Nishimura, M. and Mimura, T. (2008). Localization of myo-inositol-1-phosphate synthase to the endosperm in developing seeds of Arabidopsis. *Journal of Experimental Botany*, 59: 3069-3076.

Mohanakrishana, T., Bhaskar, C.V.S., Rao, P.S, Chandrashekar, T.R., Sethuraj, M.R. and Vijayakumar, K.R. (1991). Effect of irrigation on physiological performance of immature plants of *Hevea brasiliensis* in North Konkan. *Indian Journal of Natural Rubber Research*, 4: 36-45.

Moharam, B.A., Jantan, I., Jalil, J. and Shaari, K. (2010) Inhibitory Effects of phylligenin and quebrachitol isolated from *Mitrephora vulpina* on platelet activating factor receptor binding and platelet aggregation. *Molecules*, 15(11): 7840-7848.

Moore, S. and Stein, W.H. (1948). Photometric method for use in the chromatography of aminoacid. *Journal of Biological Chemistry*, 176: 357-388.

Morgan, J.M. (1984). Osmoregulation and water stress in higher plants. *Annual Review of Plant Physiology*. **35**: 27-34.

Mostajeran, A. and Rahimi-Eichi, V. (2009). Effects of drought stress on growth and yield of rice (*Oryza sativa* L.) cultivars and accumulation of proline and soluble sugars in sheath and blades of their different ages leaves. *American-Eurasian Journal of Agriculture and Environment Science*. 5(2): 264-272.

Mundree, S.G., Whitaker, A., Thomson, J.A. and Farrant, J.M. (2000). An aldose reductase homolog from the resurrection plant *Xerophyta viscosa* (Baker). *Planta,* 211: 693-700.

Munnik, T., Irvine, R.F. and Musgrave, A. (1998). Phospholipid signalling in plants. *Biochemical Biophysical Acta*, 1398: 222-272.

Munnik, T. and Vermeer, J.E.M. (2010). Osmotic stress induced phosphoinositide and inositol phosphate signalling in plants. *Plant Cell and Environment*, 33: 655-669.

Munns, R. (2002). Comparative physiology of salt and water stress. *Plant Cell and Environment*, 25: 239-250.

Munns, R, and Tester, M. (2008). Mechanisms of salinity tolerance. *Annual Review of Plant Biology*, 59: 651-681.

Munns, R. and Weir, R. (1981). Contribution of sugars to osmotic adjustment in elongating and expanded zones of wheat leaves during photosynthesis and moderate water deficits at two light levels. *Australian Journal Plant Physiology*, 12: 657-668.

Murakeozy, E.P., Nagy, Z., Duhaze, C., Bouchereau, A. and Tuba, Z. (2003). Seasonal changes in the levels of compatible osmolytes in three halophytic species of inland saline vegetation in Hungary. *Journal of Plant Physiology*, 160: 395-401.

Murakeozy, E.P., Smirnoff, N., Nagy, Z. and Tuba, Z. (2002). Seasonal accumulation pattern of pinitol and other carbohydrates in *Limonium gmelinii* subsp. *hungarica. Journal of Plant Physiology*, 159: 485-490.

Murphy, A.M., Otto, B., Brearley, C.A., Carr, J.P. and Hanke, D.E. (2008). A role for inositol hexakisphosphate in the maintenance of basal resistance to plant pathogens. *Plant Journal*, 56: 638-652.

Mydin, K.K. and Mercykutty, V.C. (2007). High yield and precocity in the RRII 400 series hybrid clones of rubber. *Natural Rubber Research*, 20(1&2): 39-49.

Myers, B.A. and Neales, T.F. (1986). Osmotic adjustment, induced by drought, in seedlings of three *Eucalyptus* species. *Australian Journal of Plant Physiology*, 13: 597-603.

Nair, N.U. (1992). Biochemical aspects related to rubber synthesis and yield in *Hevea brasiliensis*. Ph.D. Thesis, University of Kerala, Trivandrum, India.

Nair, N.U., 2000. Biochemistry and physiology of latex production. In: *Natural Rubber: Agromanagement and Crop Processing* (Eds. George, P.J. and Jacob, C.K.), Rubber Research Institute of India, Kottayam, India, pp. 249-260.

Nair, N.U. (2003). Latex diagnosis for optimizing production in clone RRII 105. *Proceedings of the International Worshop on Exploitation Technology*, 15-18, December 2003, Rubber Research Institute of India, Kottayam, India, pp. 191-198.

Nair, N.U., Thomas, M., Sreelatha, S., Simon, S.P., Vijayakumar, K.P. and George, P.J. (1993). Clonal variation in lipid composition in the latex of *Hevea brasiliensis* and its implication in latex production. *Indian Journal of Natural Rubber Research*, 6: 143-145.

Nair, N.U., Sreelatha, S., Thomas, M., Simon, S.P. and Vijayakumar, K.R. (2001). Latex diagnosis for assessing clonal performance. *Indian Journal of Natural Rubber Research*, 14(1): 63-65.

Narayanan, C.R., Joshi, D.D., Mujumdar, A.M. and Dhekne, V.V. (1987). Pinitol- A new anti-diabetic compound from the leaves of *Bouganinvillea spectabilis*. *Current Science*, 56(3): 139-141.

Nelson, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry,* 153(2): 375-380.

Nelson, D.E., Rammesmayer, G. and Bohernt, H.J. (1998). Regulation of cell specific inositol metabolism and transport in plant salinity tolerance. *The plant Cell,* 10: 753-764.

Ngugi, M.R., Doley, D., Hunt, M.A., Dart, P. and Ryan, P. (2003). Leaf water relations of *Eucalyptus cloeziana* and *Eucalyptus argophloia* in response to water deficit. *Tree Physiology,* 23:335-343.

Nguyen, A. and Lamant, A. (1988). Pinitol and myo-inositol accumulation in water stressed seedlings of maritime pine. *Phytochemistry*, 27(11): 3423-3427.

Ningjian, A.O. (2005). The development and use of the quebrachitol as a valuable botanical drug resource in latex from *Hevea brasiliensis*. *Journal of Yunnan Agricultural University,* 20: 467-473.

Nishibe, S. Sakusshima, A., Takumura, H., Takenaka, T. and Noguchi, Y. (2001). Cyclitols from Apocynaceae leaves. *Natural Medicine,* 55: 268-271.

Nobre Junior, H.V., Cunha, G.M., Moraes, M.O., Luciana, M.F., Oliveira, R.A., Maia, F.D., Nogueira, M.A., Lemos, T.L. and Rao, V.S. (2006). Quebrachitol (2-O-methyl-L-inositol) attenuates 6-hydroxydopamine-

induced cytotoxicity in rat fetal mesencephalic cell cultures. *Food and Chemical Toxicology*, 44: 1544-1551.

Noiraud, N., Delrot, S. and Lemonie, R. (2000). The sucrose transporter of celery: Identification and expression during salt stress. *Plant Physiology,* 122: 1447-1455.

Nuissier, G., Diaba, F. and Grignam-Dubis, M. (2008). Bioactive agents from beach waste*: Syringodium flotsam* evaluation as a new source of L-chiro-inositol. *Innovative Food Science and Emerging Technologies*, 9: 396-400.

Nurul-Husna, S., Norhaizan, M.E., Abdah, M.A., Saad Norazalina and Ismail Norsharina (2010). Rice bran phytic acid (IP6) induces growth inhibition, cell cycle arrest and apoptosis on human colorectal adenocarcinoma cells. *Journal of Medicinal Plants Research,* 4: 2283-9.

Obendorf, R.L. (1997). Oligosaccharides and galactosyl cyclitols in seed desiccation tolerance (Review Update). *Seed Science Research,* 7: 63-74.

Obianga, N.L.E., Sallé, G., Eschbach, J.M. and Salomon, M. (2009). Susceptibility of rubber trees to Loranthaceae in Gabon. *Journal of Plant Interactions,* 4: 233-240.

Ogawa, S. (1986). *Fine Chemical.* 66(5): 65.

Ogawa, S., Senda, N. and Suzuki, N. (1990). Collection of L-quebrachitol contained natural rubber serum. *JP2019332.*

Ortbauer, M. and Popp, M. (2008). Functional role of polyhydroxy compounds on protein structure and thermal stability studied by circular dichroism spectroscopy. *Plant Physiology and Biochemistry*, 46: 428-434.

Orthen, B. and Popp, M. (2000). Cyclitols as cryoprotectants for spinach and chickpea thylakoids. *Environmental and Experimental Botany*, 44: 125-132.

Orthen, B., Popp, M. and Smirnoff, N., (1994). Hydroxyl radical scavenging properties of cyclitols. *Proceedings of Royal Society*, Edinburgh 102B, pp. 269-272.

Ouseph, P.J. and O Bran, R.M. (1990). Thermoelectric power of yBa 2cu 307-δ. *Physical review* B, 41(1): 4123.

Padmavathi, T.A.V. and Rao, D. M. (2013). Differential Accumulation of osmolytes in 4 cultivars of Peanut (*Arachis hypogaea* L.) under drought Stress. *Journal Crop Science Biotechnology*, 16 (2): 151-159.

Pan, X., Welti, R. and Wang, X. (2008). Simultaneous quantification of major phytohormones and related compounds in crude plant extracts by liquid chromatography-electrospray tandem mass spectrometry. *Phytochemistry,* 69(8): 1773-1781.

Pardekooper, E.C. and Sookmook, S. (1969). Diumal variations in latex yield and dry rubber content and relation to saturation and deficit of air. *Joumal of the Rubber Research Institute of Malaya*, 21: 341347.

Pakianathan, S.W., Harridas, G. and d'Auzac, J. (1989). Water relations and latex flow. In: *Physiology of rubber tree latex* (Eds. d'Auzac, J., Jacob, J.L. and Chrestin, H.), Boca Raton, Forida, USA, CRC Press, p.233-256.

Pakianathan, S.W., Tata S.J., Low F.C. and Sethuraj M.R. (1992). Certain aspects of physiology and biochemistry of latex production. In: *Natural Rubber: Biology, Cultivation and Technology* (Eds. Sethuraj, M.R. and Mathew, N.M.), Amsterdam, Elsevier, pp. 298-323.

Papageorgiou, G. and Murata, N. (1995). The unusually strong stabilizing effects of glycine betaine on the structure and function of the oxygen-evolving photosystem II complex. *Photosynthetic Research,* 44: 243-252.

Patakas, A., Nikolaou, N., Zioziou, E., Radoglou, K. and Noitsakis, B. (2002). The role of organic solute and ion accumulation in osmotic adjustment in drought-stressed grapevines. *Plant Science,* 163: 361-367.

Pate, J. and Arthur, D. (1998). Delta ^{13}C analysis of phloem sap carbon: Novel means of evaluating seasonal water stress and interpreting carbon isotope signatures of foliage and trunk wood of *Eucalyptus globulus*. *Oecologia*, 117: 301-311.

Pate, J., Shedley, E., Arthur, D. and Adams, M. (1998). Spatial and temporal variations in phloem sap composition of plantation-grown *Eucalyptus globulus*. *Oecologia*, 117: 312-322.

Pate, J.S., Sharkey, P.J., Lewis, O.A.M. (1974). Phloem bleeding from legume fruits-Technique for study of fruit nutrition. *Planta,* 120: 229-243.

Patra, B., Ray, S., Richter, A. and Majumder, A.L. (2010). Enhanced salt tolerance of transgenic tobacco plants by co-expression of PcINO1 and McIMT1 is accompanied by increased level of myo-inositol and methylated inositol. *Protoplasma*, 245(1-4): 143-52.

Patrick, R.A., Daniel, R.V., Kristin, K.W. and Pamela, J.W. (2010). Effect of sugars on artemisinin production in *Artemisia annua* L.: Transcription and metabolite measurements. *Molecules,* 15(4): 2302-2318.

Passarinho, J.A.P., Lamosa, P., Baeta, J.P., Santos, H. and Ricardo, C.P.P. (2006). Annual changes in the concentration of minerals and organic compounds of *Quercus* suber leaves. *Physiologia Plantarum*, 127: 100-110.

Paul, M.J and Cockburn, M. (1989). Pinitol a compatible solute in *Mesembryantheum crystallimum* L. *Journal of Experimental Botany,* 40: 1093-1098.

Perera, I.Y., Heilmann, I. and Boss, W.F. (1999). Transient and sustained increases in inositol 1,4,5-trisphosphate precede the differential growth response in gravity stimulated maize pulvini. *Proceedings of the National Academy of Sciences of the United States of America,* 96: 5838-5843.

Perelló, J., Isern, B., Costa-Bauzá, A. and Grases, F. (2004). Determination of myo-inositol in biological samples by liquid chromatography-mass spectrometry. *Journal of Chromatography B*, 802: 367-370.

Peterbauer, T., Puschenreiter, M. and Richter, A. (1998). Metabolism of Galactosyl ononitol in seeds of *Vigna umbellate. Plant Cell Physiology,* 39(3): 334-341.

Phillips, D.V. and Smith, A.E. (1974). Soluble carbohydrates in soybean. *Canadian Journal of Botany*, 52: 2447-2452

Philpot, M.W. and Garth, D.R.W. (1953). Stability and mineral composition of *Hevea* latex. *Journal of Rubber Research Institute of Malaysia*, 14: 133-148.

Pinherre, C., Chaves, M.M., and Ricardo, C.P. (2001). Alteration in carbon and nitrogen metabolism induced by the water deficit in the stem and leaves of *Eupinus albus. Journal of Experimental Botany,* 52: 1063-1070.

Piotrowicz-Cieslak, A.I, Lopez, P., Macedonio, G. and Gulewicz, K. (2003). Cyclitols, galactosyl cyclitols and raffinose family oligosaccharides in Mexican wild lupin seeds. *Acta Societatis Botanicorum Poloniae,* 72 (2): 109-114.

Popp, M., Lied, W., Bierbaum, U., Gross, M., Grosse-Schulte, T., Hams, S., Oldenettel, J., Schöler, S. and Wiese, J. (1997). Cyclitols- stable osmotica in trees. In: *Trees - Contribution to Modern Tree Physiology* (Eds. Rennenberg, H., Eschrich, W. and Ziegler, H.), Backhuys Publishers, Leiden, the Netherlands. pp. 257-270.

Popp, M. and Smirnoff, N. (1995). Polyol accumulation and metabolism during water deficit. In: *Environment and Plant metabolism* (Ed. Smirnoff, N.), BIOS, Oxford, UK. pp. 199-216.

Posternak, T. (1965). *The cyclitols.* Holden-Day, San Fancisco, California.

Prabhavathi, V., Yadav, J.S., Kumar, P.A. and Rajam, M.V. (2002). Abiotic stress tolerance in transgenic eggplant (*Solanum melongena* L.) by introduction of bacterial mannitol-1- phosphate dehydrogenase gene. *Molecular Breeding,* 9: 137-147.

Premakumari, D. and Saraswathyamma, C.K., (2000). The Para rubber tree. In: *Natural Rubber Agromanagement and CropProcessing.* (Eds. George, P.J. and Kuruvilla Jacob, C.), Rubber Research Institute of India, Kottayam, India, pp. 29–35.

Prevot, J.C., Jacob, J.L. and Vidal, A. (1984). *Le potential d 'oxydoreduction. Etude de sa significantion physiologique dans le latex de l' Hevea brasiliensis. Son utilization comme critere physiologique.* Rapport 2/84, Institute Recherche Caoutch, CIRAD, Montpellier.

Pritchard, J. (2007). Solute transport in the phloem. In: *Plant solute transport.* (Eds. Yeo, A.R. and Flowers, T.J.). Blackwell Publishing, Oxford, UK.

Priyadarshan, P.M. (2011). Biology of *Hevea* rubber. Wallingford (UK): CAB International.

Pujarniscle, S. (1968). Caractere lysosomal des lutoides du latex *d'Hevea bmiliensis. Physiologie Vegetale,* 6: 27.

Pujarniscle, S. (1969). Etude de quelques facteurs intervenant sur la permeabilite et la stabilitie de la membrane des lutoides du latex *d'Hevea bmiliensis. Physiologie Vegetale,* 7: 391.

Pushparajah, E.H.Y., Chan, E.T. and Sivanadyan, K. (1983). Recent developments for reduced fertiliser applications for *Hevea. Proceedings of Rubber Research Institue of Malaysia First Conference,* Kuala Lumpur, 313.

Qiao, L.X., Hu, Y.H., Nan, F.J., Powis, G. and Kozikowski, A.P. (2000). A Versatile Approach to PI(3,4)P2,PI(4,5)P2, and PI(3,4,5)P3 from L-(-)-quebrachitol. *Organic Letters*, 2(2): 115-117.

Quan, R., Shang, M., Zhang, H., Zhao, Y.and Zhang, J. (2004). Engineering of enhanced glycine betaine synthesis improves drought tolerance in maize. *Plant Biotechnology Journal*, 2: 477-486.

Raghavendra, A.S., Sulochanamma, S., Rao, G.G., Mathew, S., Satheesan, K.V. and Sethuraj, M.R. (1984). The pattern of latex flow in relation to clonal variation, plugging and drought tolerance. In: *Proceedings of the IRRDB Symposium-Exploitation, Physiology and Improvement of Hevea*, Montpellier, France. IRRDB, Kuala Lumpur, Malaysia, pp 205-226.

Rahman, A.Y., Usharraj, A.O., Misra, B.B., Thottathil, G.P., Jayasekaran, K., Feng, Y.S., Su. H., Ong, Y., Ng, F.L., Lee, L.S., Tan, H.S., Sakaff, M.K.L.M., Teh, B.S., Khoo, B.F., Badai, S.S., Aziz, N.A., Yuryev, A., Knudsen, B., Dionne-Laporte, A., Mchunu, N.P., Yu, Q., Langston, B. J., Freitas, T.A.K., Young, A.G., Chen, R., Wang, L., Najimudin, N., Saito, J.A. and Alam, M. (2013). Draft genome sequence of the rubber tree *Hevea brasiliensis. BMC Genomics,* 14: 75.

Rahnama, H., Vakilian, H., Fahimi, H. and Ghareyazie, B. (2011). Enhanced salt stress tolerance in transgenic potato plants (*Solanum tuberosum* L.) expressing a bacterial *mtlD* gene. *Acta Physiologia Plantarum*, 33: 1521-1532.

Rajam, M.V., Dagar, S., Waie, B., Yadav, J.S., Kumar, P.A., Shoeb, F. and Kumria, R. (1998). Genetic engineering of polyamine and

carbohydrate metabolism for osmotic stress tolerance in higher plants. *Journal of Bioscience*, 23: 473-482.

Raj, S., Das, G., Pothen, J. and Dey, S.K. (2005). Relationship between latex yield of *Hevea brasiliensis* and antecedent environmental parameters. *International Jouranl of Biometeorology*, 49(3): 189-196.

Rao, G., Rao, P., Rajagopal, R., Devakumar, A.S., Vijayakumar, K.R. and Sethuraj, M.R. (1990). Influence of soil, plant and meteorological factors on water relations and yield in *Hevea brasiliensis*. *International Journal of Biometeorology*, 34(3): 175-180.

Rao, P., Saraswathyamma, C.K. and Sethuraj, M. R. (1998). Studies on the relationship between yield and meteorological parameters of para rubber tree (*Hevea brasiliensis*). *Agricultural and Forest Meteorology*, 90: 235-245.

Rao, P. S. and Vijayakumar, K.R. (1992). Climatic requirements. In: *Natural Rubber,* (Eds. Sethuraj, M.R. and Mathew, N.M.), Elsevier, Amsterdam, Netherlands, 200.

Ray, S., Patra, B., Chatterjee, D.A., Ganguli, A. and Majumdar, A.L. (2010). Identification and organization of chloroplastic and cytosolic L-myo-inositol-1-phosphate synthase coding gene(s) in *Oryza sativa*: comparison with the wild halophytic rice, *Porteresia coarctata*. *Planta*, 231: 1211-1227.

Rhodes, E. and Wiltshire, J.L. (1932). Quebrachitol – A possible by product from latex. *Journal of Rubber Research Institute of Malaya,* 3(3): 160 - 171.

Rhodes, D. and Hanson, A.D. (1993). Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 44: 357-384.

Richter, A. and Popp, M. (1992). The physiological importance of cyclitols in *Viscum album* L. New Phytologist, 121: 431- 438.

Richter, A., Thonke, B. and Popp, M. (1990). 1D-1-*O*-methyl-mucoinositol in *Viscum album* and members of Rhizophoraceae. *Phytochemistry*, 29: 1785-1786.

Richmond, M.L., Barfuss, D.L., Harte, B.R., Gray, G.I. and Stine, C.M. (1982). Separation of carbohydrates in dairy products by HPLC. *Journal of Dairy Science*, 65: 1394-1400.

Rojruthai, P., Tarachiwin, L., Sakdapipanich, J.T. and Tanaka, Y. (2009). Phospholipids associated with NR molecules inducing long-chain branching Part II. *KGKElastomereund Kunststoffe*, 399: 404.

Rolland, F., Moore, B. and Sheen, J. (2002). Sugar sensing and signalling in plants. *Plant Cell,* S185-S205.

Ruderman, S., Kongsawadworakul, P., Viboonjun, U., Mongkolporn, O. and Chrestin, H. (2012). Mitochondrial/Cytosolic Acetyl CoA and rubber biosynthesis genes expression in *Hevea brasiliensis* latex and rubber yield. *Kasetsart Journal of Natural Science*, 46: 346 - 362.

Ruinen, J. (1950). Microscopy of the lutoids in *Hevea* latex. *Ann. Bogonensis,* 1: 7.

Sacher, R.F. and Staples, R.C. (1985). Inositol and sugars in adaptation to tomato to salt. *Plant Physiology,* 77: 206-210.

Sando, T., Takaoka, C., Mukai, Y., Yamashita, A., Hattori, M., Ogasawara, N., Fukusaki, E. and Kobayashi, A. (2008). Cloning and characterization of mevalonate pathway genes in a natural rubber producing plant, *Hevea brasiliensis*. *Bioscience, Biotechnology, and Biochemistry,* 72: 2049-2060.

Salvucci, M.E. (2000). Sorbitol accumulation in white flies: evidence for a role in protecting proteins during heat stress. *Journal of Thermal Biology*, 25: 353-361.

Sanseera, D., Niwatananun, W., Liawrienngrath, B., Liawrienngrath, S., Baraee, A., Trisuwan, K. and Pyne, S.G. (2012). Antioxidant and anticancer activities from aerial parts of *Acalypha indica* Linn. *Chiang Mai University Journal of Natural Science*, 11(2): 157-168.

Sanz, M.L., Villamiel, M. and Martinez-Castro, I. (2004). Inositols and carbohydrates in different fresh fruit juices. *Food Chemistry,* 87: 325-328.

Sakdapipanich, J. (2005). The development of skim rubber and more valuable economic product from natural rubber. *National Rubber Research and Development. Mahidol University Science Technology,* 138-142.

Saraswathyamma, C.K. and Sethuraj, M.R. (1975). Clonal variations in latex flow characteristics and yield in rubber tree. *Journal of Plantation Crops*, 3: 14-15.

Satheesan, K.V., Rao, G.G. and Sthuraj, M.R. (1982). Clonal and seasonal variation in osmotic concentration of latex and lutoids serums of *Hevea brasiliensis* Muell. Arg. *Proceedings of the Vth Annual Symposium on Plantation Crops*, Kasargod, 240-246.

Sasaki, K., Hicks, K.B. and Nagahashi, G. (1988). Separation of eight inositol isomers by liquid chromatography under pressure using a calcium-form, cation-exchange column. *Carbohydrate Research*, 183: 1-9.

Saxena, S.C., Salvi, P., Kaur, H., Verma, P., Petla, B.P., Rao, V., Kamble, N. and Majee, M. (2013). Differentially expressed *myo*-inositol monophosphatase gene (*CaIMP*) in chickpea (*Cicer arietinum* L.) encodes a lithium-sensitive phosphatase enzyme with broad substrate specificity and improves seed germination and seedling growth under abiotic stresses. *Journal of Experimental Botany*, 11: 2-17.

Sayed, S.A., Gadallah, M.A.A. and Salama, F.M. (2013). Ecophysiological studies on three desert plants growing in Wadi Natash, Eastern Desert, Egypt. *Journal of Biology and Earth Sciences*, 3(1): B1 35-B1 43.

Scobell, H.D., Brobst, K.M. and Steale, E.H. (1977). Automated liquid chromatography system for analysis of carbohydrate mixtures. *Cereal Chemistry*, 54: 905.

Scott, T.A. and Melvin, E.H. (1953). Determination of dextrin with anthrone. *Analytical Chemistry,* 25 (11): 1656-1661.

Schobest, B. (1977). Is there an osmotic regulatory mechanism in algae and higher plants? *Journal of Theoretical Biology,* 68: 17-26.

Scholda, R., Billek, G. and Hofmann-Ostenhof, O. (1964). Untersuchungen uber die Biosynthese der Cyclite. 7. Mitt.: Der mechanismus der Umwandlung von meso-Inosit in Methylather des 1-Inosit in Blattchen von Artemisia vulgaris und Artemisia dracunculus. *Monatshefte für Chemie*, 95: 1305.

Schultes, R.E. (1970). The history of taxonomic studies in *Hevea. The Botanical Review,* 36(3): 197-211.

Schultes, R.E. (1977). Wild *Hevea*: An untapped source of germplam. *Journal of Rubber Research Institute of SriLanka,* 54: 227-257.

Schultes, R.E. (1987). Studies in the genus *Hevea*: 8. Notes on intraspecific variants of *Hevea brasiliensis* (Euphorbiaceae). *Economic Botany,* 41(2): 125-147.

Schultes, R.E. (1990). A brief taxonomic view of the genus *Hevea*. MRRDB Monograph 14 *Malaysian Rubber Research and Development Board*, Kula Lumpur.

Serraj, R. and Sinclair, T.R. (2002). Osmolyte accumulation: can it really help increase crop yield under drought condition? *Plant Cell and Environment*, 25:333-341.

Sethuraj, M.R. (1992). Yield components in *Hevea brasiliensis*. In: *Natural rubber: Biology, Cultivation and Technology* (Eds. Sethurj, M.R. and Mathew, N.M.), Amsterdam, Elsevier. pp. 137-163.

Sethuraj, M. and George, M.J. (1976). Multiple band application of ethephon to increase the drainage area and yield. *Proceedings of International Rubber Research and Development Board*, 1976, Bogor, Indonesia.

Sethuraj, M.R., Potty, S.N., Vijayakumar, K.R., Krishnakumar, A.K., Rao, P.S., Thapliyal, A.P., Mohanakrishna, T., Rao, G.G., Chaudhuri, D., George, M.J., Soman, T.A., and Meenattoor, J.R. (1989). Growth performance of *Hevea* in the non-traditional regions of India. In *Proceedings of the Rubber Research Institute of Malaysia, Rubber Growers' Conference*, 1989, Malacca, Malaysia, pp. 212-227.

Sethuraj, M.R. and Raghavendra, A.S. (1987). Rubber. In: *Tree Crop Physiology*. (Eds. Sethurj, M.R. and Raghavendra, A.S.), Elsevier Science Publishers, Amsterdam, pp. 193-223.

Sethuraj, M.R., Rao, G.G. and Raghavendra, A.S. (1984). The pattern of latex flow from rubber tree (*Hevea brasiliensis)* in relation to water stress. *Journal of Cellular Biochemistry, 8B (Supplement): 236.*

Sheldrake, A.R. (1978). Effects of osmotic stress on polar auxin transport in *Avena* mesocotyl sections. *Planta,* 145: 113-117.

Shen, B., Jensen, R.G. and Bohnert, H.J. (1997a). Increased resistance to oxidative stress in transgenic plant by targeting mannitol synthesis to chloroplast. *Plant Physiology.* 1123: 1177-1183.

Shen, B., Jensen, R.G. and Bohnert, H.J. (1997b). Mannitol protects against oxidation of hydroxyl radicals. *Plant Physiology,* 115: 527-532.

Shen, H.Y., Jiang H.L., Mao, H.L., Pan, G, Zhou, L. and Cao, Y.F. (2007). Simultaneous determination of seven phthalates and four parabens in cosmetic products using HPLC-DAD and GC-MS methods. *Journal of Separation Science,* 30(1): 48- 54.

Sheveleva, E., Chmara, W. Bohnert, H.J. and Jensen. R.G. (1997). Increased salt and drought tolerance by D-Ononitol production in transgenic *Nicotiana tabacum* L. *Plant Physiology,* 115: 1211-1219.

Shinozaki, K. and Shinozaki, Y.K. (2007). Gene networks involved in drought stress response and tolerance. *Journal of Experimental Botany*, 58(2): 221–227.

Sickler, C.M., Edwards, G.E., Kiirats, O., Gao, Z. and Loescher, W. (2007). Response of mannitol producing *Arabidopsis thaliana* to abiotic stress. *Functional Plant Biology*, 34: 382-391.

Siddique, Z.S., Khan, A.M., Kim, B.G., Huang, J.S. and Kwon, T.R. (2008). Physiological responses of *Brassica napus* genotypes to combined drought and salt stress. *Plant Stress,* 2(1): 78-83.

Silpi, U., Thaler, P., Kasemsap, P., Lacointe, A., Chantuma, P., Adam, B., Gohet, E., Thanisawanyangkura, S. and Ameglio, T. (2006). Effect of tapping activity on the dynamics of radial growth of *Hevea brasiliensis* trees. *Tree Physiology*, 26:1579-1587.

Silpi, U., Lacointe, A., Kasempsap, P., Thanysawanyangkura, S., Chantuma, P., Gohet, E., Musigamart, N., Clément, A., Améglio, T. and Thaler, P. (2007). Carbohydrate reserves as a competing sink: Evidence from tapping rubber trees. *Tree Physiology,* 27: 881–889.

Sinclair, T.R. (2005). Theoretical analysis of soil and plant traits influencing daily plant water flux on drying soils. *Agronomy Journal*, 97: 1148-1152.

Singh, D.P. Choudhury, B.D. Singh, P., Sharma, H.C. and Karwasra, S.P.S. (1990). Drought tolerance in oil seed *Brassica* and *Chickpea*. HAU, Hissar, pp. 10-55.

Singh, L.R., Poddar, N.K., Dar, T.A., Rahman, S., Kumar, R. and Ahamad, F. (2011). Forty years of research on osmolyte-induced protein folding and stability. *Journal of Iranian Chemical society,* 8 (1): 1-23.

Smeekens, S. (2000). Sugar-induced signal transduction in plants. *Annual Review of Plant Physiology and Plant Molecular Biology,* 51: 49-81.

Smirnoff, N. and Cumbes, Q.J. (1989). Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry,* 28: 1057- 1060.

Smith, R.H. (1954). The phosphatides of the latex of *Hevea brasiliensis. Biochemistry Journal,* 57: 130 - 140.

Soedjanaatmadja, U.M.S., Subroto, T. and Beintema, J.J. (1995). The effluent of natural rubber factories is enriched in the antifungal protein hevein. *Bioresource Technology*, 53: 39-41.

Somasundar, P., Riggs, D.R., Jackson, B.J., Cunningham, C., Vona-Davis, L. and Mc Fadden, D.W. (2005). Inositol hexaphosphate (IP6): A novel treatment for pancreatic cancer. *Journal of Surgical Research*, 126, 199-203.

Southorn, W.A. (1969). Physiology of *Hevea* (Latex Flow). *Journal of Rubber Resarch Institute of Malaya*, 21(4): 494-512.

Southorn, W.A. and Yip, E. (1968). Latex flow studies: Electrostatic considerations in the colloidal stability of fresh *Hevea* latex. *Journal of Rubber Research Institute of Malaya,* 20(4): 201-215.

Sperry, J.S., Adler, F.R, Campbell, G.S and Comstock. J.P. (1998). Limitation of plant water use by rhizosphere and xylem conductance: results from model. *Plant Cell and Environment*, 21: 347-359.

Sreelatha, S., Sheela P.S. and James J. (2004). On the possibility of using ATP concentration in latex as an indicator of high yield in *Hevea brasiliensis. Journal of Rubber Research,* 7(1): 71-78.

Sreelatha, S. (2003). Biochemical factors influencing latex flow during stress, tapping frequency and stimulation in *Hevea brasiliensis.* Ph. D. Thesis, Mahatma Gandhi University, Kottayam, India, 204. p.

Stephanie, B., Ruth, G.R., Johann, H., Renato, A. and Hans, N. (1986). Isolation and identification of cyclitols in carob pods (*Ceratonia siliqua* L.). *Journal of Agricultural and Food Chemistry,* 34 (5): 827-829.

Stevenson, J.M., Perera, I.Y., Heilmann, I., Peresson, S. and Boss, W.F. (2000). Inositol signalling and reproductive traits in soybean. *Crop Science*, 43: 464-473.

Stieglitz, K.A., Yang, H., Roberts, M.F. and Stec, B. (2005). Reaching for mechanistic concensus across life kingdoms: Structure and insights into catalysis of the myoinositol-1-phosphate synthase (MIPS) from *Archaeoglobus fulgidus*. *Biochemistry,* 44: 213-224.

Stinson, E.E., Dooley, C.J., Purcell, J.M. and Ard, J.S. (1967). Quebrachitol - A New component of *Maple* sap and syrup. *Journal of Agricultural and Food Chemistry*, 15(3): 394-397.

Stoneman, G.L., Turner N.C., and Dell, B. (1994). Leaf growth, photosynthesis and tissue water relations of greenhouse-grown *Eucalyptus marginata* seedlings in response to water deficits. *Tree Physiology,* 14: 633-646.

Stoop, J.M.H. and Mooibroek, H. (1998). Cloning and characterization of NADP-mannitol dehydrogenase cDNA from the button mushroom, *Agaricus bisporus*, its expression in response to NaCl stress. *Applied Environment Microbiology,* 64: 4689-4696.

Steinitz, B. (1999). Sugar alcohols display non osmotic roles in regulating morphogenesis and metabolism in plants that do not produce polyols as primary photosynthetic products. *Journal of Plant Physiology,* 155: 1-8.

Streeter, J.G. (1980). Carbohydrates in soybean nodules. II. Distribution of compounds in seedlings during the onset of nitrogen fixation. *Plant Physiology*. 66: 471-476.

Streeter, J.G. (2001a). Simple partial purification of D-pinitol from soybean leaves, *Crop Science*, 41(6): 1985-1987.

Streeter, J.G. and Strimbu. C.E. (1998). Simultaneous extraction and derivatization of carbohydrates from green plant tissues for analysis by gas-liquid chromatography. *Analytical Biochemistry,* 259: 253-257.

Streeter, J.G., Lohnes, D.G. and. Fioritto, R.J. (2001b). Patterns of pinitol accumulation in soybean plants and relationships to drought tolerance. *Plant, Cell and Environment,* 24: 429-438.

Sultanbawa, Y. and Li-Chan, E.C.Y. (1998). Cryoprotective effects of sugar and polyol blends in Ling Cod surimi during frozen storage. *Food Research International*, 31: 87-98.

Sumesh, K.V., Satheesh, P.R., Annamalainathan, K., Krishnakumar, R., Molly Thomas and Jacob, J. (2011). Physiological evaluation of a few modern *Hevea* clones for intrinsic drought tolerance. *Natural Rubber Research*, 24(1): 61-67.

Sureshan, K.M., Ikeda, K., Asano, N. and Yutaka, W. (2008). Efficient synthesis of optically pure chiro and allo-inositol derivatives, azidocyclitols and aminocyclitols from myo-insoitol. *Tetrahedron Letters*, 64: 4072-4080.

Taiz, L. and Zeiger, E. (1991). Plant Physiology. Benjamin/Cummings. Redwood City, California, USA, 792p.

Takahashi, H., Kittaka, H., and Ikegami, S. (2001). Novel synthesis of enantiomerically pure natural inositols and their diastereoisomers. *Journal of Organic Chemistry,* 66: 2705-2716.

Tan, J., wang, W., Xiang, B., Han, R. and Guo, Z. (2013). Hydrogen peroxide and Nitrous oxide mediated cold and dehydration induced myo-iositol phosphate synthase that confer multiple resistances to abiotic stresses. *Plant Cell and Environment*, 36: 288-299.

Tang, C., Huang, D., Yang, J., Liu, S., Sakr, S., Heping, L., Yihua, Z. and Yunxia, Q. (2010). The sucrose transporter *HbSUT3* plays an active role in sucrose loading to laticifer and rubber productivity in exploited trees of *Hevea brasiliensis* (para rubber tree). *Plant Cell and Environment*, 33: 1708-1720.

Tanret, C. (1889). *Compt. Rend. Hebd. Seaces Acadmeic. Science,* 109 - 908.

Tarczynski, M.C., Jensen, R.G. and Bohnert, H.J. (1992). Expression of a bacterial *mtlD* gene in transgenic tobacco leads to production and accumulation of mannitol. *Proceedings of National Academy of Science United States of America*, 89: 2600-2604.

Tarczynski, M.C., Jensen, R.G. and Bohnert, H.J. (1993). Stress protection of transgenic tobacco by production of the osmolyte mannitol. *Science,* 259: 508-510.

Tardieu, F. and Simonneau. T. (1998). Variability among species of stomatal control under fluctuating soil water status and evaporative demand: Modeling isohydric and anisohydric behaviour. *Journal of Experimental Botany*, 49: 419-432.

Tausz, M., Merchant, A., Kruse, J., Samsa, G. and Adams, M.A. (2008). Estimation of drought-related limitations to mid-rotation aged plantation grown *Eucalyptus globulus* by phloem sap analysis. *Forest Ecology and Management* 256:844–848.

Thapa, G., Dey, M., Sahoo, L. and Panda, S.K. (2011). An insight into the drought stress induced alterations in plants. *Biologia Plantarum*, 55: 603-613.

Thomas, M., Sathik, M.B.M., Luke, L.P., Sumesh, K.V., Satheesh, P.R., Annamalainathan, K. and Jacob, J. (2012). Stress responsive transcripts and their association with drought tolerance in *Hevea brasiliensis*. *Journal Plantation Crops*, 40: 180-187.

Thomas, M., Sathik, M.B.M., Saha, T., Jacob, J., Schaffner, A.R., Luke, L.P., Kuruvilla, L., Annamalainathan, K. and Krishnakumar, R. (2011). Screening of drought responsive transcripts of *Hevea brasiliensis* and identification of candidate genes for drought tolerance. *Journal of Plant Biology*, 38&39: 111-118.

Thomas, J.C., Sepahi, M., Arendall, B. and Bohnert, H. J. (1995). Enhancement of seed germination in high salinity by engineering mannitol expression in *Arabidopsis thaliana*. *Plant Cell and Environment*, 18: 801-806.

Thomas, M., Nair, D.B., Gopalakrishnan, J., Sreelatha, S. and Jacob, J. (1999). Ethephon application increases the rubber yield from *Hevea brasiliensis* by altering the water realtions of the latex. *Journal of Plant Biology*, 26(3): 191-198.

Turner, N.C. (1997). Further progress in plant water relations. *Advances in Agronomy*, 58: 293-338.

Turner, N.C. and Jones, M.M. (1980). Turgor maintenance by osmotic adjustment: A review and evaluation. In: *Adaptation of plants to water and high turgor stress*. (Eds. Turner, N. C. and Karmer, P. J.), John Wiley and sons, Chichester. pp. 87-103.

Tungngoen, K., Kongsawadworakul, P., Viboonjun, U., Katsuhara, M., Brunel, N., Sakr, S., Narangajavana, J. and Chrestin, H. (2009). Involvement of HbPIP2;1 and HbTIP1;1 aquaporins in ethylene stimulation of latex yield through regulation of water exchanges between inner liber and latex cells in *Hevea brasiliensis*. Plant *Physiology*, 151: 843-856.

Tungngoen, K, Viboonjun, U., Kongsawadworakul, P., Katsuhara, M., Julien, J.L., Sakr, S., Chrestin, H. and Narangajavana, J.(2011). Hormomal treatment of the bark of rubber trees (*Hevea brasiliensis*) increases latex yield through latex dilution in relation with the differential expression of two aquaporin genes. *Journal of Plant Physiology,* 168: 253-262.

Tupý, J. (1973). Influence de la stimulation hormonale de la production sur le teneur de saccharose du latex d'*Hevea brasiliensis*. *Rev. Gener. Caouthouc et Plastiques* 50: 311-314.

Tupy, J. and Primot, L. (1976). Control of carbohydrate metabolism by ethylene in latex vessels of *Hevea brasiliensis* Muel. Arg. in relation to rubber production. *Biologia Plantarum*, 18: 373-383.

Tupý, J.and Primot, L. (1982). Sucrose synthetase in the latex of *Hevea brasiliensis* Muell. Arg. *Journal of Experimental Botany*, 33: 988-995.

Tyree ,M.T. and Jarvis, P.G .(1982). Water in tissues and cells. In: *Physiological Plant Ecology II: Water Relations and Carbon Assimilation II,* (Eds. Lange, O.L., Nobel, P.S., Osmond, C.B. and Ziegler, H,) Springer-Verlag, Berlin, pp 35-77.

Udagawa, Y., Machida, M. and Ogawa, S. (1991). Method of recovering L-quebrachitol from rubber latex serums. *US Patent No. 5041689.*

Valliyodan, B. and Nguyen, H.T. (2006). Understanding regulatory networks and engineering for enhanced drought tolerance in plants. *Curent Opinion in Plant Biology,* 9: 189-195.

Valluru, R. and Van den Ende, W. (2011). Myo-inositol and beyond - emerging networks under stress. *Plant Science,* 181: 387-400.

Venkatachalam, P., Geetha, N., Sangeetha, P. and Thulaseedharan, A. (2013). Natural rubber producing plants: An overview. *African Journal of Biotechnology,* 12(12): 1297-1310.

Vernon, D.M., Ostrem, J.A. and Bohnert, H.J. (1993). Stress perception and response in a facultative halophyte: The regulation of salinity induced genes in *Mesembryanthemum cystallinum. Plant Cell and Environment*, 16: 437-444.

Vernon, D. M. and Bohnert, H. J. (1992). A novel methyl transferase induced by osmotic stress in the facultative halophyte *Mesembryanthemum crystallinum.* The *EMBO Journal,* 11: 2077-2085.

Verslues, P.E. and Sharp, R.E. (1999). Proline accumulation in maize (*Zea mays* L.) primary roots at low water potentials. II. Metabolic source of increased proline deposition in the elongation zone. *Plant Physiology*, 119: 1349-1360.

Vijayakumar, K.R., Dey, S.K., Chandrashekar, T.R., Devakumar, A.S., Mohankrishna, T., Rao, P.S. and Sethuraj, M.R. (1998). Irrigation requirement of rubber trees (*Hevea brasiliensis*) in the subhumid tropics. *Agricultural Water Management,* 35(3): 245-259.

Vijayakumar, K.R., Rao, G.G., Rao, P.S., Devakumar, A.S., Rajagopal, R., George, M.J. and Sethuraj, M.R. (1988). Physiology of drought tolerance of *Hevea. Compte-Renfu du Collque Exploitation-physiologie et Amelioration de l' Hevea*, 1988, Paris, pp. 269-281.

Vijayakumar, K.R., Sulochanamma, S., Thomas, M., Sreelatha, S., Simon, S.P. and Sethuraj, M.R. (1991). The effect of intensive tapping on induction of tapping panel dryness and associated biochemical changes in two clones of *Hevea.* Physiology and Exploitation of *Hevea brasiliensis*: *Proceedings of IRRDB symposium*, Kumming, China, pp. 103-110.

Vitrac, X., Larronde, F., Krisa, S., Decendit, A., Deffieux, G. and Mérillon, J.M. (2000). Sugar sensing and Ca^{2+}-calmodulin requirement in *Vitis vinifera* cells producing anthocyanins. *Phytochemistry,* 53: 659-665.

Wanek, W. and Richter, A. (1997). Purification and characterization of myo -inositol 6-*O*-methyltransferase from *Vigna umbellata* Ohwi et Ohashi. *Planta,* 197(3): 427-434.

Wang, Z. and Stutte, G.W. (1992). The role of carbohydrates in active osmotic adjustment in apple under water stress. *Journal of American Society of Horticultural Science*, 117: 816-823.

Wang, Z., Quebedeaux, B. and Stutte, G. W. (1995). Osmotic adjustment: Effect of water stress on carbohydrates in leaves, stems and roots of apple. *Australian Journal of Plant Physiology*, 22(5): 747-754.

Wang. Z., Quebedeaux, B. and Stutte, G. W. (1996). Partitioning of (^{14}C) glucose into sorbitol and other carbohydrates inapple under water stress. *Australian Journal of Plant Physiology,* **23**: 245-251.

Wang, W.T., Safar, J. and Zopt, D. (1990). Analysis of inositols by high performance liquid chromatography. *Analytical Biochemistry*, 188(2): 432-435.

Watson, G.A. (1989). Climate and soil. In: *Rubber.* (Eds. Webster, C.C. and Baulkwill, W.J.), Longman, New York. 614 p.

Webster, C.C. and Paadekooper, E.C. (1989). Botany of the rubber tree. In: *Rubber.* (Eds. Webster, C.C. and Baulkwill, W. J.), Longman Scientific and Technical, Essex, England. Pp 57-84.

White, D.A., Turner, N.C. and Galbraith, J.H. (2000). Leaf water relations and stomatal behavior of four allopatric *Eucalyptus species* planted in Mediterranean southwestern Australia. *Tree Physiology,* 20: 1157-1165.

Wimmer, R., Olsson, M., Petersen, M.T.N., Hatti-Kaul, R., Pettersen, S.B., and Muller, N. (1997). Towards a molecular level understanding of protein stabilization: the interaction between lysozyme and sorbitol. *Journal of Biotechnology*, 55: 85-100

Wingler, A. (2002). The function of trehalose biosynthesis in plants. *Phytochemistry,* 60: 437-440.

Wititsuwannakul, D., Rattanapittayaporn, A. and Wititsuwannakul, R. (2003). Rubber biosynthesis by *Hevea* latex bottom fraction membrane. *Journal of applied Polymer Science,* 87: 90-96.

Wititsuwannakul, R., Pasitkul, P., Kanokwiroon, K. and Wititsuwannakul, D. (2008a). A role for a *Hevea* latex lectin-like protein in mediating rubber particle aggregation and latex coagulation. *Phytochemistry,* 69: 339-347.

Wititsuwannakul, R., Rukseree, K., Kanokwiroon, K. and Wititsuwannakul, D. (2008b). A rubber particle protein specific for *Hevea* latex lectin binding involved in latex coagulation. *Phytochemistry,* **69:** 1111-1118.

Wititsuwannakul, D. and Wititsuwannakul, R. (2001). Biochemistry of natural rubber and structure of latex. In: *Biopolymers.* (Ed. Steinbuchel, A.), vol. 2. Wiley–VCH, Weiheim, Germany, pp. 151-202.

Woodman, R.M., and Rhodes, E. (1932). Notes on quebrachitol and the lipin from *Hevea* latex. *Journal of Rubber Research Institute of Malaya,* 4 (2): 153-155.

Wu, Y. Jiang, S.K., Li, G.H., Zhang, G.M., Zou, J.Y. and Xu, R. (2013). Method of large-scale extracting L-quebrachitol from natural rubber factory waste water. *PCT Patent Application No. PCT/ CN20131075778.* (in Chinese).

Wychereley, P.R. (1992). The genus *Hevea*: Botanical aspects. In: *Natural Rubber: Biology, Cultivation and Technology*. (Eds. Sethuraj, M.R. and Mathew, N.M.), Elsevier, Amsterdam, Pp 50-66.

Yancey, P.H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *Journal of Experimental Biology*, 208: 2819-2830.

Yancey, P.H, Clarrk, M.E., Hand, S.C., Bowlus, R.D. and Somero, G.N. (1982). Living with water stress, evolution of osmolyte system. *Sceince,* 217: 1201-1222.

Yang, B., Huang, D.B., Liu, S.J. and Tang, C.R. (2007). Molecular cloning and sequence analysis of six sucrose transporter genes from *Hevea brasiliensis* (Para rubber). *Chinese Journal of Tropical Crops,* 28: 32-38.

Yang, B.R., Kallio, H., Judin, V.P. and Korteniemi, V. M. (2009). Extract with high amount of quebrachitol and its use. *PCT Patent Application No. PCT/FI2009/050273.*

Yang, B., Zhang, J. and Kellio, H. (2011). Inositols and methyl inositols in sea buckthorn berries: Influence of origin, harvesting date and growth conditions. *Food Chemistry*, 125: 388-396.

Yaojun, D. (1997). A brand new material in pharmaceutical industry-quebrachitol, its characteristics, development and utilisation. *Chinese traditional and Herbal drugs*, 28(8): 500-502.

Yaojun, D and Donghua, D. (1999). Study on extraction of quebrachitol from Natural rubber. In: *Proceedings of International Rubber Research Development Board symposium*, 1999, China, pp. 1-12.

Yaojun, D. and Donghu, D. (2000). Study on extraction of quebrachitol from natural rubber. *Natural product Research and Development,* 12(6): 61-65.

Yaojun, D., Ningjian, A.O., Hong, J. and Yinzhi, O.U. (2004). Effects of different latex preservatives on quebrachitol in natural rubber latex. In: *Proceedings of International Rubber Research Development Board symposium: NR Industry: Responding to Globalization,* 7-8 September 2004, Kunming Yunnan, China, pp. 463 - 466.

Yeang, H.Y. and Paranjothy, K. (1982). Initial physiological changes in *Hevea* latex flow characteristics associated with intensive tapping. *Journal of Rubber Research Institute of Malaya,* 30: 31-36.

Yip, E. and Chin, H.C. (1977). Latex flow studies X distribution of metallic ions between phases of Hevea latex and the the effect of yield stimulation in this distribution. *Journal of Rubber Research Institute of Malaysia*, 25(1): 31-49.

Yoonram, K., Takahashi, S., Rattanapittayaporn, A., Koyama, T., Wititsuwannakul, D. and Wititsuwannakul, R. (2008). cDNA, from *Hevea brasiliensis* latex, encoding 1-deoxy-Dxylulose-5-phosphate reductoisomerase. *Plant Science,* 175: 694-700.

Yoshida, K.T., Wada, T., Koyama, H., Mizobuchi-Fukuoka, R. and Naito, S. (1999). Temporal and spatial patterns of accumulation of the transcript of myo-inositol-1-phosphate synthase and phytin containing particles during seed development in rice. *Plant Physiology*, 119: 65-72.

Yoshida, K.T., Fujiwara, T. and Naito, S. (2002). The synergistic effects of sugar and abscisic acid on myo-inositol-1-phosphate synthase expression. *Physiologia Plantarum*, 114: 581-587.

Yusof, F., Chow, K.S., Ward, M.A. and Walker, J.M. (2000). A stimulator protein from *Hevea brasiliensis* latex. *Journal of Rubber research,* 3(4): 232-249.

Zhao, C.X., Shao, H.B. and Chu, L.Y, (2008). Aquaporin structure-function relationships: Water flow through plant living cells. *Colloids Surface,* 62: 163-172.

Zhu, X., Gong, H., Chen, G., Wang, S. and Zhang, C. (2005). Different solute levels in two springwheat cultivars induced by progressive field water stress at different developmental stages. *Journal of Arid Environments*, 62: 1-14.

Zhu, J. and Zhang, Z. (2009). Ethylene stimulation of latex production in *Hevea brasiliensis*. *Plant Signalling and Behaviour*, 4(11): 1072-1074.

Zinselmeier, C., Jeong, B. R. and Boyer, J. S. (1999). Starch and the control of kernel number in maize at low water potentials. *Plant Physiology,* 121: 25-36.

Appendices

APPENDICES

Reagents and Buffer solutions

1. **Solvents for extraction and processing of latex serum**

   Acetic acid 3%

   Acetone

   Acetonitrile

   HPLC grade water

   Ethyl alcohol 80%

   Chloroform

   MilliQ water

   Silica gel – Supercosil silica gel.

   Amberlite mixed bed resin - Amberlite MB 150 ion exchange resin

2. **Reagents for biochemical estimation**

   Ethyl alcohol 80%

   Sodium acetate buffer 1M

   Sodium metaperiodate 0.01M

   Anthrone reagent

   0.1g anthrone in a solution containing 29 ml water and 100 ml sulphuric acid

   Copper reagent

   A- Dissolve 25 g sodium carbonate, 25 g sodium potassium tartrate, 20g sodium bicarbonate and 200 g sodium sulphate in 800ml distilled water and make up volume to 1000 ml.

   B- 15% copper sulphate solution containing 1 or two drops of concentrated sulphuric acid.

   C- Freshly prepare 25 ml Copper reagent A + 1 ml Copper reagent B for estimation

Arsenomolybdate solution

Dissolve 25 g Ammonium molybdate in 450 ml distilled water and add 21 ml concentrated sulphuric acid and mix well. Add sodium arsenate solution (3 g in 25 ml distilled water) to this solution and stir well.

Citrate buffer (0.2M)

Dissolve 10.504 g citric acid monohydrate salt in 100 ml of 1N sodium hydroxide (1N NaOH) solution (4g/100 ml). Make up the volume to 250 ml. Check pH and adjust to 5.5.

Ninhydrin reagent

1. Dissolve 1g ninhydrin in 25 ml methyl cellosolve solution
2. Dissolve 40 mg stannous chloride ($SnCl_2$. $2H_2O$) in 25 ml of 0.2M citrate buffer.

Add stannous chloride solution to ninhydrin containing methyl cellosolve solution

Diluent - water: propanol, 1:1 (V/V)