Isolation and Characterization of Certain Biomolecules from *Euphorbia caducifolia* Haines

Thesis submitted to the University of Hyderabad for the degree of

DOCTOR OF PHILOSOPHY

By

KUSUMA VENUMADHAV

12LPPH04

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad-500046

July 2017

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad – 500046, Telangana, India

DECLARATION

I, **Kusuma Venumadhav,** hereby declare that the thesis entitled **"Isolation and Characterization of Certain Biomolecules from *Euphorbia caducifolia* Haines"** submitted to University of Hyderabad has been carried out by me under the supervision of **Prof. Kottapalli Seshagirirao**, Department of Plant Sciences, School of Life Sciences, University of Hyderabad, Hyderabad – 500046, India. I also declare that the work has not been submitted for any other degree or diploma to any University or Institution and is free from plagiarism.

Kusuma Venumadhav
12LPPH04

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
Hyderabad – 500046, Telangana, India

CERTIFICATE

This is to certify that this thesis entitled "**Isolation and Characterization of Certain Biomolecules from *Euphorbia caducifolia* Haines**" is a record of Bonafide work done by **Kusuma Venumadhav**, a research scholar for Ph. D. programme under the Department of Plant Sciences, School of Life Sciences, University of Hyderabad under my guidance and supervision. This thesis is free from plagiarism and has not been submitted in part or in full to this or any other University or Institution for the award of any degree or diploma.

Parts of the thesis have been:

A. Published in the following publications:
   1. International Journal of Pharma Sciences and Research, 8(5), 92-96, 2017
   2. Roxburghia, 6(1-4), 111-119, 2016

B. Presented at the following conferences:
   1. "International Conference on Biotechnology & Bioengineering Trends 2017" held at JNTU, Hyderabad, 23-25th March 2017.
   2. "Plant Diversity: Challenges in Conservation and Sustainable Utilization" held at Kakatiya University, Warangal, India, 4-6th February 2016.
   3. "6th Asian Community of Glycoscience and Glycotechnology" held at University of Hyderabad, Hyderabad, 9-12th December 2014.

Further, the student has passed the following courses towards the fulfillment of the coursework requirement for Ph.D.

Sl.No.	Course Code	Name	Credits	Pass/Fail
1.	PL 801	Research Methodolgy	4	Pass
2.	PL 802	Research Ethics & Management	2	Pass
3.	PL 801	Lab Work	4	Pass
4.	PL 801	Biostatistics	2	Pass

Supervisor **Head** **Dean**

ACKNOWLEDGEMENTS

I express my deep feelings to my research supervisor **Prof. K. Seshagirirao**, for giving me the opportunity to work under his guidance. I express my deep gratitude to his helpful attitude and back-up without which I would have ever completed this work.

I am very much grateful to my Doctoral committee members **Prof. P. B. Kirti** and **Prof. S. Rajagopal** for tracking my progress and for valuable suggestions.

My sincere thanks to Prof. Reddanna, In-charge Dean School of Life Sciences and former Deans Prof. A.S. Raghavendra, and Prof. R. P. Sharma, Prof. Ramandam, Present and former Head, Department of Plant Sciences, Prof. Ch. Venkata Ramana and Prof. A.R. Reddy for providing necessary facilities during my research work.

I am extremely thankful to Prof. V. S. Raju, Kakatiya University, for his valuable suggestions. I must thanks to Young Scientist Dr. Sateesh Suthari for his support.

My special thanks to my Frienoves **Srikanth Battu** and **Sumbul Afroz** for being with me in all there for me through thick and thin. Thank you all for always being there to cheer me up when I was down, helped me remain stable in times of instability, and guide me in my moments of confusion. Words are not enough to express how grateful I am to you all

I would like to extend exceptional thanks to my companions Nani Babu, Dr. Hari Krishna, Kizukala Jamir, Sindhur, Rathnamma, Rutuparna, Kunal, Shiva, Bindiya, Tejaswini, Tulasi for their help, moral support and constructive suggestions.

Sincere appreciation to my friends Yasin, Daniel, Vanaja, Naresh, Padmarao, Srinivas pyata, Ravi Kiran, Samu and Chaithanya for their support in all situations

I would also like to thank CIL, University of Hyderabad for the DSC, XRD and SEM facilities; also thank Ruska laboratories for the TEM facilities.

I am highly thankful to Deverder, Dasarath, and Shrawana for technical and analytical support.

This work would not have been possible without the financial assistance of the **UGC-RGNF**, University of Hyderabad, IRTG – MCGS (DFG), DST (SERB) and UGC-SAP

I thank one and all for the affection I got during my stay in the university.

Last but not least, I am deeply indebted to my parents **Kusuma Yashoda** and **Mallesh** whose dream has enabled me to take this path, I owe everything to them for whatever I am, and family members emotional support and unforgettable movements.

Kusuma Venuamdahv

ABBREVIATIONS

µg	micro gram
µl	microliter
ATR-IR	Attenuated transmission resonance Infra-red
BHT	Butylated Hydroxy Toulene
BSA	Bovine serum albumin
CD	Circular dichroism
CLGG	Cross linked guar gum
CLLSG	Cross linked leucaena seed gum
cm	Centimeter
db	Dry basis
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulfoxide
DPPH	1,1-Diphenyl-2-picryldydrazyl
DSC	Differential scanning calorimetry
ECP	Euphorbia caducifolia polysaccharide
g	Gravitational force
Gal	Galactose
GC-MS	Gas chromatography coupled with mass spectroscopy
GlcNAc	N-acetyl Glucosamine
Glu	D-glucose
gm	Gram
HCl	Hydrogen chloride
HRBC	Human red blood cells
IL-6	Interleukin-6
kDa	kilo Dalton
M	Molarity
Man	Mannose
MIC	Minimum inhibitory concentration
Min	Minute
ml	milliliter
mM	milli molar
MS	Mass spectrometry

MTT	3-5,5-dimethylthyazol-2-yl-2,5-diphenyltetrazolium bromide
MW	Molecular weight
N	Normality
ng	Nano gram
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pH	-log(H+) concentration
PMF	Peptide mass fingerprinting
ROS	Reactive oxygen species
RPM	Rotations per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
TCA	Trichloroacectic acid
TEMED	N'N'N'N' tetra methyl ethylene diamine
TFA	Trifluoro Acetic acid
TEM	Transmission Electron Microscopy
TG	Thermo gravity
TNFα	Tumor necrosis factor alpha
Tris	tris(hydroxymethyl)aminoethane
UV	Ultra violet
W/V	Weight/Volume
XRD	X-ray diffraction

TABLE OF CONTENTS

CHAPTER I

INTRODUCTION

Natural products are the substances produced by living organisms. Secondary metabolites gained attention as natural products where primary and secondary metabolites are considered to be natural products by organic chemists. Secondary metabolites have been leading the drug discovery in early stages of Natural product chemistry (Harvey, 2008). From the discovery of an antibiotic "Penicillin" in 1928, the "Golden Age of Antibiotics" started but later on the interest in the drug discovery from natural products has been reduced and the novel lead compounds are producing with the help of combinational chemistry. However, Combinational Chemistry gives low molecular structural analogues of the natural products (Butler, 2004; Mishra & Tiwari, 2011; Lewis, 2012). David J. Newmann reported 1328 only New chemical entities from 1981 to 2014 in various categories (Fig. 1.1) (Newman & Cragg, 2016). Natural products are still a source of novel drugs and plants have been a major contributor of such products.

These are products derived from nature includes micro-organisms, plants, and animals part of terrestrial and marine sources. The microbial world produced active compounds like antibiotics, anticancer, antidiabetic and other after the exploration of penicillin. Major natural products from the animal sources are "venoms and toxins" apart from other small contributions. Marine environment places the second role after the plant n producing Novel Natural Products. Marine region covers 70% of the earth with unique biodiversity (Haefner, 2003). Algae, sponges, tunicates, ascidians, and bryozoans

are the marine organisms explored with potential drugs (Mayer et al., 2010). Plants are the greater source of natural products.

Life has relied on nature for its basic needs mostly on plants, majorly for food and medicine. The plants are the core source of the creation of the traditional medicine system all over the world (Cragg & Newman, 2013). History of Medicine says the role of plants in treating infections and diseases. Natural products from plants always remain as a valuable source contributing a revolutionary green approach to the discovery of novel compounds (Table 1.1). Around 70,000 plants were screened for their potential activity till now which are using in different medical systems across the world. In the global market, Europe contributed a significant number of natural products (Fig. 1.2) (Dev, 1999).

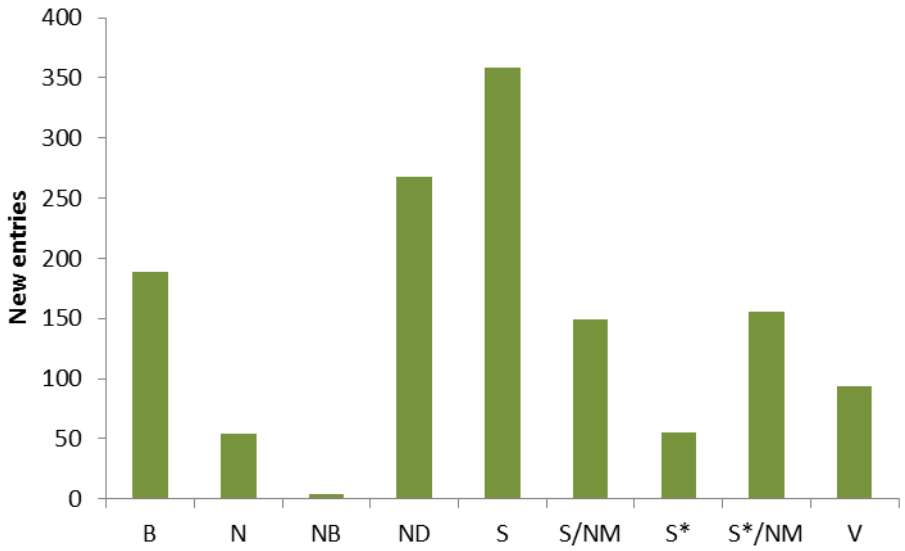


Fig. 1.1. New chemical entries of drugs from 1981 to 2014

B-Biological; N-Natural products; NB-Botanical Natural product; ND-Derived Natural product; S-Synthetic; S/NM-Natural product mimic; S*-Synthetic with natural pharmacore; V-Vaccine

Table 1.1. Some examples of commonly available Plant product

Category	Plant name	Therapeutic uses
Diosgenin, Hecogenin, Stigmasterol	*Dioscorea spp.*	Oral contraceptives
Digoxin, Digltoxin	*Digitalis purpurea* L, *D. lanata*	Cardiotonic
Tropane Alkaloids Atropine Ahyoscyamine Scopolamine	*Atropa belladonna* L, *Datura spp.* *Hyoscyamus niger* L, *Mandragora officinarum* L	Anticholinergics
Opium Alkaloids Codeine Morphine	*Papaver somniferum* L	Analgesics Antitussive
Reserpine	*Rauvolfia serpentina* (L.) Bentham ex Kurz	psychotropic, Antihypertensive
Vinca Alkaloids (Catharanthus) Vinblastine Vincristine	*Catharanthus roseus* (L) G. Don	Anticancer
Physostigmine	*Physostigma venenosum* Balfour	Cholinergic
Pilocarpine	*Pilocarpus jaborandi* Holmes	
Cinchona alkaloids Quinine Quinidine	*Chincona spp.*	Antimalarial, antiarrythmic
Colchicine	*Colchicum autumnale* L.	Antigout
Cocaine	*Erythroxylum coca* Lamarck	Local anesthetic
d-Tubocurarine	*Strychnos toxifera* Bentham	Skeletal muscle relaxant
Taxol	*Taxus brevifolia* Nutt.	Anticancer

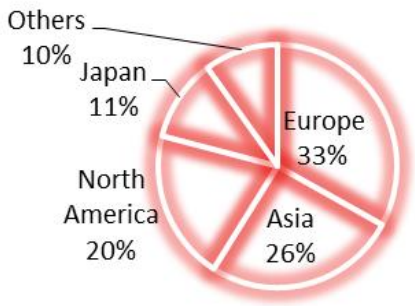


Fig. 1.2. Contribution of natural products in the market

In India, plants have been used for many therapeutic applications by our traditional system of medicines such as Ayurveda, Unani, Homeopathy, and Siddha. Traditional medicine system gave birth to many active compounds in early years of natural chemistry, but reports of natural products from different sources has been decreased rapidly in last decade and looking for new active compounds. According to National Medicinal Plant Board (NMPB), 6000 species are using in different medical systems and a maximum number of plants using in the folk medicine compared to other documented medicine systems (Fig. 1.3)(FRLTH INDIA. 2017). Now it needs to explore new natural compounds from potential folk medicinal plants and ethnomedicinal plants for better therapeutic applications. Detailed use plants in the medical systems documented for the use of future reference along with identification. In Ayurveda, Charaka Samhita is the guide for the plant formulations. Like, modern medicine attained documentation regarding the plant materials and their identifications along with medical importance called Pharmacognosy.

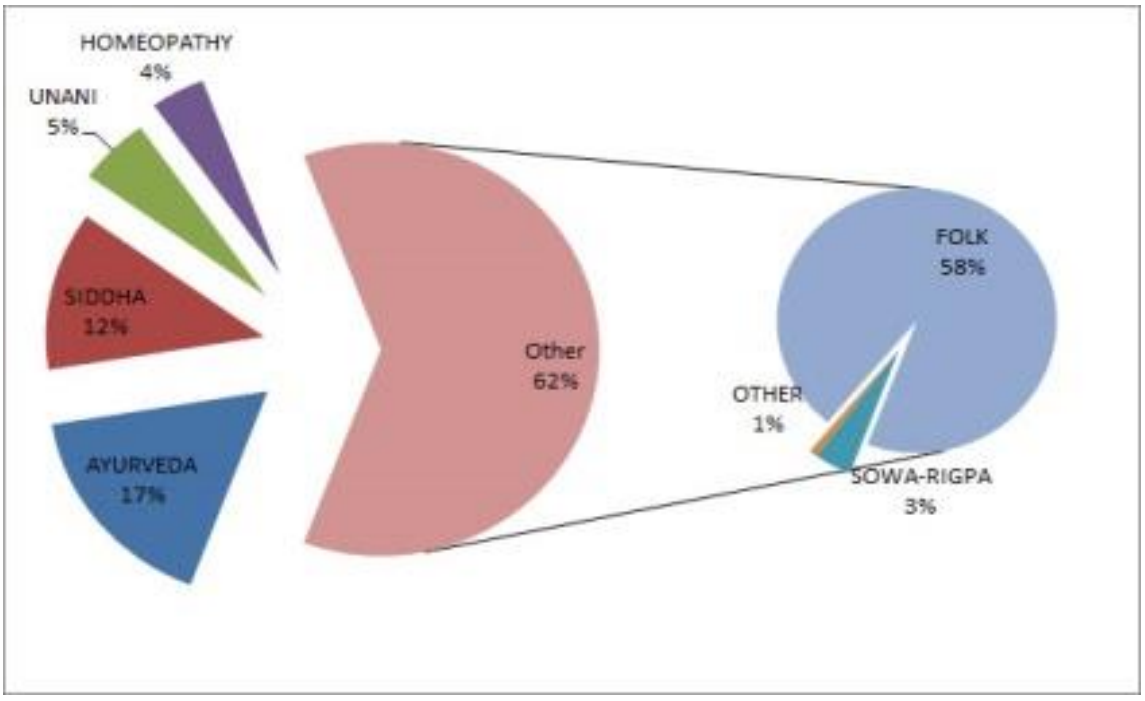


Fig. 1.3. Distribution of medicinal plants in Indian Medicine System

Pharmacognosy is a study of crude drugs or knowledge of crude drugs on chemical as well as structural characterization and also metabolic pathways leading to pharmacologically active compounds. The horizon of pharmacognosy has enlarged from classic morphological characters to the modern study of bioactive compounds with their mechanism of action and therapeutic uses (Kato & Pezzuto, 2017). Pharmacognosy gained attention due to, search for new leads for drug development, biotechnology for the production of pharmaceuticals and nutraceuticals, increased interest on phytotherapy and validation of traditional medicines. Apart from the study of secondary metabolites, Primary metabolites and their small molecules advanced for the therapeutics. Primary metabolites include polysaccharides, proteins, nucleic acids, fatty acids and secondary metabolites majorly includes alkaloids, flavonoids, terpenoids, glycosides, saponins, phenolic compounds, anthraquinones, tannins and others.

Polysaccharides

Polysaccharides are biological macromolecules with the repetitive arrangement of monosaccharides as basic units. These are one of 4 major biological macromolecules along with proteins, nucleic acids, and fatty acids. Polysaccharides can serve as an energy source and structural components to the cell with its highly complex structure (Z. Wang, Luo, & Ena, 2007). Cellulose, Chitin, and starch are the abundant polysaccharides in the earth. Applications of polysaccharides on industrial orientation are enormous. Majorly they are as thickening agents, chelating agents, emulsifying agents, stabilizing agents, suspending agents, flocculating agents and also used to form gels and membranes. Polysaccharides are biocompatible and biodegradable in nature along with bioavailability. Gums and their derivatives are considered as a major class of the polysaccharides as part of industrial applications (Lapasin & Pricl, 1995). Marine origin polysaccharides are gaining interests along with microbial polysaccharides. Agar,

Agarose, alginates, and carrageenan are the seaweed polysaccharides employing in the various fields (Laurienzo, 2010). Exopolysaccharides of the microorganisms like xanthan, dextrans are currently using the market in addition to many novel immunomodulatory polysaccharides (Sutherland, 1998).

Botanical polysaccharides

Polysaccharides from higher plants show significant therapeutic properties by its non-toxic nature and ability to not cause any side effects compared to bacterial and fungal polysaccharides. Thus, botanical polysaccharides are ideal therapeutics for immunomodulatory, antitumor and wound healing activities (Schepetkin & Quinn, 2006). Apart from other activities, most polysaccharides are considering for immunomodulatory activity as they are the non-immune origin. Botanical polysaccharides affect the macrophages and stimulate the production of cytokines, ROS, chemokines and along with cell proliferation (Fig. 1.4). Plant polysaccharides from the different plant species have shown immunomodulatory activity on various types of macrophages.

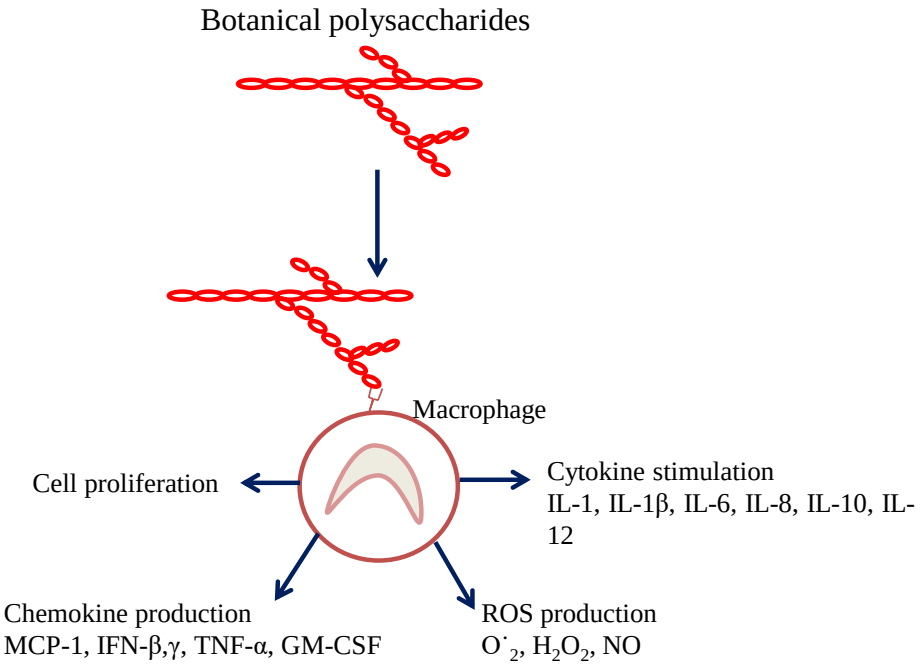


Fig. 1.4. Action of botanical polysaccharides on Macrophages

Lectins

"Lectin is a sugar-binding protein or glycoprotein of non-immune origin which agglutinates cells and/or precipitates glycoconjugates" (Goldstein et al., 1980). In 1888 Stillmark demonstrated a protein which agglutinates the RBC called "Ricin" from *Ricinus communis*. Later noticed the "Abrin" in *Abrus precatorius* by H.Hellin in 1891. Karl Landsteiner in 1900 discovered the ABO blood group using lectin. Lectin "Concanavalin A" crystallized and revealed the sugar specificity of lectins by inhibiting the haemagglutination.

Lectin Classes

Lectins were classified on sugar specific nature by Goldstein and poretz in 1986. Classes are based binding of the lectin to the sugars mannose or glucose, galactose or N-acetyl galactosamine, N-acetyl glucosamine, fucose and sialic acid (Table 1.2). Further, based on their overall structure and subunits, lectins are classified as Merolectins, Hololectins, Super lectins and Chimerolectins. They are grouped on evolutionary relations into different families, i.e., Chitin binding lectins, Curcubitaceae phloem lectins, Amaranthin lectins, Monocot mannose binding lectins, Legume lectins, Jacalin related lectins and Ribosome inactivating protein type 2 lectins (Hamid et al., 2013) (Table.1.2 **)**

Table 1.2 Classification of lectins on sugar basis with some examples

Class/Binding Lectins	Examples	Predominant plant families
Mannose	Concanavalin A, Lentil lectin, Snowdrop lectin	Fabaceae
Galactose / N-Acetyl Galactosamine	Ricin, Peanut agglutinin, Jacalin	Euphorbiaceae, Fabaceae, Loranthaceae
N-acetylglucosamine	Wheat germ agglutinin	Poaceae, Solanaceae, Fabaceae
N-acetylneuraminic acid	Elderberry lectin, Maackia amurensis hemoagglutinin and leucoagglutinin	Nelumbonaceae, Fabaceae
Fucose	Ulex europaeus agglutinin, Aleuria aurantia lectin	Fabaceae

Table 1.3 Lectins from *Euphorbia* genus

Plant Name	Part	Sugar Specificity	Authors	Year
Euphorbia heterophylla	Seeds	N-acetyl galactosamine	NsimbaLubaki et.al	1983
Euphorbia charcias	Latex	Galactose	Babieri et al.	1983
Euphorbia calcina L	Latex	N-acetyl galactosamine	NsimbaLubaki et al.	1986
Euphorbia dalberi L	Latex	N- acetyl galactosamine	NsimbaLubaki et al.	1986
Euphorbia coerulscens	Latex	Galactose	Lynn & Clevette-Radford	1986
Euphorbia hermentiana	Latex	Galactose	Lynn & Clevette-Radford	1986
Euphorbia escula	Latex	Galactose	Lynn & Clevette-Radford	1986
Euphorbia globose	Latex	Galactose	Lynn & Clevette-Radford Lynn et al.	1986
Euphorbia lactea	Latex	Galactose	Lynn & Clevette-Radford Lynn et al.	1986
Euphorbia lactea cristata	Latex	Galactose	Lynn & Clevette-Radford Lynn et al.	1986
Euphorbia lathyris	Latex	Galactose	Lynn & Clevette-Radford et al.	1986
Euphorbia mammillaris	Latex	Galactose	Lynn & Clevette-Radford et al.	1986
Euphorbia splendens	Latex	Galactose	Lynn & Clevette-Radford et al.	1986
Euphorbia stapilioides	Latex	Galactose	Lynn & Clevette-Radford et al.	1986
Euphorbia marginata	Latex	Galactose	Stirpe et al.	1993
Euphorbia neriifolia	Latex	Galatose	Seshagirirao & Prasad	1995
Euphorbia milli	Latex	Galactose	Seshagirirao	1999
Euphorbia milii, var. milii	Latex	GalNac	M. Dias-Baruffi et al.	2000
Euphorbia antiquorum	Latex	Galactose	Seshagirirao & Prasad	2001
Euphorbia nivulia	Latex	Galactose	Seshagirirao	2001
Euphorbia milii var. hislopii	Latex	Galactose	K.C. Fonseca et al.	2010
Euphorbia helioscopia	Leaves	Fructose	Rafiq et al.	2014
Euphorbia tirucalli	Whole plant	Mannose	Santana et al.	2014
Euphorbia trigona	Latex	Galactose	Villanueva J et al.	2015
Euphorbia tithymaloides	Leaves	Galactose	Jawade et al.	2016
Euphorbia pulcherima	Whole plant	Galactose	Torky et al.	2016

Lectins from *Euphorbia*

Latex, a colloidal suspension of laticifer cells flows with cellular components, nuclei, mitochondria, amyloplast, rubber, sterols, alkaloid, carbohydrates, proteins, free amino acids, phenolics (K. Seshagirirao & Prasad, 2001). Euphorbiaceae is also one among the 20 families of latex-producing plants. Biochemical as well as biosystematics studies have been investigated using Euphorbiaceae lattices. Apart from screening of metabolites, proteins also present in the latex. Lennox and Ellis (1945) reported a protease "Euphorbin" in the latex of Euphorbia lathyris. 18 proteases were isolated from different *Euphorbia* species by Lynn and Clevette-Radford (1984, 1985, 1988). In 1983, Barbieri et al., purified galactose-specific lectins from the latex of Euphorbia characias and Hura crepitans. Most of the lectin from the latex of the genus Euphorbia has been reported galactose-specific in nature (Table. 1.3). Lectins are using for therapeutics, pharmaceutical and food industries.

Euphorbia L. Genus

Euphorbia genus belongs to Euphorbiaceae family and cosmopolitan distribution. This family is one of the largest family in angiosperms and consist of over 300 genera, 37 tribes, three subfamilies and 7500 species (Webster, 1994; Mwine & Van Damme, 2011). The diversity of unusual secondary metabolites in the family is accountable for the poison nature of some members. Abdel-Fattah in 1987 attempted for classification of family poisons. Listed as:

Action of Poison on	**Examples**
Fish	*E. scheffleri* Pax, *E. tirucalli* L., *E. inaequilatera* Sond
Human	*E. ledienii* A.Berger, *E. heterophylla L.*, *E. cooperi* N.E.Br. ex A. Berger, *E. candelabrum* Kotschy, *E. virosa* Willd., *E. poissonii* Pax, *E. unispina* N.E.Br. *E. venenifica* Tremaux ex Kotschy
Domestic animals	*E. caput-medusae* L., *E. silenifolia* Sweet *E. ingens* E.Mey. *ex* Boiss

Euphorbia L is the largest genus of the family with almost 2000 species. Members of the genus distributed in temperate, tropical and subtropical regions and range from herbs to trees. Despite poison nature and irritant ability, plants are greatly used in traditional medicine. For example, *E. hirta, E. heterophylla* as antibacterial (Chika C et.al., 2007; Falodun et.al., 2008)*; E. kansui*, *E. hyberna*, *E. tirucalli*, *E. thymifolia* as antiviral (Bedoya et al., 2009; Betancur-Galvis et al., 2002; Lin et al., 2016.; Zheng et al., 1998); *E. nivulia* Buch.-Ham. as an antifungal (Annapurna et al., 2004); *E. helioscopia*, *E. pulcherrima* as nematicidal (Devi & Pamila, 2000); *E. splendens*, *E. conspicua* N.E. Br.as Molluscicidal (De Oliveira Dias, Dos Santos Machado, Migliolo, & Franco, 2015; Mauricio Carvalho de Vasconcellos, 2003); *E. hirta* as insecticidal (Murakami et al., 2007); Euphorbia genus limited to *E. ficheriana* and *E. kansui* for polysaccharide extraction among a large number of species.

Euphorbia caducifolia Haines

Euphorbia caducifolia Haines succulent milk hedge (Fig. 1.5), a folk medicinal plant belongs to Euphorbiaceae. The term "caducifolia" means falling leaf, leaves of the plants are caducus in nature. *E. caducifolia* is a latex-producing plant in Euphorbia genus and found in rocky areas of the tropical region. The plant latex is used for wound healing, leucoderma, and skin eruptions. Latex and root of the plant used to treat cancer. Root also used for snakebite in Maharashtra. Leaf extracts of the plant were reported for its antimicrobial activity. Apart, there are not many reports on phylloclade of the plant which is a rich source of biofuel and other compounds. Though the plant has been using for many medical applications but limited to little exploitation (Table.1.4). Looking the medicinal importance of the plant, it was considered worthwhile to evaluate the plant for the biological activity scientifically.

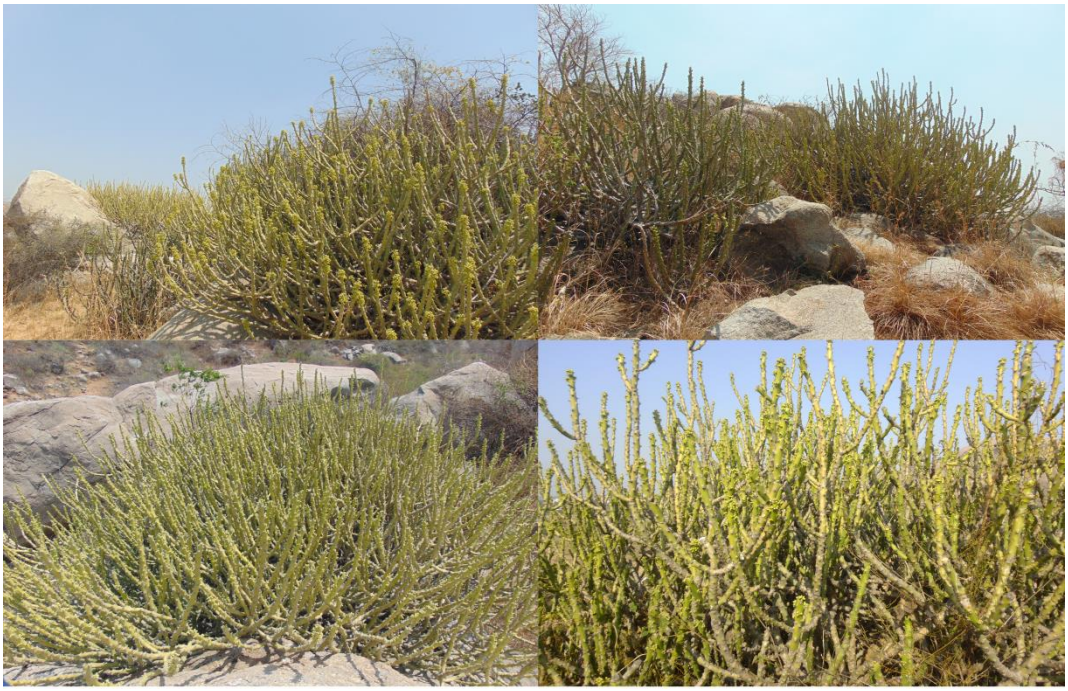


Fig. 1.5. Distribution of *E. caducifolia* Haines in the wild (Bhongiri area, Yadadri dist. Telangana)

Table 1.4. List of publications on *E. caducifolia*

Part	Study	Authors	Year
Plant	Autecological Study	Hussain et al.	1970
Plant	CAM metabolism	Sen et al.	1970
Plant	Ecology and Seedlings	Sen et al.	1970
Latex	Triterpenes isolation	Govardhan et al.	1984
Latex	Latex Biochemistry	Lynn & Clevette-Radford	1986
Latex	Antiparasitic Activity	Maqbool et al.	1987
Phylloclade	Milk clotting Activity	Umar et al.	1990
Latex	Latex Biochemistry	Seshagirirao & Prasad	2001
Plant	Ethnobotany	Mohammed et al.	2004
Latex	Tumor promoting Terpene	Baloch et al.	2005
Plant	Ethnobotany	Meena et al.	2009
Latex	Co-carcinogenic Diterpene	Baloch et al.	2010
Latex	Antimicrobial Activity	Goyal et al.	2012
Leaves	Antimicrobial Activity	Kapoor, B.B.S et al.	2013
Latex	Snakebite treatment	Chavre	2013
Plant	Biofuel	Rajeswari et al.	2013
Latex	Phytochemical Study	Bhattacharyya et al.	2013
Phylloclade	Ultrastructure of Laticifers	Rajeswari et al.	2014
Aerial part	Anticancer Activity	Bano et al.	2017
Plant	Ethnomedicine	Gupta et al.	2017
Latex	Case report: Ocular injury	Taviad et al.	2017

Based on the literature survey the present investigation we framed the following objectives:

Objectives

1. Pharmacognosy and Phytochemical screening of *E. caducifolia*
2. Isolation and characterization of polysaccharide from phylloclade of *E. caducifolia*
3. Isolation and characterization of Lectin from latex serum of *E. caducifolia*
4. Isolation and characterization of unique starch granules from latex of *E. caducifolia*

Bioprospecting study

Sample collection
(Based on Ethnobotany or Chemotaxanomy or Random selection)

Place:
Jangati vari guttalu
(Bandasomaram)
Bhongiri, Telangana, IN

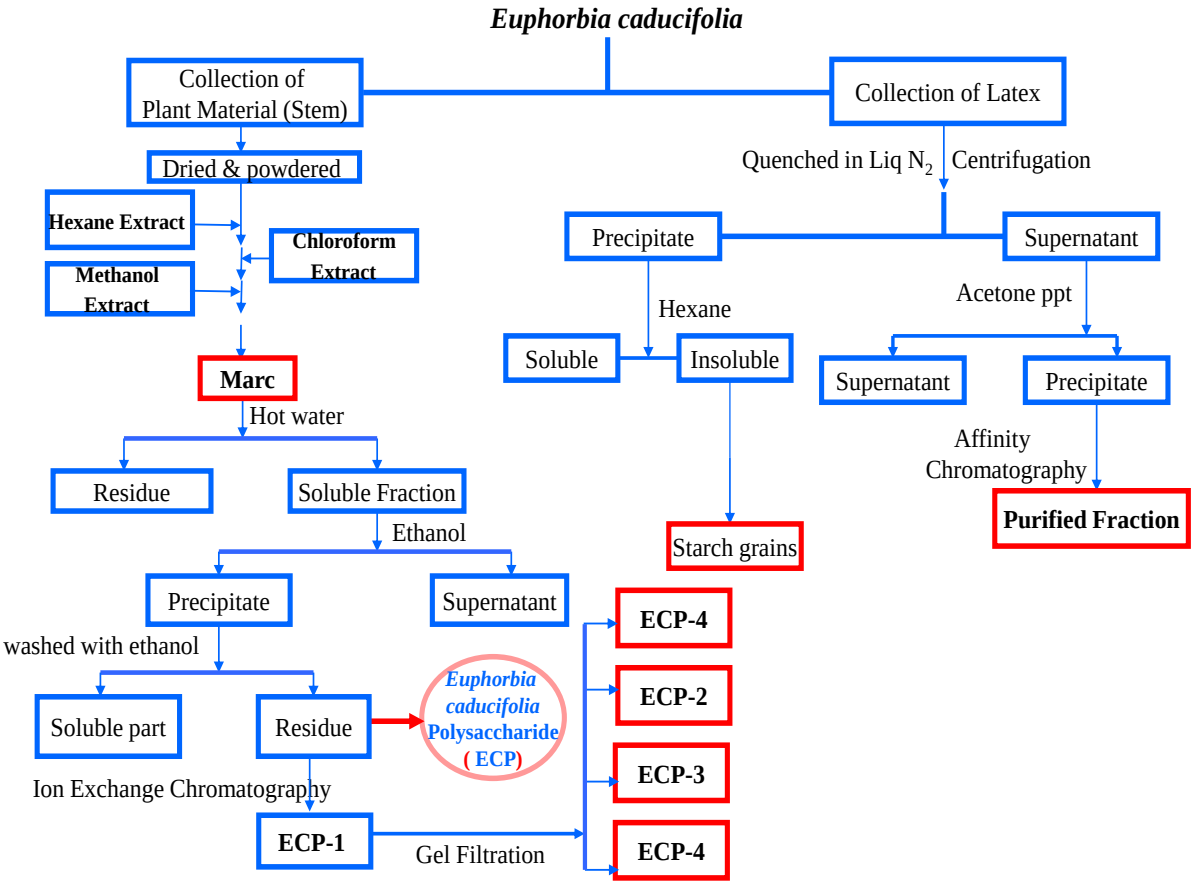
Coordinates:
17°30'19"N 78°54'35"E

Collected Time:
Flowering Season
(Feb-March)

Taxonomic Identification of Plant by Curator Prof. K. Seshagirirao
University of Hyderabad Herbarium (World Code – UH)

Germ plasm
Ex situ or /and Invitro

Herbarium
Vouche Specimen Nos. Venu-2027 & 2028



Isolation and purification Process of Biomolecules from *Euphorbia caducifolia*.

CHAPTER II

PHARMACOGNOSY
&
PHYTOCHEMICAL SCREENING OF *E.caducifolia*

Pharmacognosy is a study of crude drugs or knowledge of crude drugs on chemical as well as structural characterization and also metabolic pathways leading to pharmacologically active compounds. Pharmacognosy gained attention due to, search for new leads for drug development, biotechnology for the production of pharmaceuticals and nutraceuticals, increased interest on phytotherapy and validation of traditional medicines (Verpoorte, 2000).

Phytochemical screening is necessary to know the presence of metabolites and others in the extracts to produce the evidence for the ethnobotanical or ethnomedical surveys. High throughout screenings gives birth to the new moieties. Now it needs to explore new natural compounds from potential folk medicinal plants and ethnomedicinal plants for better therapeutic applications.

All living organisms contain an antioxidant defensive mechanism to counter the free radicals, reactive oxygen species (ROS), reactive nitrogen species (RNS) and other oxidants produced as byproducts of the metabolism. Most of the diseases caused by overproduction and reactive mechanism of free radicals (Alam et al., 2013; Valko et al., 2007). Many medicinal plants, spices, aromatic plants using as food supplements and medicines are natural antioxidants (Veeru et al., 2009). It has been an upsurge of interest

for potential antioxidants from plants sources. Therefore, this study was conducted to establish pharmacognosy of the plant and to reveal the phytochemical compounds exist in the phylloclade and to explore the ability of free radical scavenging activity as an antioxidant.

A. PHARMACOGNOSY OF *Euphorbia caducifolia:* *Euphorbia caducifolia,* collected from hills of the bongiri village, Yadadri District, Telangana State, India. The plant specimen was authenticated by Prof. K. Seshagirirao, Curator, University of Hyderabad Herbarium and deposited herbarium with voucher numbers Venu - 2027 and Venu-2028.

Plant Name (Scientific Name) : *Euphorbia caducifolia* Haines
(Common Names) : Short time leaf succulent plant
Danda Thor (Hindi)
Kada jemudu or Katti jemudu (Telugu),
Family : Euphorbiaceae
Distribution : India, Pakistan & Afghanistan
India (Rajasthan, Gujarat, Madya Pradesh, Bihar, Jharkhand, Chattisgarh, Odisha, Telangana, Andhra Pradesh, Karnataka, Maharastra) (Fig. 2.1)
Morphological Characters (Fig. 2.2) : Plants are terete branching shrubs with height up to 2-3 m and 5-10 m broad, it can form Large Landscape
Phylloclade : Modified stem, alternative spines
Leaves : Caducus, Obovate, Fleshy, 3 to 6 cm long
Flower : Cyathia inflorescence, Red & Yellow colour, Very dense, 1 cm across, Triad/single, central sessile
Capsule (Fruit) : 3 lobed, 1 -1.2 cm, glabrous, dark pink on mature
Seeds : Rounded, 0.2 -0.3 mm diameter, greyish, smooth

Microscopical Characters : (Fig. 2.3)

Phylloclades : T.S and L.S Phylloclades showed Laticifers

Leaves : Paracytic stomata, Vein islets: 5-7,
Vein termination:7-10

Seeds : Shows oil globules and starch granules,
Papillate surface

Latex : contain starch granules and calcium oxalate crystals.

Powder Microscopy : Phylloclade powder showed starch grains, Laticifers, Calcium oxalate crystals

Chemical constituents : Phylloclades and latex contains Caducinol, Cyclo caducinol, Epicyclo caducinol (Afza et al., 1989) Acid polysaccharides (Uronic acid polysaccharides)

Similar look like species/Substitutions/Adulteration possibilities:

: *Euphorbia nivulia, Euphorbia gokakensis*

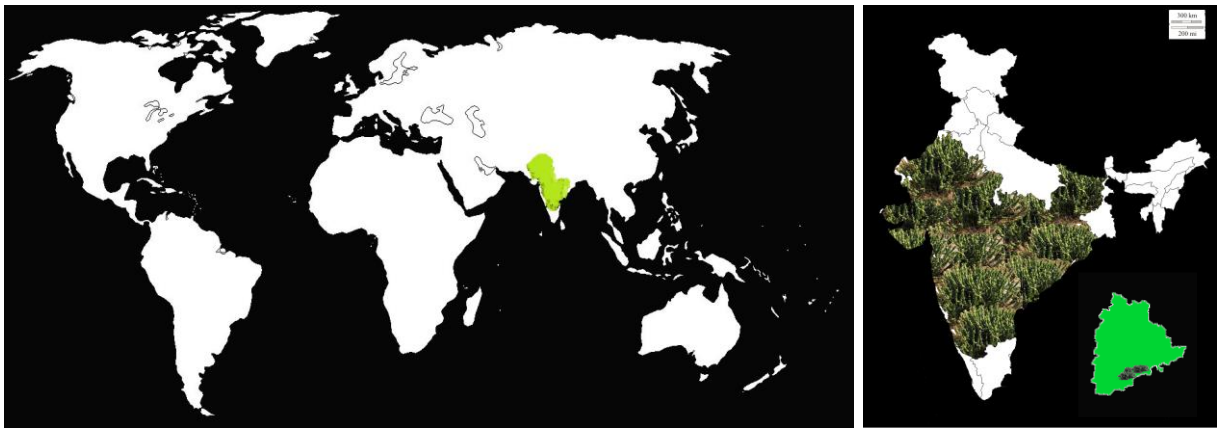


Fig. 2.1. Geographical distribution of *Euphorbia caducifolia*

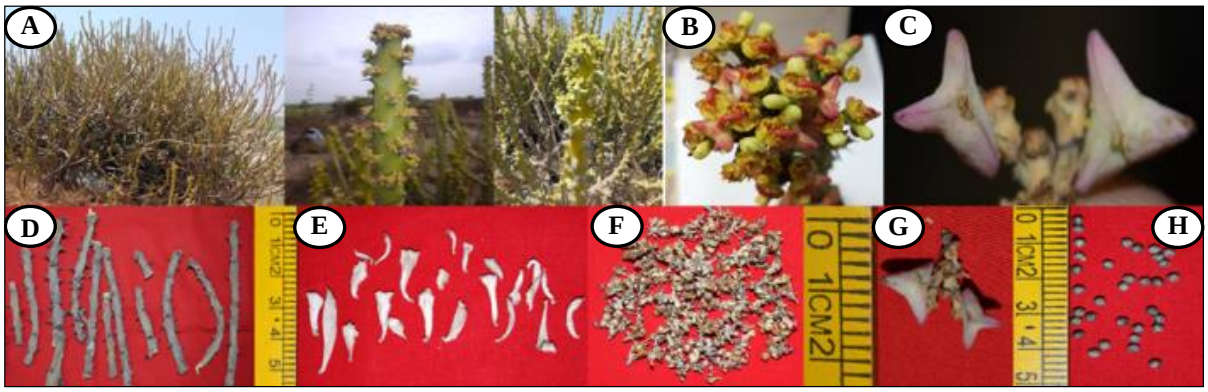


Fig. 2.2. Morphological characters of *E. caducifolia:*
A) Wild distribution, B) Cyathia with capsule, C & G)Capsule,
D) Dried phylloclades, E) Dried leaves, F) Dried flowers, H) Seeds

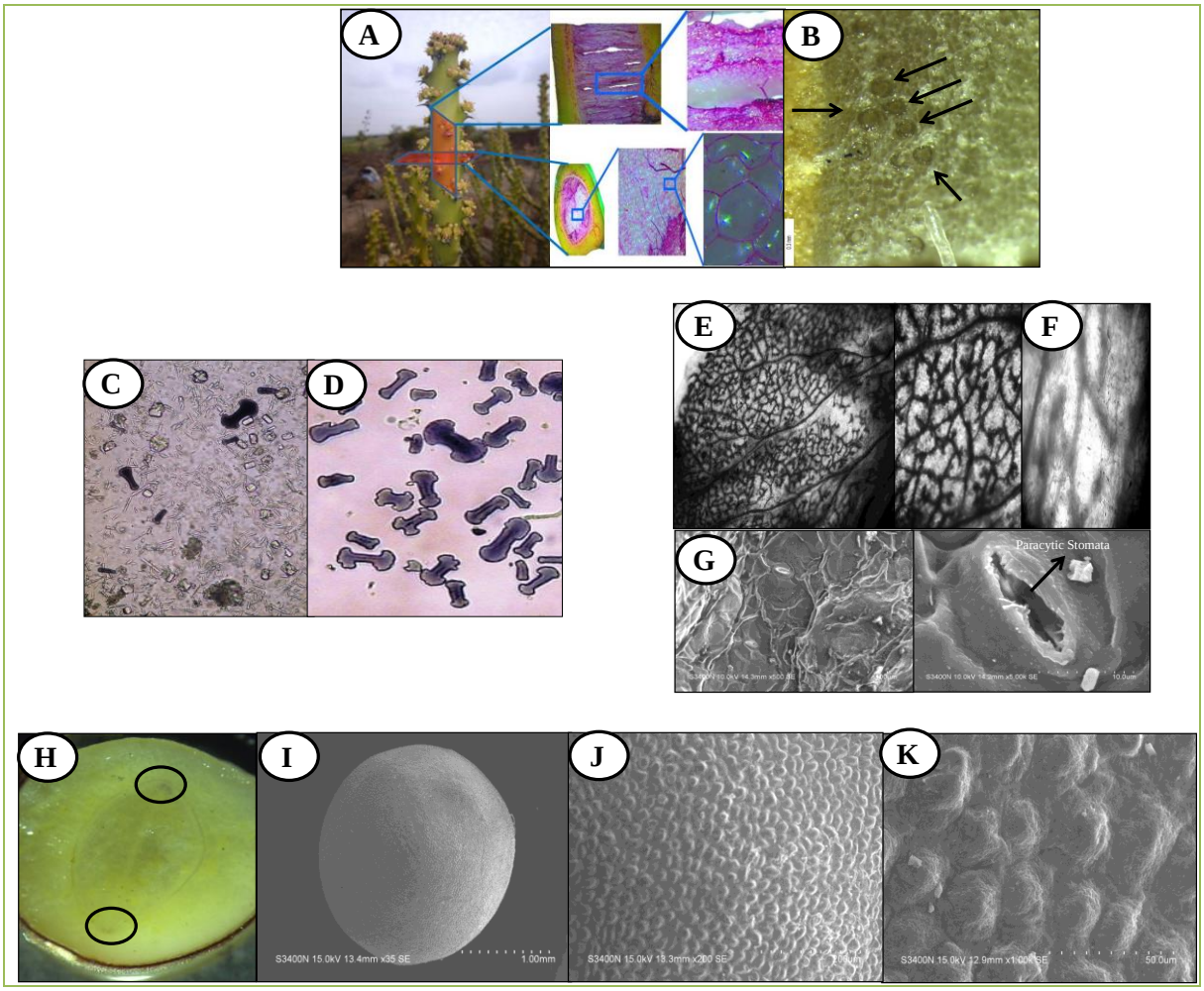


Fig. 2.3. Microscopic characterization of plant specimens: A) T.S & L.S of Phylloclade, B) Laticifers; Latex: C) Calcium oxalate crystals, D) Starch granules; Leaf: E) Venation, F & G) Paracytic stomata; Seed : Cross section with oil globules and starch (circles), SEM of seed: I) seed, J) Lower magnification (Papillate), K) Higher magnification (Undulate network)

B. PHYTOCHEMICAL SCREENING OF *E. caducifolia*

Materials and Methods

Preparation of Extracts: The dried plant material was made into powder and used for extraction. The powder was extracted with hexane, methanol, and water in successive order. The extracts were concentrated at temperature 40°C under vacuum and used for further analysis.

Phytochemical screening: Extracts were analyzed for alkaloids, carbohydrates, glycosides, flavonoids, phenolic compounds, quinones, tannins, saponins, and terpenoids (Santhi & Sengottuvel, 2016).

Estimation of Total Phenolic Content (TPC): The TPC of the extracts was determined using the Folin-Ciocalteu colorimetric method. 1 ml of extract was mixed with 1 ml of 1 N Folins reagents and well shaken. After 5 min, 1 ml of 10% sodium carbonate was added and incubated for 1 h at room temperature. The absorbance was taken after incubation at 760 nm against blank. Total phenolic content was given as Gallic acid equivalents (GAE) (Basma et al., 2011).

Estimation of Flavonoids: 0.1 ml of extract was added to 0.3 ml 5% sodium nitrite and mixed with 3 ml of 1% aluminum chloride. After 5 min incubation, 2 ml of 1 M NaOH was added and made up the volume to 10 ml with water. The solution was mixed well and measured absorbance at 510 nm against blank. The flavonoid content was calculated using rutin as standard (Basma et al., 2011).

Antioxidant Activity

DPPH Radical Scavenging Assay: DPPH (2,2-diphenyl 1-picrylhydrazyl) free radical used to determine the scavenging activity of extracts (Molyneux, 2004). DPPH (1 ml, 0.1mM) added to various concentrations of extracts and BHT (3 ml) and 0.5 h incubated in the dark and measured absorbance at 517 nm against the blank (n=3). The activity was calculated by the following equation (Kumar et al., 2005):

Percentage inhibition = [(Abs control – Abs sample)/Abs control] × 100

Nitric oxide radical scavenging Activity: Nitric oxide (NO) produced by Sodium Nitroprusside at pH 7.2-7.4 was determined by Griess reagent (Griess diazotization reaction). The reaction mixture (3 ml) was incubated at 25°C for 2 h containing Sodium Nitroprusside (10 mM) and extracts with different concentrations. An Aliquot (0.5 ml)

was added to Griess reagent (0.5 ml) and measured at 546nm (n=3). The activity was calculated by comparing control and test sample. Ascorbic acid used as a reference compound (Boora, Chirisa, & Mukanganyama, 2014).

Results and Discussion

Phytochemical analysis: Phytochemical studies of extracts showed the presence of various classes of metabolites. *E. caducifolia* showed the high quantity of terpenoids in methanol and hexane extracts as *Euphorbia* species are predominant with cyclic and acyclic terpenoids (Piazza, & Calvin, 1983). Test for the presence of alkaloids shown no significant colouration. Water extract was rich in carbohydrates, glycosides, tannins, phenols, flavonoids, and saponins. Further experiments revealed that the presence of acidic polysaccharides and saponin glycosides. Methanol extract contained reducing sugars, glycosides and some phenolic compounds whereas in hexane extract showed positive for reducing sugars and glycosides along with terpenoids (Table 2.1).

Table 2.1. Phytochemical Screening of *E. caducifolia* extracts

Fraction	Water	Methanol	Hexane
Alkaloids	--	--	--
Carbohydrates	++	++	++
Glycosides	++	++	++
Flavanoids	++	+	--
Phenolics	++	+	--
Tannins	++	--	--
Saponins	++	--	--
Quinones	--	++	++
Terpenoids	--	++	++

Estimation of total phenolic and flavonoid content: Phenolic compounds such as flavonoids and tannins are excellent free radical scavengers and protect the plant from different types of oxidative damages (Tadesse et al., 2016). Water extract showed the high phenolic and flavonoid contents whereas no significant amounts observed in hexane

extract. Methanol extract showed good amounts of flavonoids compared to phenolics (Table 2.2). Antioxidant activity of the extracts might be due to the presence of phenols and flavonoids present Procházková et al., 2011).

Table 2.2. Total Phenolic and Flavonoid contents in *E. caducifolia* plant extracts

Fraction	Phenolics	Flavonoids
	mg of GAE/g of Extract	mg of RE/g of Extract
Water	68.740 ± 0.025	50.04 ± 0.125
Methanol	00.926 ± 0.06	13.534 ± 0.754
Hexane	00.080 ± 0.014	00.004 ± 0.0002

Antioxidant activity: Free radicals are the most reactive molecules and damage the cells. Aerobic organisms protect themselves from free radical by the defensive antioxidant mechanism, and it is necessary to provide external antioxidants in failure defensive system (Aksoy et al., 2013). The extracts were analyzed for their antioxidant capacity to use as a free radical scavenger. Most of the members of the castor family are a good source of natural antioxidants such as *Euphorbia hirta* (Basma et al., 2011), *E. heterophylla* (Okeniyi, Adedoyin, & Garba, 2012). *E. caducifolia* extracts also exhibited antioxidant activity. The potentiality of the extract to scavenge the free radical produced was considered for antioxidant activity (Fig. 2.4). DPPH and NO are the two radicals used for the experiments. All extracts were showed activity on concentration dependency. Water extract exhibited more scavenging activity towards DPPH (86±1.5%) as well NO (39.23±4.7%) to hexane extract. More free scavenging activity of water extract might be due to the presence of the high phenolic and flavonoid contents. Methanol extract also exhibited similar (DPPH-77.81±1.3% and NOSA-36.03±2.71%) to water but dropped activity frequency at high concentration of the extract. Higher antioxidant activity was

observed at higher concentrations. Extracts showed less ability to scavenge the NO free radical as compared to DPPH.

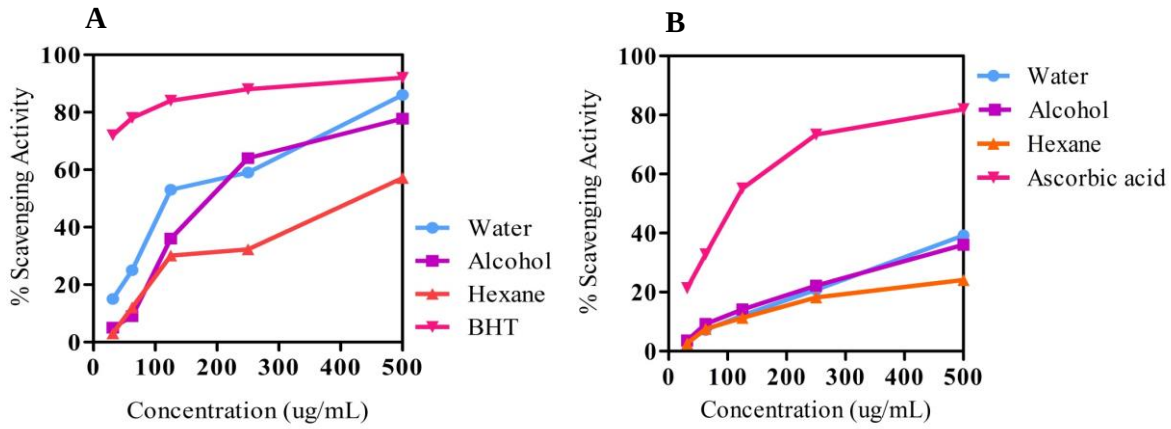


Fig. 2.4. Antioxidant activity of *E. caducifolia* plant extracts

A) DPPH Scavenging activity, B) Nitric Oxide Scavenging activity

Conclusions

Euphorbia caducifolia is a promising plant for the various therapeutic applications with rich contents of terpenoids, polysaccharides, tannins, flavonoids and phenolic compounds. The presence of significant amount of total phenolics and flavonoids contents in water extract might be responsible for strong antioxidant activity as compared with methanol and hexane extract. Hexane and methanol extract can be used for the terpenoid sources and water extract for phenolics and flavonoids. High contents of terpenoids in methanol and hexane extracts are useful in various applications. A further study of isolation and characterization of bioactive molecules from the plant is required for therapeutic and multiple applications.

CHAPTER III

ISOLATION
&
CHARACTERIZATION OF POLYSACCHARIDE FROM PHYLLOCLADE OF *E.caducifolia*

1. Optimization of extraction process, Structural and Biological characterization of Polysaccharide

Polysaccharides can serve as an energy source and structural components to the cell with its highly complex structure (Z. Wang et al., 2007). Polysaccharides from higher plants show significant therapeutic properties by its non-toxic nature and ability to not cause any side effects compared to bacterial and fungal polysaccharides. Thus, botanical polysaccharides are ideal therapeutics for immunomodulatory, antitumor and wound healing activities (Schepetkin & Quinn, 2006).

Euphorbia caducifolia Haines, a Euphorbiaceae species grows extensively on rocky areas of arid and semi-arid regions with extensive branching and clumps of phylloclades (Shaik et al., 2013). Phylloclades are fleshy, succulent with spiny stipules. Milky and sticky natured latex flows through laticiferous cells along with proteins, phytosterols, and terpenoids (Afza et al., 1989). Plant leaves were consumed as Food in arid areas. Ethnomedical uses of the plant were reported, latex for wound healing activity and other skin infections (Goyal, Nagori, & Sasmal, 2012), root extracts for snakebite

(Chavre, 2013), leaves for antimicrobial activity (Kapoor et al., 2013) and phylloclades as biofuels (Shaik et al., 2013).

Response Surface Methodology (RSM) is known to be the best technique to evaluate the relationship between parameters and the yield (D. Ye et al., 2015). Extraction temperature, solid to liquid ratio and time are the common variables which affect the yield. In RSM, Box-Behnken experimental design is more efficient and easier method to analyze and interpret the variables affect the yield (Chaiklahan et al., 2013).

The objective of the study was to evaluate and optimize the variables affecting the polysaccharide yield using a single factorial (RSM), three level-three variable design (BBD). In addition, reporting its structural and biological studies as therapeutic interests.

Materials and Methods

Plant material: The collected stems were dried at room temperature until it gains constant weight. Dried stems were ground to pass 2 mm sieve and proceeded for the extraction.

Extraction process: The powder was freed from oils, fats, fatty acids, and other phytochemicals by sequential extraction using the soxhlet apparatus with hexane, chloroform, and methanol. The residual marc was dried and used for polysaccharides extraction (Huang et al., 2010).

Water extraction: Response surface methodology was used to extract crude polysaccharides in coercion with hot water method. In brief, dried marc was suspended in established volume of distilled water at designed temperature and time. The suspension was centrifuged, and collected supernatant was concentrated under vacuum at 45°C. The polysaccharides from the supernatant was precipitated with absolute ethanol and garnered

the pellet by centrifugation. The precipitated pellet was washed with different percentages of ethanol to remove soluble material and deproteinized using sevag method (Zou et al., 2015). A brown coloured crude polysaccharide was obtained (C.-L. Ye & Lai, 2015) and the concentration was determined by using Phenol-Sulphuric acid method (DuBois et al., 1956). The yield was calculated using the following equation:

Yield % (w/w) = [content of polysaccharide (g)/powder weight (g)] × 100

Response Surface Methodology (RSM) Experimental Design: Box-Behnken design (BBD), a three-level three-factor design was used to optimize the polysaccharides extraction. Parameters like temperature (A), solid to liquid ratio (B) and time (C) were the independent variables, and the polysaccharide yield was taken as response function (R) to the independent variables. The BBD matrix consisted of total 17 experimental points in random order with five replications of center points (D. Ye et al., 2015). Each variable was set at three levels and coded -1, 0, +1 for low, intermediate and high values respectively (Table 3.1). The fitted polynomial equation was expressed as surface and contour plots for visualizing the relationship between three independent variables and the response variable (Yuan et al., 2015).

Table 3.1 Factors and levels in Box-Behnken Design used to analyze extraction yield of polysaccharides from *Euphorbia caducifolia.*

Variables for Extraction	Factor Level		
	-1	0	1
A: Temperature (°C)	40	80	120
B: Solid to liquid ratio (g/L)	25	50	75
C: Time (h)	1	2	3

Purification and Monosaccharide analysis: Crude polysaccharide obtained was redissolved in water, and applied on DEAE Cellulose-52 column (2.5×30) and equilibrated with distilled water. The polysaccharides were eluted with stepwise NaCl solution (0, 0.1, 0.5 and 1M). The eluates containing polysaccharides were pooled, concentrated and dialyzed against distilled water. Dialyzed fraction was lyophilized (considered as EC polysaccharide or ECP) and stored at -20°C (Ye et al., 2008). EC polysaccharide was analyzed for monosaccharide composition using GC-MS. The polysaccharide was hydrolyzed in 2.5 M TFA for 3 h at 121°C in a sealed tube, and residual acid was removed with methanol under N_2 stream. The residue was dissolved in pyridine and derivatized with silylating reagent (BSTFA and 1% TMCS) in 1:2 ratio at 40°C for 2h. Monosaccharide derivatives were extracted with dichloromethane and analyzed for composition using GC-MS (Pitthard & Finch, 2001).

Spectral Analysis: Spectra of EC polysaccharide was taken by using Chemito UV2100, UV-Visible spectrometer and Bruker Alpha FTIR, Fourier transform infrared spectrometer. For UV-Visible spectrum, polysaccharides dissolved in distilled water and spectrum recorded at a frequency range 1100-190 nm. The FTIR spectrum was measured at a range of 4000-500 cm^{-1} (Yuan et al., 2015).

Thermal Analysis (TG-DTA): Mettler TGA/DSC1 was used to determine the thermal properties of polysaccharides. EC polysaccharide was weighed to 4.1 mg and heated at a rate of 10°C/min from 40°C - 600 $^{\circ}$C under the flow of N_2 atmosphere (Janarthanan, Zin Wan Yunus, & Ahmad, 2003).

Antioxidant activity

a) DPPH-free radical scavenging activity: Blois method was used to determine the free radical scavenging activity of EC polysaccharide using DPPH radical with minor modifications (Molyneux, 2004). 1 ml of DPPH (0.1mM) was added to 3ml of various concentrations of polysaccharides (0.125-2 mg/mL) and incubated for 30 min in the dark and absorbance was measured at 517 nm. Ascorbic acid was used as a standard. Three separate experiments were performed, and the percentage inhibition of the polysaccharides was calculated by the following equation: (Kumar et al., 2005)

Percentage inhibition = [(A control – A sample)/A control] × 100

Where A- Absorbance.

b) Nitric Oxide radical scavenging activity: Sodium Nitroprusside gives nitric oxide ($NO^{\cdot}$) in aqueous solution at pH 7.2-7.4, which reacts with oxygen to produce nitrite ions and the formed nitrite ions determined by Griess reagent (Griess diazotization reaction). 3 ml reaction mixture containing 10mM Sodium Nitroprusside in phosphate buffered saline pH 7.2 and EC polysaccharide with different concentrations (0.125-2 mg/mL) were incubated at 25°C for 2 h. 0.5 ml aliquot of reaction mixture was added to 0.5 ml of Griess reagent (equal parts of 1% sulphanilamide in 5% H_3PO_4 and 0.1% naphthyl ethylenediamine dihydrochloride), and the absorbance was measured at 546nm. BHT (Butylated hydroxytoluene)used as reference compound. All three experiments were done separately, and the percentage inhibition was calculated by comparing control and test samples (Green et al., 1982).

c) Reducing power: 1 ml of EC polysaccharide (0.125-2 mg/mL) was added to 2.5 mL of phosphate buffer (pH 6.6, 0.2 M) and 2.5 ml of 1% potassium ferricyanide

[$K_3Fe(CN)_6$] and incubated at 50°C for 30 min. 2.5 ml of 10% TCA was added to the mixture and centrifuged for 10 min at 3000 rpm. To the 2.5 ml of supernatant, 2.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$ added. BHT used as reference compound. The absorbance was measured at 700 nm. The increase in the absorbance indicates the high reducing power (Yildirim et.al., 2001).

Emulsifying activity: The emulsifying activity of EC polysaccharide was performed by turbidimetry method (Benhura & Chidewe, 2004). 3ml of different concentrations of EC polysaccharide in water was added to the 1 ml of vegetable oil (castor oil) and homogenized for 10 min. A 50 µl aliquot was diluted with 5ml of 0.1% Sodium dodecyl sulfate, and the absorbance was measured at 500 nm. The ability of the emulsion was determined by recording the absorbance at 0 h, and the stability of the emulsion was determined by allowing the emulsion at room temperature for 24 h. The turbidity of the emulsion was calculated by:

$$T = 2.303A/l;$$

where T- Turbidity; A- Absorbance; *l*- Path length of the cuvette;

MTT Assay: Murine macrophages (RAW 264.7) was used for the experiment. The cells (1×10^4) were inoculated in 96 well plate and incubated for 24 h in an incubator (5% CO_2, 37°C). The cells were treated with different concentrations EC polysaccharide and incubated for 24 h. After incubation, 3(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added as 5 mg/mL and incubated further for 4 h. 100 µl of DMSO added to the wells and absorbance taken at 590nm. Percentage of cell viability was calculated with untreated cells as a control (Khan et al., 2006).

Cytokine Measurement: RAW 264.7 cells (5×10^5 cells/mL) were stimulated with EC polysaccharide and the levels of cytokines, Tumor necrotic factor –α (TNF-α) and Interleukin-6 (IL-6) were measured after 24 h of incubation (Schepetkin et al., 2008; Xu et al., 2006). TNF-α and IL-6 concentrations were determined by extrapolation from the standard curve, according to the manufacturer's protocol (BD Bioscience).

Results and Discussions

Polysaccharides extraction: Response surface methodology, a statistical technique used to analyze the relationship between parameters and the yield (D. Ye et al., 2015). The polysaccharide yield obtained with an experimental matrix of the Box-Behnken design was calculated and showed the yield in the range of 0.9% to 2.4% (Table 3.2). As single factor analysis, temperature played a significant role in the percentage yield of the polysaccharides compared with solid to liquid ratio and time by enhancing diffusion coefficient of the polysaccharide at high temperatures (Ros et al., 2004). High temperatures (>90°C) were used to extract polysaccharides from algae and plant sources (Chaiklahan et al., 2013). The response surface curves (3D) and contour plots (2D) of the design gives a visual relation between parameters to the percentage of yield (Fig. 3.1.1). Although the rise in temperature increases the polysaccharides yield, its interaction with solid to liquid ratio and time also affect the yield percentage. The influence of temperature on the yield with solid to liquid ratio shows statistical significance but not with time, albeit longer time affect the yield (Table 3.2). At 120°C, 2.36% of yield was obtained with 50 g/L of solid to liquid ratio and 3 h of time whereas 2.30% of yield was obtained with 75 g/L of solid to liquid ratio and 2 h of time, and the insignificant difference of the yield (0.06%) shows the interchangeable effect of the other two parameters on the yield. From RSM results it observed that yield saturation reached to 1.5% & 2.4 % at below and above 90°C respectively. The interactions between the

variables and yields were related with the help of multiple regression analysis by following second-order polynomial equation:

$$Yield\,(Y) = 1.44 + 0.57A + 0.062B = 0.055C + 0.15AB + 0.070AC + 0.020BC + 0.13A^2 + 0.071B^2 + 0.005C^2$$

Table 3.2: The Box-Behnken Design matrix and results of extraction yield (%) of polysaccharides from *Euphorbia caducifolia*

Run	A: Temperature (°C)	B: Solid to liquid ratio (g/L)	C: Time (h)	Yield (%)
1	80	25	1	1.39
2	80	75	1	1.62
3	80	75	3	1.69
4	40	50	1	0.93
5	80	25	3	1.38
6	40	75	2	0.96
7	80	50	2	1.46
8	80	50	2	1.53
9	80	50	2	1.40
10	40	25	2	1.29
11	120	75	2	2.30
12	40	50	3	0.98
13	80	50	2	1.40
14	80	50	2	1.43
15	120	50	3	2.36
16	120	25	2	2.01
17	120	50	1	2.03

From the regression analysis, the high value of the determination coefficient R^2 (0.97) and the adjusted R^2 (0.97) explains the high fitness of the regression model and correlation between the values. The Analysis of Variance (ANOVA) of the model suggests that the temperature variable affects the yield significantly as well as its interaction with solid to liquid ratio variable influence the yield ($P<0.05$). The optimum conditions for the better extraction of the polysaccharides are 120°C of temperature, 75 g/L of solid to liquid ratio and 2 hr of time which correlates with predicted yield (2.425%) and experimental yield (2.30%).

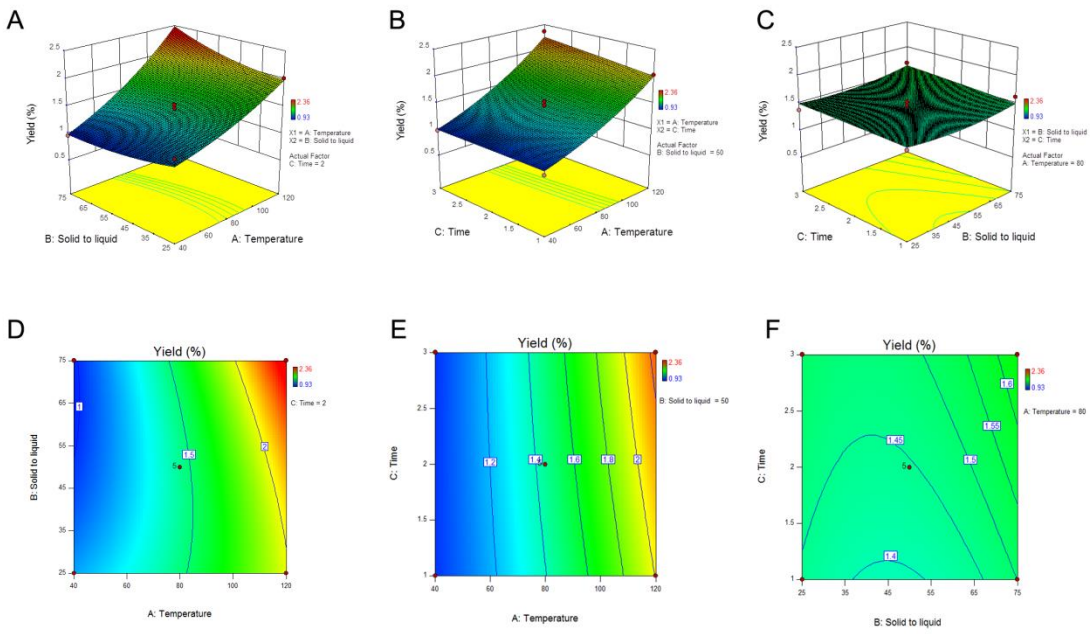
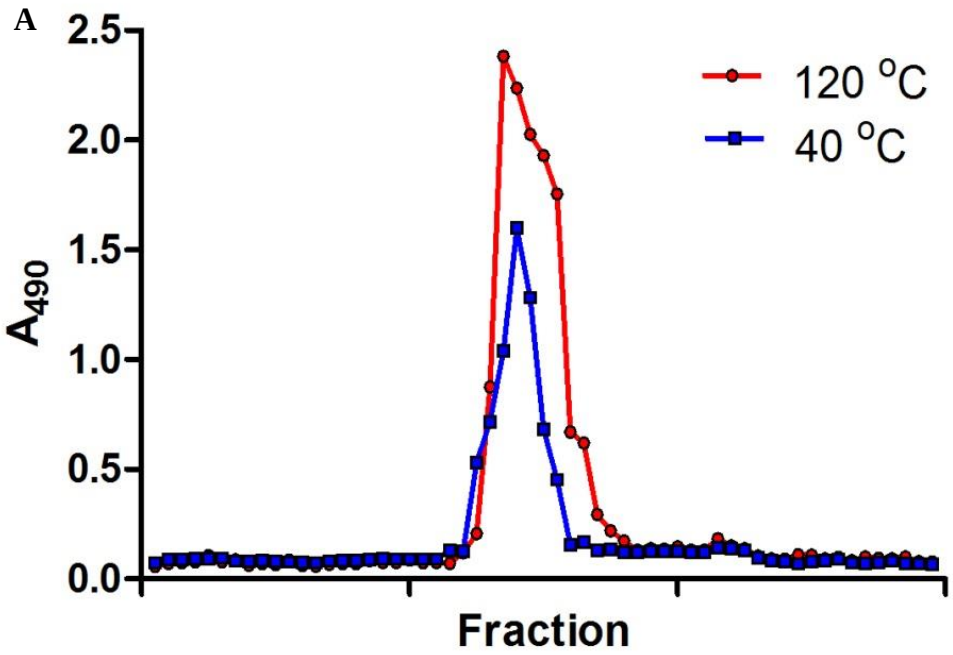


Fig. 3.1.1: Response surface curve (3D) showing the relation between parameters and polysaccharide yield; A) temperature and solid to liquid ratio, B) temperature and time, C) solid to liquid ratio and time. Contour plots (2D) showing the relation between parameters and polysaccharide yield; D) temperature and solid to liquid ratio, E) temperature and time, F) solid to liquid ratio and time.

Purification and Monosaccharide analysis: The lyophilized crude polysaccharide was chromatographed on an anion exchange column to get acidic polysaccharide fraction. Use of an anion exchange chromatography to elute the acidic polysaccharides has been

reported from wheat, sugar beet by Neukom et al. (1960) (Jermyn, 1962). From crude polysaccharide, the only fraction eluted with 1 M NaCl contains mannose, rhamnose, galactose, glucose, galacturonic acid, galactaric acid, arabinose, ribose, talose and xylose as monosaccharide constituents (Fig. 3.1.2). Previously, it has been reported that the presence of acidic polysaccharides with uronic acids and mucic acids in the leaves of *A. rosea*, *H.syriacus*, *C.olitorous* (Ohtani, Okai, Yamashita, Yuasa, & Misaki, 1995). Interestingly, similar compositions were observed with *E. caducifolia* as reported in roots of its family member *E. fisheriana* (Liu et al., 2011). Acidic polysaccharides extracted from plant sources have been reported for their extensive biological activities like hypoglycemic, anticomplementary, immunomodulatory (Ohtani et al., 1995). Similar to that of other acidic polysaccharides, EC polysaccharides was also expected to show biological activities like antioxidant and immunomodulatory activities.



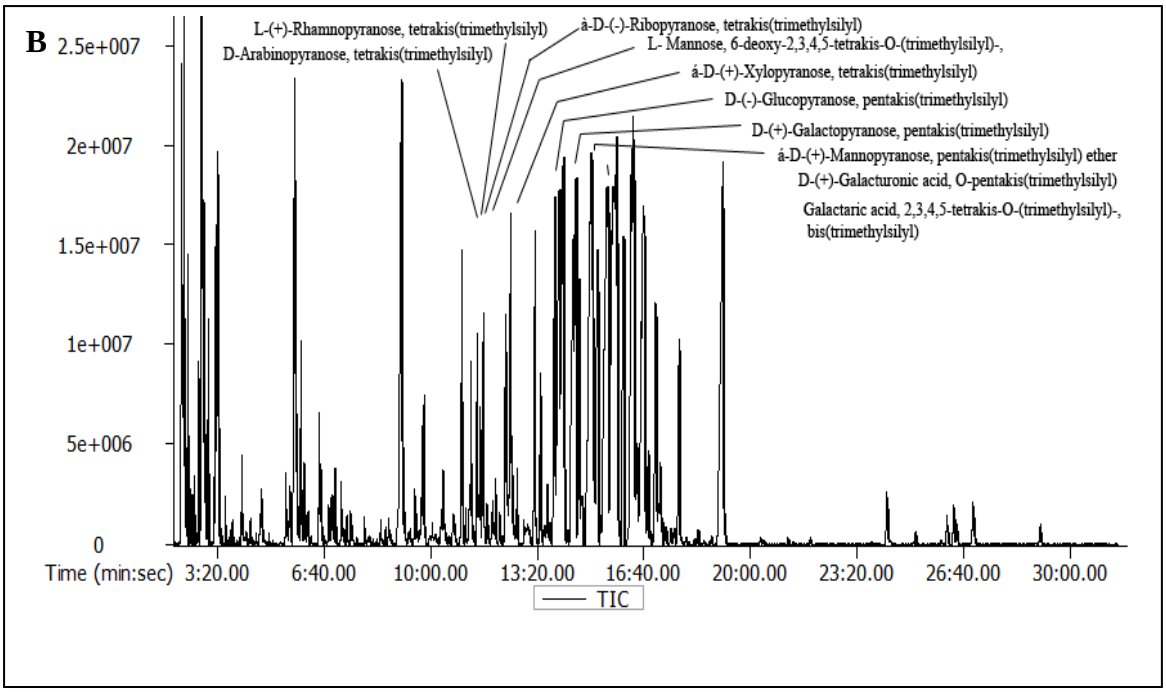


Fig. 3.1.2 Chromatograms of the EC polysaccharide;

A) Elution profile of EC polysaccharides purification on DEAE cellulose column at 120°C and 40°C, B) GC-MS profile of the EC polysaccharide for monosaccharide analysis: Trimethylsilyl derivatives of sugars

Spectral analysis: UV–Visible and infrared spectra of the EC polysaccharides exhibited typical characters of the polysaccharide. The UV-visible spectrum (Fig. 3.1.3A) of the polysaccharide did not show any absorption peaks at 260 nm and 280 nm which correlates with the absence of protein and nucleic acid respectively (Bian, Xie, & Chen, 2010). Forbye the lack of sharp peaks at amine stretch in IR spectrum of the EC polysaccharides (Fig. 3.1.3B) mount the evidence that no contamination in purified polysaccharides. A characteristic broad peak at around 3330 cm^{-1} assigned to the hydroxyl group(-OH) present in the polysaccharide. An aliphatic C-H stretching vibrations at 2921 cm^{-1} and weak absorption at 1418 cm^{-1} is from aliphatic C-H bending of CH_2 (Parikh & Madamwar, 2006). The two peaks towards the 1738 cm^{-1} and 1605 cm^{-1} in the spectra resulted from the COO^- deprotonated carboxylic group and two medium

broad peaks at 1075 and 1018 cm^{-1} suggest the presence of C-O bonds. β-configuration of the monomers present in EC polysaccharides displayed with a peak around 800 cm^{-1} (Zhou et al., 2000). Merged peaks in the fingerprint region indicate the linkages between monosaccharides.

Thermal analysis; Thermal nature of the polysaccharides was analyzed using TG-DTA. The thermogravimetric curve (Fig. 3.1.3C) of the EC Polysaccharides showed decomposition with three differentiated steps. The first mass loss event occurred from 50°C to 120°C with the loss of mass 10-15 % due to the diminution of adsorbed water which represents the hydrophilic nature of the polysaccharide (Zohuriaan & Shokrolahi, 2004). The Second stage starts at 230°C retains till 350°C and observed a loss of weight ca.36% at 285°C because of thermal depolymerization.The carbonization of polysaccharide causes sustained weight loss in the last stage occurred above 350°C to 600°C. The presence of charged cations like Na^{+}, K^{+}, Ca^{2+} and complex configuration of the polysaccharide resist the degradation process and left the solid residue around 35% after 600°C (Cerqueira et al., 2011; De, Ruiz-bermejo, Menor-salván, & Osuna-esteban, 2011). DTA thermogram of the EC polysaccharide showed early endotherm peak at 78°C with an onset temperature 48°C and end set point 114°C. Enthalpy change (ΔH) of 135.15 J/g was noticed at early stages. No glass transition (T_g) temperature was exhibited in the thermogram which attributed to interference of early endothermic peak (Parikh & Madamwar, 2006).

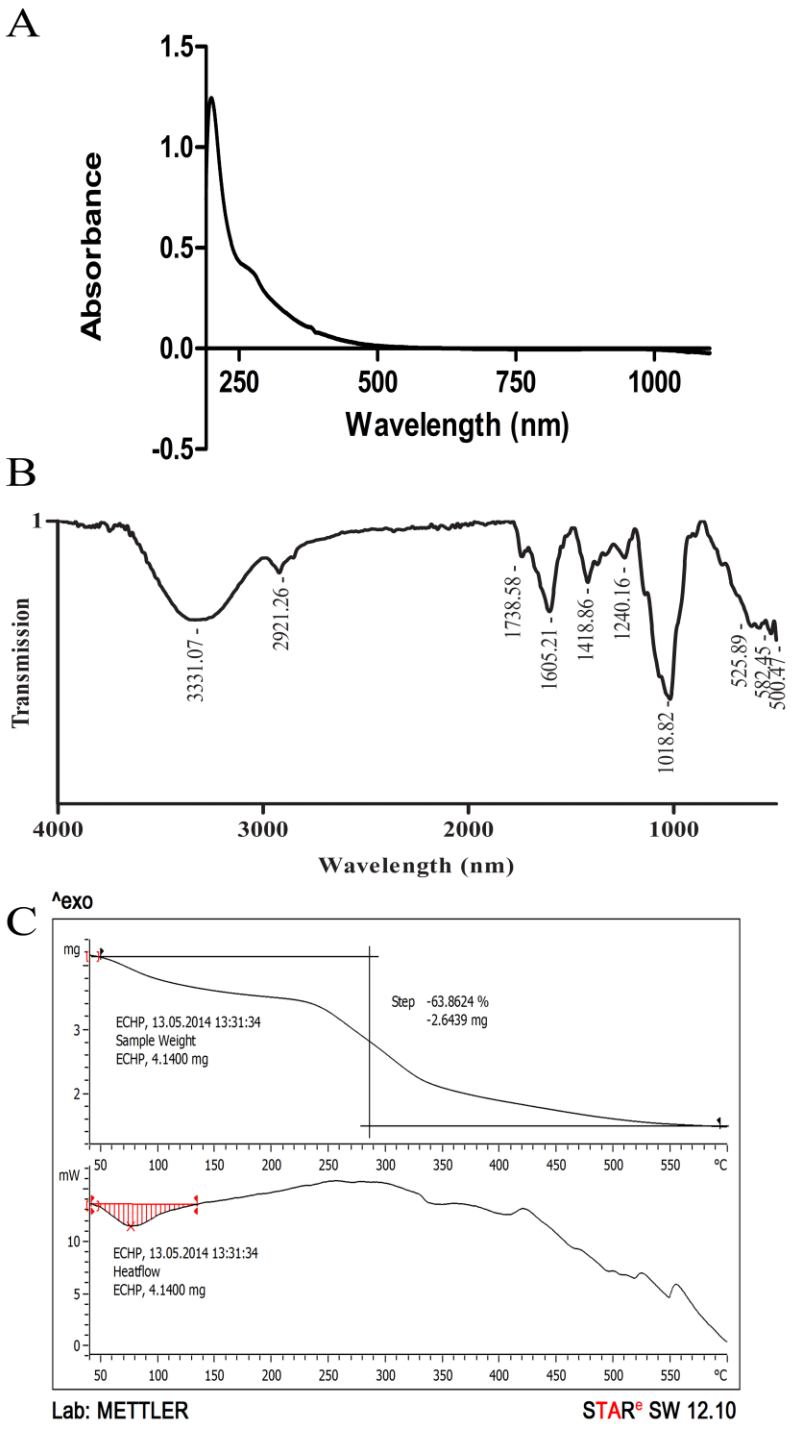


Fig. 3.1.3 Spectra and thermogram of EC polysaccharides;

A) UV-Visible Spectrum, B) ATR-IR Spectrum, C) TGDTA thermogram at heating rate 10°C min^{-1} under a nitrogen atmosphere.

Antioxidant Activity: A simple and sensitive, DPPH free radical scavenging activity was performed to observe the antioxidant activity of polysaccharides. Antioxidant activity was

increased as the concentration of the polysaccharides increases and Ascorbic acid used as a standard to compare EC polysaccharides. Free radical scavenging activity of the polysaccharide gained momentum of activity after 0.5 mg/ml concentration to greater 35% to 65% (Fig. 3.1.4A). Besides DPPH, Nitric Oxide ($NO^{\cdot}$) free radical scavenging activity was also measured (Fig. 3.1.4B). $NO^{\cdot}$ react with oxygen to give free radicals nitrite and proxy nitrite, the reason for many inflammatory processes and cause of several diseases (Luyen et al., 2014). EC Polysaccharides, a competitor for oxygen to react with $NO^{\cdot}$ and thus inhibits the production of nitrates and nitrites. EC polysaccharides at 2 mg/ml concentration showed the free radical scavenging activity 65% with DPPH and 55% with Nitric Oxide. The ability of the polysaccharides to scavenge the free radicals in the reaction mixture more than 50% suggests as prospective antioxidants.

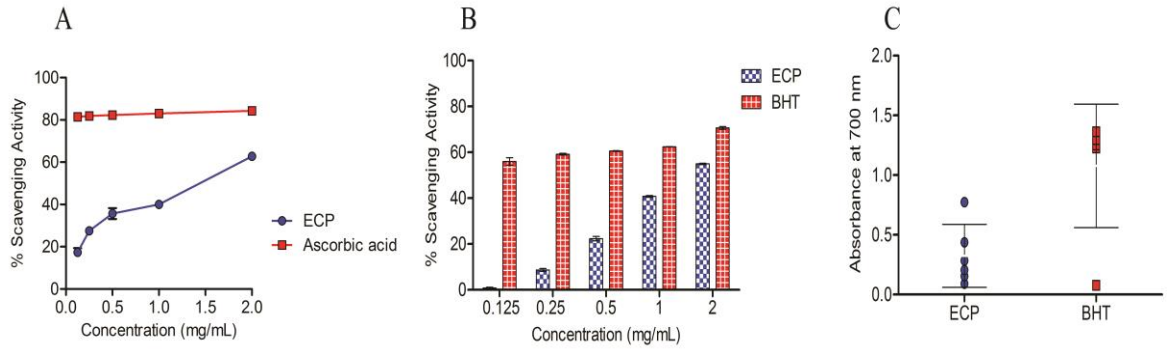


Fig. 3.1.4 Antioxidant activity of EC polysaccharides;

A) DPPH free radical scavenging activity, B) Nitric oxide radical scavenging activity, and C) Reducing power.

(values are mean ± SE, n=3)

The reducing ability of a compound to donate electron can serve as a potential antioxidant. Apart from free radical scavenging activity, the antioxidant activity of a compound can also be evaluated by its capacity to reduce the ferric cyanide complex (Fe^{3+}) to ferrous cyanide form (Fe^{2+}). Thereby the colour change in the reaction mixture

to different shades of green to blue indicates the antioxidant activity (Irshad, Zafaryab, Singh, & Rizvi, 2012). Similarly, green to blue shades were observed with EC polysaccharides, and prussian blue with BHT, where prussian blue indicates strong reducing activity. The absorbance was measured, as it proportionately correlates with the reducing power. Even though polysaccharide showed less degree of absorbance compared to BHT (Fig. 3.1.4C), its reducing ability as an electron donor evinced with increasing polysaccharide concentration.

Emulsifying Activity; Emulsions are the mixture of immiscible liquids where droplets of one liquid dispersed in other with the help of an emulsifier. Most of the acidic polysaccharides like gum arabic, gum karaya, gum tragacanth are using as emulsifiers in the pharmaceutical and food industries (Benhura & Chidewe, 2004). In an interest to that, the efficacy of the EC polysaccharide as an emulsifying agent was tested by turbidimetry method. The emulsification ability of the EC polysaccharide was observed under a microscope, further measured the turbidity with a spectrophotometer (Fig. 3.1.5). The undiluted emulsion showed flocculation of droplets into various sizes. Further dilution with 0.1% sodium dodecyl sulfate, resulted in more dispersed globules and aggregates of floccules on standing for 24 h. In turbidimetry, increasing the concentration of the polysaccharide to oil shown linearity of the emulsification, and observed 36% of turbidity shift at high concentration and 88% of shift at lower concentrations of polysaccharide on long standing. Stabilization of dispersed phase in continuous phase even after 24 h suggests that the EC polysaccharide as a good emulsifier. Phase separation of the liquids occurred within 1 hour in the control where no polysaccharide was added.

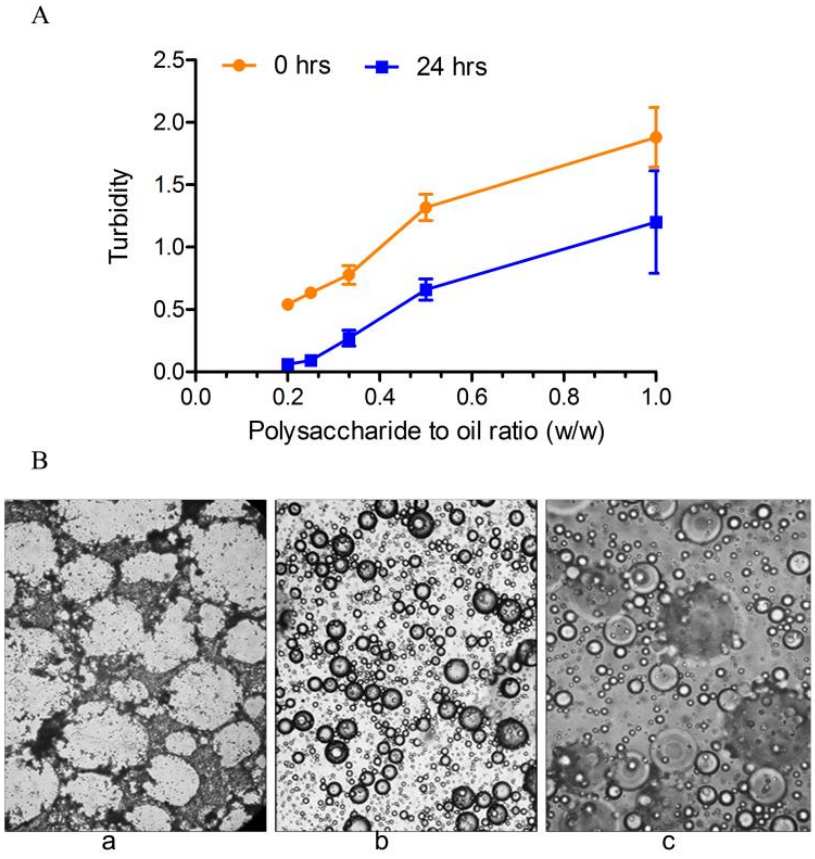


Fig. 3.1.5 Emulsion ability of the EC polysaccharides;

A) The turbidity of emulsion measured at 500 nm at 0 h and 24 h, B) Photomicrograph of Emulsion: a) Control (without polysaccharide), b) Emulsion at 0 h, c)) Emulsion at 24 h.

(Values are mean±SE, n=3)

MTT Assay and Cytokine measurement: Polysaccharides from most of the plant sources are non-toxic in nature and modulate cytokine and chemokine production as an immunomodulator (Schepetkin & Quinn, 2006). Non-toxicity and immunomodulatory activities place the polysaccharides as ideal therapeutic candidates. The EC polysaccharides showed non-toxic nature and also directly stimulate the proliferation of RAW 264.7 cells (Fig. 3.1.6A). The proliferation effect was observed in the range of 50 ng/mL-1 µg/mL, i.e., from 107% to 150%, a further increase in the concentration depicted no significant cytotoxicity on cells compared to untreated cells.

Most of the botanical polysaccharides activate several signal transduction pathways of macrophages and stimulate Nitric oxide (NO), Reactive oxygen species (ROS), and production of inflammatory (IL-1, IL-6, IL-12) as well as anti-inflammatory cytokines (IL-10) along with chemokines (TNF-α) (Schepetkin & Quinn, 2006). Treatment of macrophages (RAW 264.7 cells) with various concentrations of EC polysaccharides significantly enhanced the production of the pro-inflammatory cytokines such as IL-6 and TNF-α whereas untreated macrophages produced negligible amounts (Fig. 3.1.6B, C). The maximum IL-6 production observed with dose effective from 500 ng/mL to 100 µg/mL. Cell proliferation observed with EC polysaccharides at lower concentrations may be due to the initial production of IL-6, which stimulates the extracellular signal-regulated kinases and Mitogen-activated protein kinase pathways (Ogata et al., 1997). Increased levels of TNF-α and IL-6 at higher concentrations might be responsible for the certain level of cell death (<40%). These results evidence that the EC polysaccharides at lower concentrations can be used as a therapeutic agent for promoting wound healing and regeneration activities.

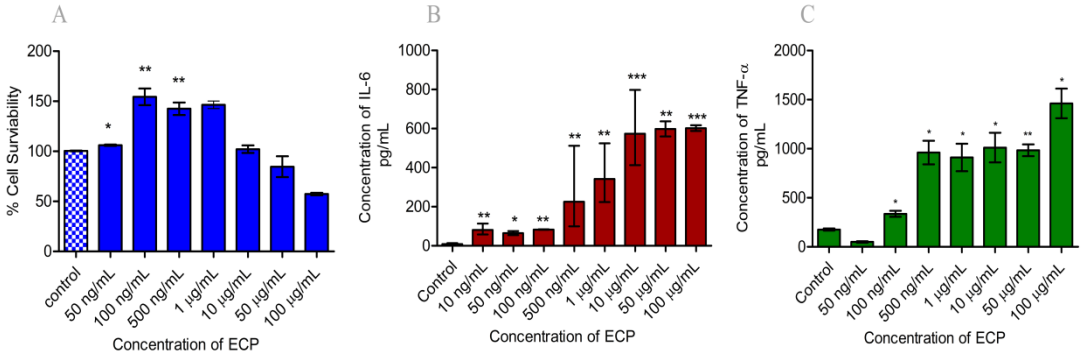


Fig. 3.1.6. Effect of the EC polysaccharides on the RAW 264.7 at a different concentration; A) Cell viability, and B) Concentration of the IL-6, C) TNF-α measured by ELISA in the culture supernatant.

(n=3, values= mean ± SE, * =P<0.05, **=P<0.01, ***=P<0.001)

Conclusions

Altogether this study suggests that manipulation of the temperature influence the polysaccharide yield along with solid to liquid ratio and time. Polysaccharides extracted to a maximum yield of 2.3% at 120°C, and the optimum conditions were identified using the response surface methodology. Purified acidic polysaccharides with uronic acids shown typical nature of spectral as well as thermal characteristics. EC Polysaccharides act as potential antioxidants and as a good emulsifier. Moreover, polysaccharides exhibited immunomodulatory activity with non-toxic nature on RAW 264.7 cells.

2. Chemical modifications of isolated polysaccharide and its characterization

Polysaccharides are the carbohydrates composed of same or different types of saccharides in an organized manner with glycosidic bonds. These are used for various industrial applications mainly in food and pharmaceutical companies. Modifications of the polysaccharides change its structure and lead to the physical as well as functional differences (Cumpstey, 2013). Mostly, chemical methods are used to modify its structure to study the structure-activity relationship in interest towards its physical properties like solubility and biological activities among various types of molecular modifications. Polysaccharide hydroxyl groups are major contributors for chemical modifications. Apart, Alginates and pectins are composed of carboxylic groups called uronic acids. In modifications, Saccharide hydroxyl oxygen act as nucleophile and carbon serve as an electrophile. Nucleophile oxygen can undergo chemical reactions like Etherification (carboxymethyl ether, hydroxyethyl ethers), Esterification (acetyl, carboxyl esters, sulphonate esters) and electrophile carbon can undergo introducing the groups like sulfate and halide groups (Cumpstey, 2013). Additionally, oxidation of sugars, nitrogen group reactions of amino sugars, esterification and other substitutions on carboxylic groups of uronic acid sugars are also considered. All together gives change in chemical, physical and biological changes.

Materials and Methods

Sulfonation: 50 mg of polysaccharide dissolved in 10 ml of formamide and 10 ml of pyridine mixture. After, added 4 ml of chlorosulfonic acid on ice bath about 2 hrs. Placed the reaction mixture for 15 hrs at 4°C and ended the reaction by adding cold water. Sodium hydrogen carbonate used to neutralize the reaction mixture until it ceases the

effervescence. Then, dialyzed the solution about 7 days against distilled water and concentrated under reduced pressure and lyophilized (O Neill, 1956).

Carboxymethylation: Yang et al. 2011 method was followed with minor modifications (Yang et al., 2011). 10 mg of polysaccharide suspended in 4 mL of isopropanol and kept under stirring at RT for 15 min, followed by the slow addition of 1.5 mL of 20% NaOH with 1 h stirring. Then the solution of 150 mg/ ml of chloroacetic acid was added to the reaction mixture with stepwise under constant stirring. The reaction was continued for 4 h at 60°C. Cooled down to RT, and 0.5 M of hydrochloric acid was added to neutralize the mixture. The derived polysaccharide was dialyzed against normal water for 6 h and then with distilled water for 12 h. Dialysate was precipitated with ethanol and lyophilized.

Acetylation: 50 mg of ECP dissolved in 7 ml of formamide and further added 12.5 ml of pyridine. After, added 10 ml of acetic anhydride at 4°C about 2 h at frequent intervals. The reaction was continued for 20 h at RT and ceased the reaction by adding cold water. Sodium hydrogen carbonate used to neutralize the reaction mixture until it ceases the effervescence. Then, dialyzed the solution about 7 days against distilled water and concentrated under reduced pressure and lyophilized (Mellerowicz, 2013)

Phosphorylation: 50 mg of polysaccharide was suspended in 10 ml of N, N-Dimethylformamide (DMF) along with 4 g of urea and stirred for 1 h to homogenize the mixture. 50 mg of phosphoric acid was added and stirred for 4 h at 130°C. Reaction mixture was brought to RT, filtered, and washed with a mixture of 1-propanol, and distilled water followed by 0.1mM hydrochloric acid, and with distilled water. The obtained filtrate was lyophilized (Oshima et al., 2008).

Morphological and elemental changes: Morphological changes of the modified polysaccharides were studied under scanning electron microscopy (SEM), and the change

in the elemental composition was investigated with energy dispersive x-ray spectroscopy (EDS).

Functional group studies and PCA analysis: Chemical modifications of the polysaccharide further confirmed with functional group analysis by IR spectroscopy. Minor changes in the chemical modifications and major distributions of the different functional groups of the modified polysaccharides were studied under the statistical analysis of principal components. PC1 and PC2 were considered for the analysis (Szymanska-Chargot & Zdunek, 2013).

Biological significance: Change in the chemical structure always changes its biological activity. Antioxidant activity and cytotoxicity of the modified polysaccharides were studies with previous methods.

Results and discussions

Morphological and elemental analysis: Modified polysaccharides changed their morphology as they introduced by other functional groups. Sulfated, carboxymethylated polysaccharide looks like fabric like structure and flakes like structures are observed with acetylated and phosphorylated polysaccharides whereas the native form of ECP is crystalline like structure (Fig. 3.2.1). Change in the elemental composition of the carbon and oxygen of the native form by introducing acetyl and carboxymethyl groups are not much significant. However, the important observation can make from the composition that, decrease in the "C" element from native 51% to 48% and 45% of acetyl and carboxymethyl respectively propose the etherification of the polysaccharides. 8% of the sulfur element and 4% of phosphorous elements confirm the substitution of sulfate groups and phosphate groups on the polysaccharide (Fig. 3.2.2).

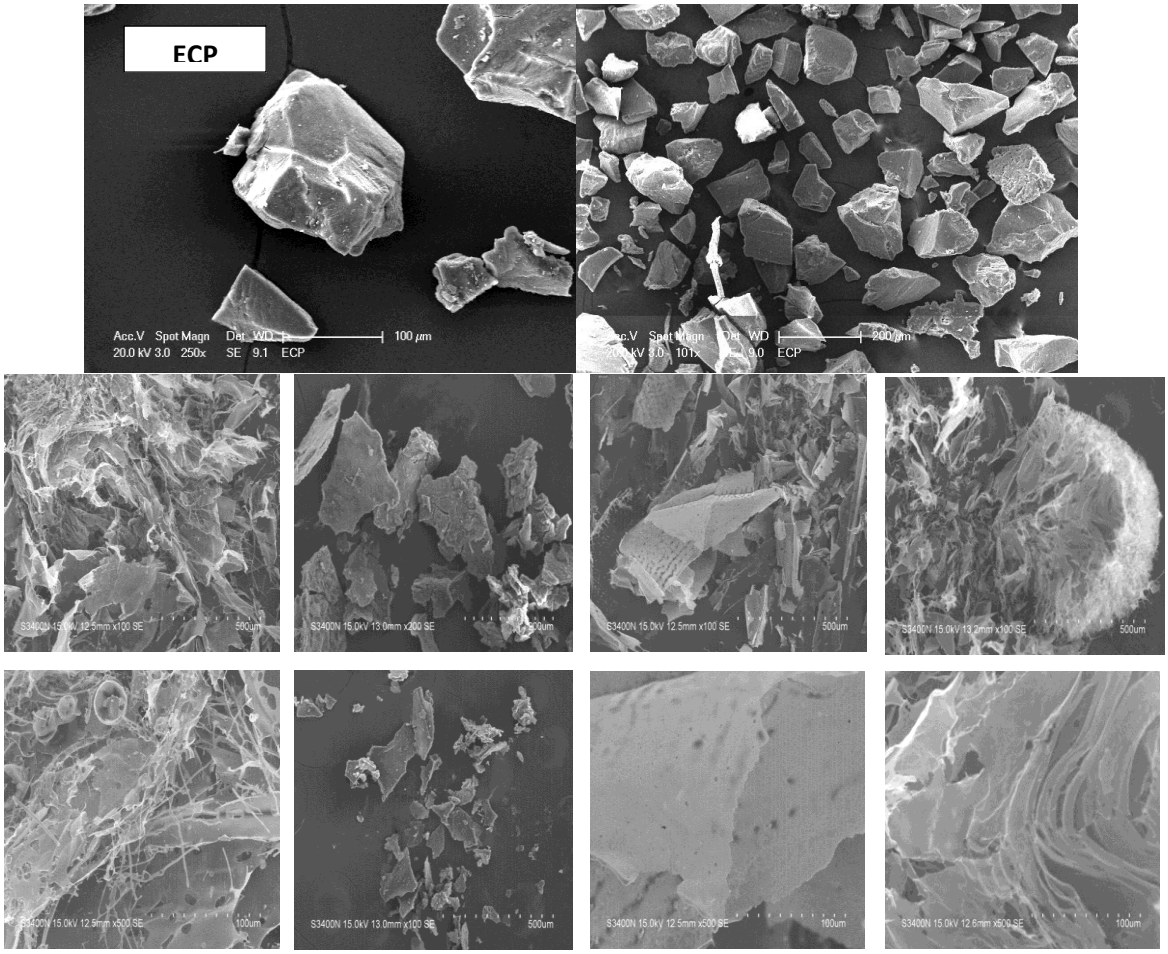


Fig. 3.2.1. Scanning Electron Microscopy images of native and modified polysaccharides

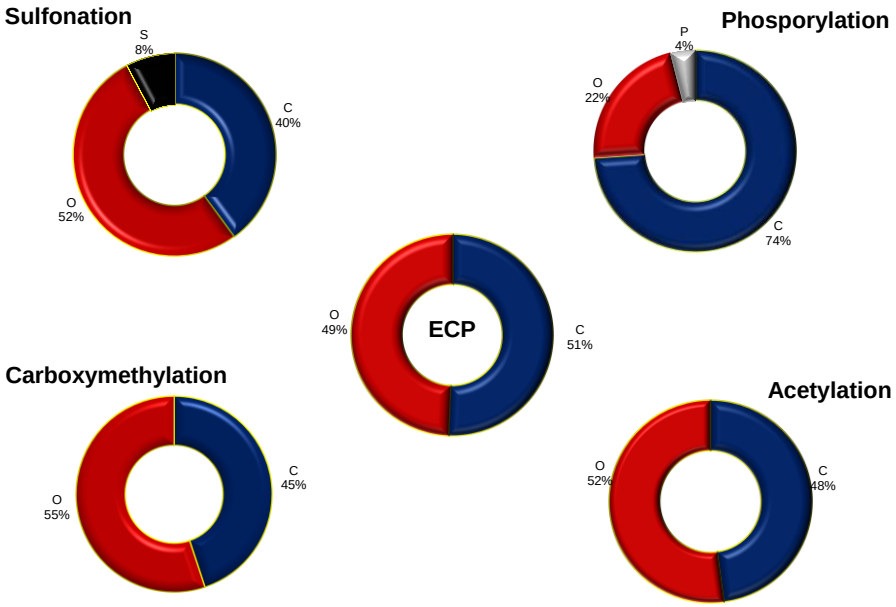


Fig. 3.2.2. Elemental composition of native and modified polysaccharide

Functional group studies and PCA analysis: An IR spectrum of the modified polysaccharides confirms the presence of relative functional groups respective to the modifications. Carrageenan, a natural sulfated polysaccharide obtained from marine algae. Sulfated polysaccharides are familiar with many biological activities like anti-tumour, antithrombin, anti-viral and mostly for anticoagulant activity (Yuan et al., 2005). Heparin know to be a natural anticoagulant, activity depends on the degree of sulphation of heparin (O Neill, 1956). A peak at 1260-1210 cm^{-1} confirms the sulphation of the polysaccharide along with sugar sulfate peals around 800 cm^{-1} (Gomez-Ordonez & Ruperez, 2011). Phosphate of the polysaccharide gives P=O, P-O stretch vibrations at 1200-1100 cm-1. For carboxymethylation and acetylation, CO stretches at 1900-1550 cm^{-1} due to the carbonyl group and stretch at 1800-1740 cm^{-1} assigned to carboxylic groups. Similar stretches were noticed with respectively modified polysaccharide in comparison to native form (Fig. 3.2.3). Change in the intensity of OH stretch of alcoholic hydroxyl groups confirms the derivative forms of esters.

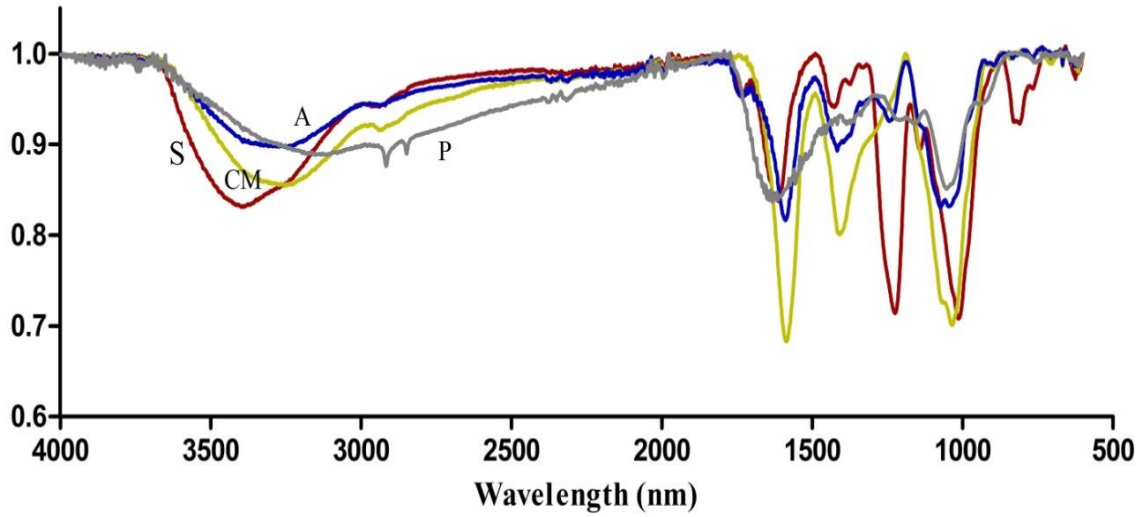


Fig. 3.2.3. ATR spectra of modified polysaccharides; A-Acetylated, CM- Carboxymethylated, S-Sulphated, P-Phosphorylated polysaccharides.

Multiple variation analysis is used to analyze the large set of data to limited variable interpretation like IR spectral data. PCA is one of the methods used to characterize the modified spectra from its native form. Total 75.68% of variation was observed with first 2 principal components PC1 and PC2 and considered to categorize the observations. A negative correlation was noticed with sulfated and phosphate forms whereas togetherness of native form with carboxymethylated and acetylated forms (Fig. 3.2.4A). Biplot shows the influence of variables on observations with particular stretch levels of vibrations at respective wavelengths (Fig.3.2.4B). The combination of the various observations concerning their modifications alters the variation to the 79.94% from 75. 68%. (Fig. 3.2.4C).

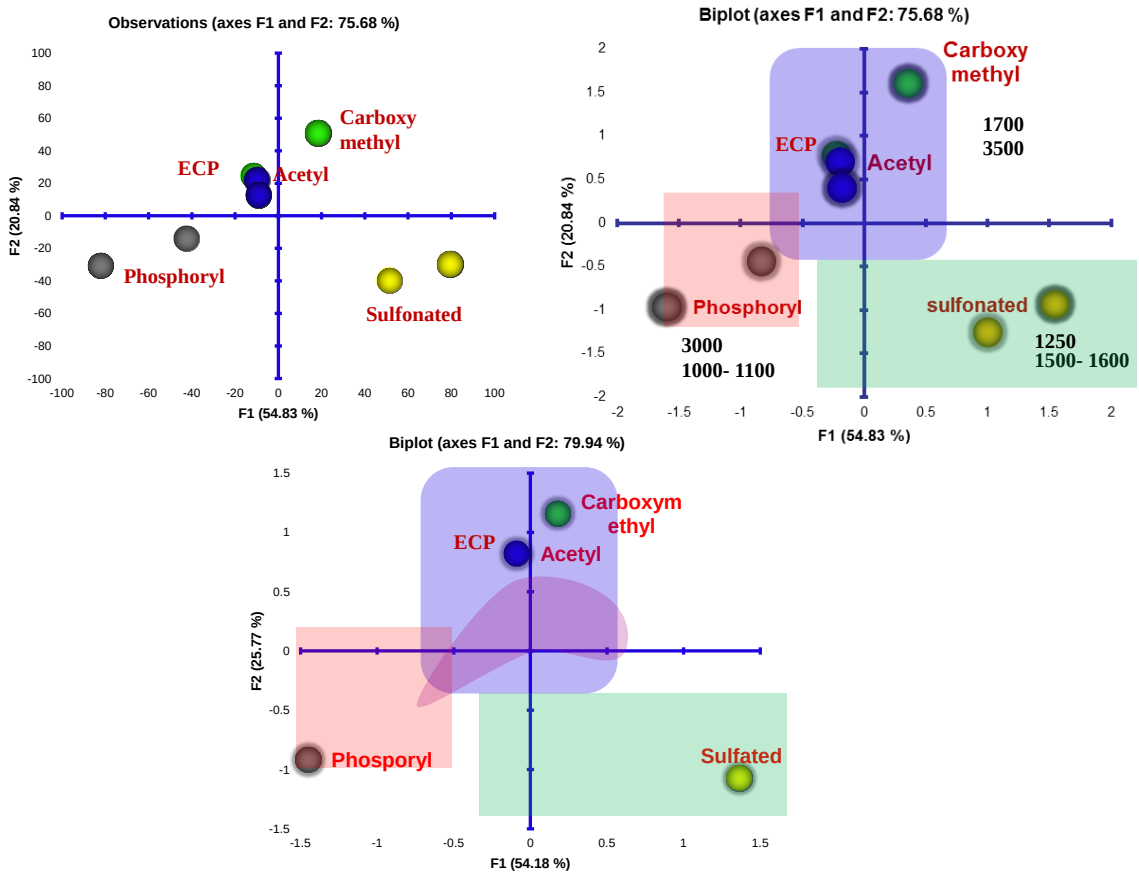


Fig. 3.2.4. Principal Components analysis of modified polysaccharide spectra; A) Plot of PC1 and PC2 of observations, B) Plot of PC1 and PC2 observations with variables, C) Average plot of observations.

Biological Significance: Structural changes always modify the biological activity. Structural changes employed to improve the physicochemical barriers like solubility, flow properties, and others. In addition, Biological significance also greatly mentioned. The addition of sulfate group improves the anticoagulant activity and antioxidant activity (O Neill, 1956). The ability of free radical scavenging activity of increased with the addition of carboxymethyl, sulfate and acetyl groups to the native form of polysaccharide. In contrast, phosphate introduced polysaccharide decreased its ability to scavenge the DPPH free radicals. A polysaccharide isolated from the plant does not show any toxicity towards macrophages. The same results were observed with carboxymethylated and acetylated ECP whereas cell death was noticed with sulfated and phosphorylated ECP (Fig. 3.2.5).

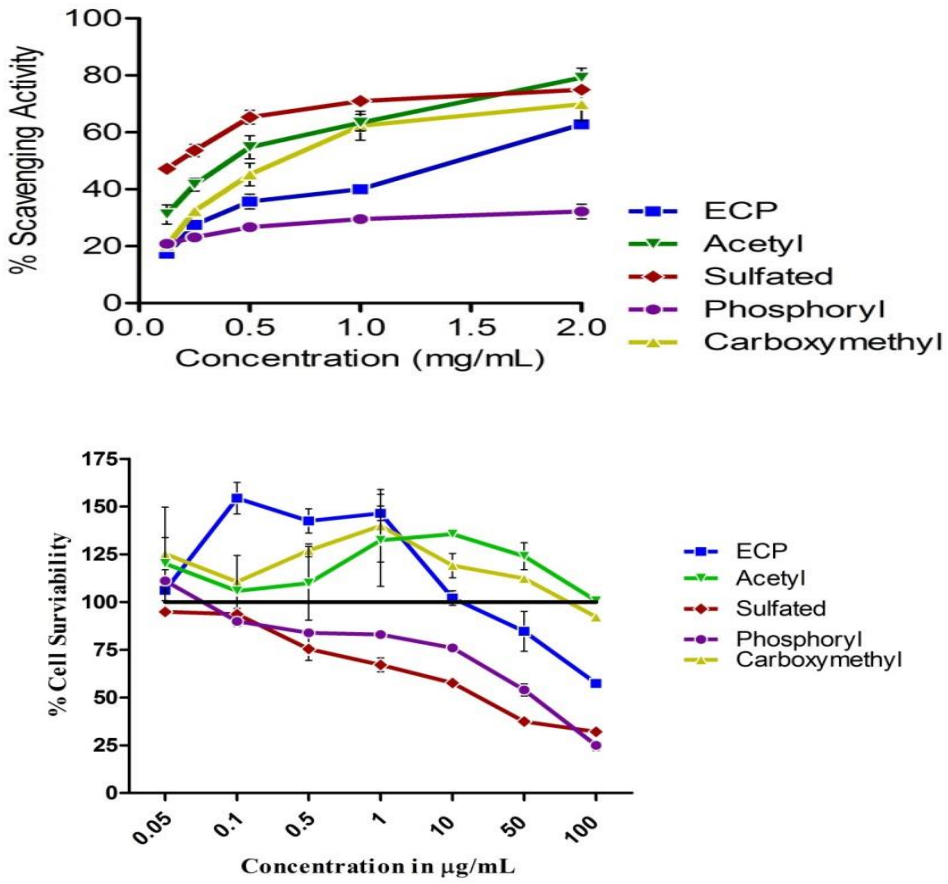


Fig. 3.2.5. The biological significance of Modified polysaccharides:
A) Antioxidant activity, B) Cytotoxic activity.

Conclusions

Change in the structural aspects of the molecules alters their nature towards physicochemical and biological properties. 8% of sulfate and 4% of phosphate groups introduced. Change in the elemental composition of C and O gives the alteration carboxymethyl and acetyl polysaccharides. Principal components analysis of ATR spectra discriminate the respective polysaccharides each other and with native form. Biological significance greatly altered with modified polysaccharides to native form.

CHAPTER IV

ISOLATION
&
CHARACTERIZATION OF LECTIN FROM LATEX OF *E.caducifolia*

Lectins have been used to study the immunology of Red Blood Cells. Seed lectins from Euphorbiaceae were studied by Stillmark (1888) initiates the immunology of lectins. Further elevates the lectin studies on isolation, purification and their biological applications. Lectins have been detected and isolated from different species of all living sources but the majority from angiosperms of the plant. *Azolla caroliana*, a pteridophyte has been reported for the presence of a lectin (Mellor et al., 1981). In gymnosperms, lectin activity was noticed with extracts of leaves and stems of *Taxus baccata* (Jermyn & Yeow, 1975) and *Cycas siamensis* megagametophyte walls extracts (Pettitt, 1977). Seeds of angiosperms are the major contributors of the lectins though several lectins have been also detected and isolated from various parts of the plants. For example, from leaves of *Aloe arborescens* (Suzuki et al., 1979) , *Dolichos biflorus* (Carter & Etzler, 1975), *Griffonia simplicifolia* (Lamb et al., 1983); Barks of *Robinia pseudoacacia* (Horejsi et al., 1978), *Sambucus nigra* (Shibuyas et al., 1987); Sieve saps of *Castanea sativa*, *Salix alba* (Gietl et al., 1979) Bulbs of *Galanthus nivalis* (Van Damme et al., 1987), *Leucojum aestivum* (Van Damme et al., 1988), *Narcissus spp*. (Van Damme & Peumans,1990),

Tulipa gesneriana (Oda & Minmi, 1986); Tubers of *Bryonia dioica* (Peumans et al., 1984), *Trichosanthes kirilowii* (Yeung et al.,1980).

Haemagglutination was also detected in the latex of the plants by Richet in 1909 from *Hura crepitans* and subsequently purified. Although the study of lectins from Euphorbiaceae was done in 1888 by Stillmark, the study was limited to few species later years. Further, the latex biochemistry and isolation of lectin from Euphorbiaceae was reported by Seshagirirao in 2001 (Seshagirirao & Prasad, 2001). In extension to the previous work done by Seshagirirao, this study emphasizes the isolation of high yield lectin, biochemical characterization, and biological activities.

Materials and Methods

Purification of Galactose-binding Protein:

Latex serum: The latex of *Euphorbia caducifolia* was collected and frozen in liquid nitrogen, frozen serum was thawed and centrifuged at 20,000 x g for 1 hour at 4°C. The liquid portion was separated and repeated the freezing, thawing and centrifugation process to get clear serum. The separated clear serum was used for experiments or stored below -20°C until required. The crude serum proteins were precipitated with cold acetone (overnight at -20°C) and centrifuged at 10,000 x g for 10 minutes. The protein pellet was dissolved in phosphate buffer saline and used for experiments or stored at -20°C.

Preparation of Affinity matrix: The Affinity matrices were prepared according to Seshagirirao method (Seshagirirao et al., 2005) In brief, 100 g of seeds of (Guar gum and Leucaena leucocephala seed gum) was blended at low speed for few seconds. The seed hulls were removed and kernels (60g) were milled into fine powder. The powder was further sieved through 1mm mesh. Next, the powder was mixed with an emulsion of Epichlorohydrin (99%) in 3 M NaOH. The mixture was incubated for 24 h at 40°C and

later for 6 h at 70°C. The formed matrices were soaked and washed several times with distilled water. Finally, the recovered gel was transferred to 10 mM phosphate buffer saline, pH 7.2 containing 0.02% of sodium azide.

Purification of Galactose-Binding Protein: The purification of galactose binding protein by affinity chromatography was performed at 4°C. 2 mL of latex serum (50µg) was loaded onto matrix column (3 X 7 cm, ca 50 mL), previously equilibrated with phosphate buffer saline pH 7.2 with a flow rate 15 mL/h. The column was washed with it until the effluent gets < 0.02 absorbance at 280 nm. The protein adsorbed was eluted with 0.2M lactose in Phosphate buffer saline. The purity of high protein fractions was verified by SDS-PAGE, pooled and dialysed against phosphate buffer saline.

Protein Estimation: Protein was estimated by Lowry et al., (1951) with minor modifications. Reagent A, was 4% Na_2CO_3 in 0.2 N NaOH, B was 1% $CuSO_4$, C was 2% Sodium potassium tartarate and D was 1 N Folin's reagent. The working reagent is a mixture of A, B, C in the ratio of 23:1:1 and used within 24 h of preparation. 1 mL of the working reagent was added to 1 mL of the protein sample, mixed well and allowed to stand for 10 min. 0.2 mL of reagent D was added rapidly while vortex the sample. After incubation for 30 min, absorbance was recorded at 750 nm. BSA (Fraction V) was used as standard protein (5-50 µg).

Carbohydrate Estimation and monosaccharide composition: Total carbohydrate was estimated using Dubois method (DuBois et al., 1956). The glycosylated carbohydrates to the protein were studied using GC-MS analysis. Lectin (50 µg) was hydrolysed with 4 M TFA by incubating for 6 h at 80°C. Hydrolysate was dried with methanol under speed vac. The residue was dissolved in pyridine and derivatized with silylating reagent (BSTFA and 1% TMCS) in 1:2 ratio at 40°C for 2h. Monosaccharide derivatives were extracted with

dichloromethane and analyzed for composition using GC-MS (Pitthard & Finch, 2001). Quatitized the monosaccharides using a set of standards.

Haemagglutinating activity: The haemagglutinating activity of the galactose binding protein was determined with minor modifications of (Lis & Sharon, 1972) method.

a) Preparation of Alsevere's solution: The Alsevere's solution was prepared by dissolving 2.05g of glucose, 0.8g of Sodium chloride in 80 mL of distilled water. The pH was adjusted to 6.1 with citric acid and the volume was made to 100 mL of distilled water. The solution was autoclaved, cooled and stored at 4°C.

b) Preparation of Erythrocytes: Venous whole blood was added to the equal volume of Alsever's solution. The blood suspension can be stored as long as two weeks in the alsevere's solution. The erythrocytes were isolated from the stock blood suspension by centrifugation at room temperature using a table top centrifuge (1000 x g for 5 min). The packed erythrocytes at the 1000 x g considered as 100%. The packed cells were washed with cold saline (0.9% NaCl) for 3-4 times (5 mL saline for each ml of packed erythrocytes) and finally, 4% cells were made in saline.

c) Preparation of trypsin treated erythrocytes: The erythrocytes were treated with trypsin on the day of the experiment. The 4% erythrocyte suspension incubated with 0.1% trypsin 1:250 (1000-1500 BAEE units/mg) for one hour at 37°C. The trypsin treated erythrocytes were washed 5-6 times with cold saline to remove the traces of trypsin and 4% cells were made and used for the experiments.

d) Agglutination assay: 100 µl of the protein sample (final conc. 1µg) was serially diluted in a microtitre plate and 100 µl of trypsinized 4% erythrocytes were added to each well. The agglutination was observed visually after incubation of the plate for one hour at

37°C. The highest dilution which showed positive haemagglutination was considered as the Titre. The amount of protein presents in this dilution represents the minimum quantity of protein required for agglutination and is defined as one unit. The specific activity is the number of units per mg of protein.

Sugar Inhibition assay: 50 µl (0.4 M) of serially diluted carbohydrate solution was mixed with 50 µl protein containing 8 haemagglutination units in a microtitre plate and incubated at room temperature for 30 min. 100 µl of 4% trypsinised human O group erythrocyte suspension was added to the incubated solution for 1 h at 37°C. The inhibition concentration of the sugar was recorded as the minimum concentration of sugar required for complete inhibition of 2 haemagglutinating units with 2% of the erythrocytes.

Temperature and pH stability: The lectin (50 µg) was incubated at different temperatures (4°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C and 90°C) for 10 minutes and was brought to room temperature. The ability of haemagglutination was tested to determine the temperature. The lectin (50 µg) was incubated at different pH (5, 6, 7, 8, 9 and 10) for 30 minutes and the haemagglutination activity was tested to determine optimum pH.

CD spectroscopy studies: Circular dichroism spectra were recorded to study the secondary confirmations of the lectin using JASCO spectrometer. The lectin was placed in the 2 mm Quartz cuvette with a concentration of 2 HU of protein and recorded spectra. Spectra measured for Far UV region (250-190 nm) and Near UV region (300-250 nm) with 50 nm per minute and accumulated 3 scans. The secondary structure was analyzed using Dichroweb online software. In addition, the effect of the temperature and pH on the unfolding of the lectin was also studied.

Binding Studies: *E. caducifolia* lectin is a galactose specific lectin. The ability of its affinity to the sugar was studied using UV, CD and Fluorescence spectroscopy. 2 mM of lactose was used for the study. Lectin (OD<0.1) was incubated with lactose and studied the binding ability by recording the spectra 300-250 for UV spectrum and 250 -190 for CD spectrum. And also, change in the intrinsic fluorescence of the lectin was studied with the addition of gradient concentrations of lactose (2mM- 6mM) to the lectin and change on the intrinsic fluorescence was recorded using JASCO spectrometer. The lectin was excited at 285 nm with fixed bandwidth 5 nm. Emission was recorded from 300-400 nm with same bandwidth up to 3 scan repetitions. The binding ability of the lectin was calculated using stern-volmer equation.

Biological activities

MTT Assay: RAW 264.7 was used for the experiment. The 1×10^4 cells were inoculated and incubated for 24 h in the CO_2 incubator. The lectin was added to the cells at different concentrations and incubated for different time points. After incubation, MTT, 5 mg/mL was added and incubated in the dark for 4 h. Crystallized formazan was extracted with DMSO and estimated at 590nm of Absorbance. Cell proliferation was calculated with control cells (without lectin) (Khan et al., 2006).

Mouse monocyte macrophages (J774a.1), Human cervical cancer cells (HeLa) and Human breast cancer cells (MCF-7) were used for the experiment. The cells (10,000) were inoculated in 96 well plates and incubated for 24 h in the CO_2 incubator. Then, the cells were treated with different concentrations protein and incubated for different time points. After time points, 3(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added as 5 mg/mL and incubated further for 4 h. 100 ul of DMSO was added to the wells and absorbance taken at 590nm.

Adherent capacity Assay, Cell Aggregation Assay: The cells ($1x10^4$/ well) were seeded along with protein (50 µg/ml) in 96 well plates and incubated for 24 h. After incubation, media was removed and tapped on a paper towel to remove loosely attached cells. The cells were stained with 0.1% crystal violet in phosphate buffer saline for 10 min. Further, the plates were washed with water and dried at room temperature. The well images were taken by the microscope and counted the cells aggregations by using the ImageJ software. The adherent capacity was determined by crystal violet quantification at 595 nm.

Cell migration Assay: Monolayer cells of HeLa cells were grown to confluence in a 12 well plate. A scratch was created by using PCR micro tip in the middle of the monolayer well. Detached cells were removed by washing with incomplete media twice. Then the cells were treated with EC lectin with 50, 100 and 200 µg /ml and untreated was considered as control. The plate was incubated and pictures of the gap area were taken at different time points to observe the migration of the cells towards the gap.

Results and Discussions

Purification of Galactose Binding Lectin: Lectins are glycoproteins with carbohydrate moieties and have the ability to agglutinate the blood. Lectins show specificity towards various sugars. Galactose-binding lectins are specific to the galactose and their derivatives as the name says. Galactose-binding lectins can be purified by affinity chromatography using galactose-specific matrices. The presence of galactose-specific lectins in *Euphorbia caducifolia* and other *Euphorbia* species were reported by Seshagirirao and Prasad in 2001. Lectins from *Euphorbia caducifolia* latex was isolated by applying on galactose affinity matrices. Affinity matrices were prepared and evaluated with snake gourd lectin. Applied crude serum was eluted with 0.2 M Lactose and

collected the protein (Fig. 4.1). Two matrices were used to assess the yield of the protein. Purified protein with a yield of 60% was observed with CLGG matrix, and 82% of yield was noted with CLLSG (Table.4.1). CLLSG shows more affinity and gives high purification folds compared to the other matrices like Guar gum, cashew nut and egg matrices (Kottapalli Seshagirirao et al., 2005) and also with Sepharose 4B.

Table 4.1. The yield of galactose-binding protein from *Euphorbia caducifolia* latex on different affinity matrices.

Purification step	Protein (mg)	Specific Activity	Total Activity (titre X mg)	Yield (%)
Crude Protein	50	2560	128000	100
CLGG	4.5	17066	76797	60
CLLSG	6.2	17066	105809.2	82

Previously, it has been reported the isolation of ECL using Sepharose 4B column with a yield of (%) which is more as observed with CLGG and less with CLLSG. It shows that the use of CLLSG for isolation or purification of galactose binding lectin is more efficient than another column (). Further purified protein was run over SDS-PAGE to check the lectin purity (Fig. 4.1 (insert)), and lyophilized until the studies its biochemical properties.

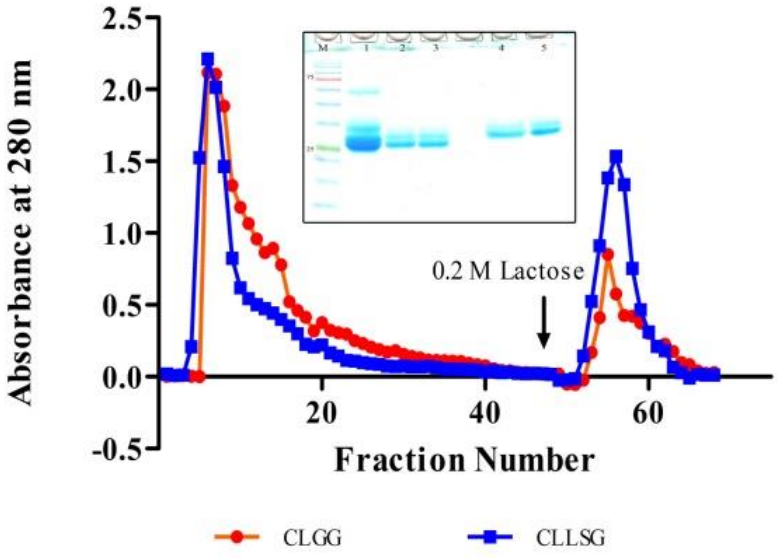


Fig. 4.1. Affinity Chromatography of *Euphorbia caducifolia* Latex serum on CLGG, CLLSG column (3X7 cms) at 4 ^{0}C. ; Insert SDS-PAGE of Purified Galactose-binding protein from *Euphorbia caducifolia* latex : M- Protein marker, 1- Crude protein, Purified protein 2-CLGG, 3- CLLSG, and purified protein with reducing agent β-Mercaptol4- CLGG, 5- CLLSG.

Haemagglutinating activity and carbohydrate specificity: The haemagglution activity of ECL was observed more with O compared to other blood groups (Table.4.2). ECL did not show much difference in agglutinating trypsinized red blood cells of Rh positive A, B and AB groups but agglutinated almost 6 folds with Rh O group. It indicates the specificity of the ECL towards the O type as other Euphorbia spp. (Lynn & Clevette-Radford, 1986b).

All lectin are carbohydrate specific and inhibition the agglutination of RBC in the presence of their specific sugar where binding sites of the lectin blocked. ECL was observed specificity with different carbohydrates (Table.4.3), galactose and galactose derivatives (Table.4.4). We noticed the delay in agglutination with melibiose and raffinose where the galactose in 1→6 glycosidic linkage but observed no agglutination with lactose where the glycosidic linkage is involved is the 1→4 type. Apart from the specificity of the lectin to the sugar, and also express its specificity with linkages of

specific monomer in oligosaccharides. O-Nitrophenyl galactoside also inhibits the agglutination as galactose majorly binds with lectin sites. Reports say that most of the latex lectin from Euphorbia are specific in galactose moiety either direct or its derivatives (Barbieri et al., 1983; Lynn & Clevette-Radford, 1986a).

Table 4.2. Haemagglutination of Lectin with trypsinized human Rh^+ Erythrocytes.

Erythrocyte	Specific Activity (U/mg)	
	Crude Protein	Purified Protein
A^+	400	2560
B^+	400	2560
AB	400	2560
O^+	2560	17066

Table 4.3. Sugar Inhibition Assay with different sugars and oligosaccharides.

S.No	Carbohydrate	Agglutination Activity	Galactose present (Yes/No)	Saccharide
1	Agar	Present	Yes	Polymer
2	Agarose	Present	Yes	Polymer
3	Arabinose	Present	No	Mono
4	Dulcitol	Present	Yes	Mono
5	Fructose	Present	No	Mono
6	Fucose	Present	Deoxy Galactose	Mono
7	Galactosamine	Absent	Yes	Mono
8	Galactose	Absent	Yes	Mono
9	Glucose	Present	No	Mono
10	Inositol	Present	No	Mono
11	Mannose	Present	No	Mono
12	N-Acetyl galactosamine	Absent	Yes	Mono
13	N-Acetyl glucosamine	Present	No	Mono
14	Rhamnose	Present	No	Mono
15	Salicin	Present	No	Mono
16	Xylose	Present	No	Mono
17	Lactose	Absent	Yes	Di
18	Melibiose	Present (Slow)	Yes	Di
19	Raffinose	Present (Slow)	Yes	Tri
20	O-Nitrophenyl galactopyranoside	Absent	Yes	Derv.

Table 4.4. Sugar Inhibition Assay with different Galactose derivatives and their MIC .

Carbohydrate	Agglutination	Galactose present (Yes/No)	Saccharide	MIC (mM)
Lactose	Absent	Yes	Di	3.13
Galactose	Absent	Yes	Mono	25
O-Nitrophenyl galactopyranoside	Absent	Yes	Derv.	25
Galactosamine	Absent	Yes	Mono	50
N-Acetyl galactosamine	Absent	Yes	Mono	50
Melibiose	Present (Slow)	Yes	Di	-
Raffinose	Present (Slow)	Yes	Tri	-

pH and temperature effect: Lectin isolated was observed for the influence of pH and temperature on activity (Fig. 4.2). ECL shows a broad range of activity in pH as well as temperature. Lectin activity notified at pH range 6.0-9.0 but showed maximum activity at pH 8.0. Maximum activity or association constants of the several lectins were found at neutral or slightly alkaline pH (Duk & Lisowska, 1984). Like most of the Euphorbiacae latex lectins, ECL also inactive above pH 10.0. Temperature plays a good role on activity as well as the stability of a protein apart from pH as physical conditions. *E. caducifolia* lectin is heat stable and shown maximum activity at 60°C and 70°C, and gradually activity ceased above 90°C.

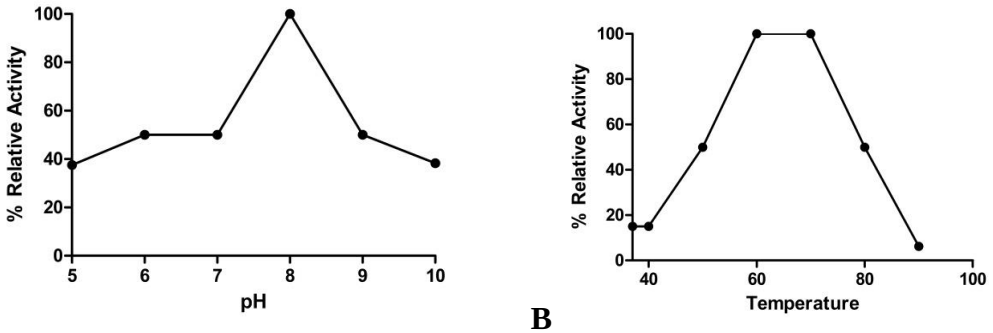


Fig. 4.2. Physicochemical parameters influence on Lectin haemagglutinating activity (A) pH, (B) Temperature

Glycoprotein characterization: Glycoprotein character of the lectin was identified by the Schiff periodic base on PAGE. The carbohydrate portion of the lectin was estimated to be 11.82% and this was almost nearer to the previous reports. Carbohydrate content of the lectin was very similar to the amount present in other species of the genus. Further studies revealed the presence of monosaccharides covalently bound to the protein (Table.4.5). Sugars present in the protein allude to structural conformations of protein as well as its biological importance.

Table 4.5. Sugar composition in the Lectin

Sugar	Residue/Molecule
Fuc	11.3
Gal	8.3
Glu	0.6
Man	8.9
GLcNac	12.7
Xyl	1.4
Total sugar (%)	11.4

CD Spectroscopy Studies: Structural confirmation of the lectin was studied by Circular Dichroism spectrometry. Spectra of the native lectin in the near UV and far UV was recorded at 25°C. Far UV spectrum observed with 2 positive peaks at 195 and 230 nm, and 2 negative peaks at 210 and 218 nm. Secondary structure information of the lectin was analyzed by software available at DICHROMEWEB. The secondary structural content obtained are 1% of regular α helix and 6% of disordered α helix (Total α helix of 7%), 21% of regular β sheet and 14% of disordered β sheet (Total β sheet of 35%), 24% β turns and 34% of unordered structures (Fig. 4.3). Overall CD spectrum of the lectin indicates that the predominant of β sheet protein to minor α helical structure of the protein. Influence of the temperature and pH on the secondary structure of ECL also studied using CD spectroscopy. As affecting the RBC agglutination, temperature, as well as pH, alters the structure of the lectin.

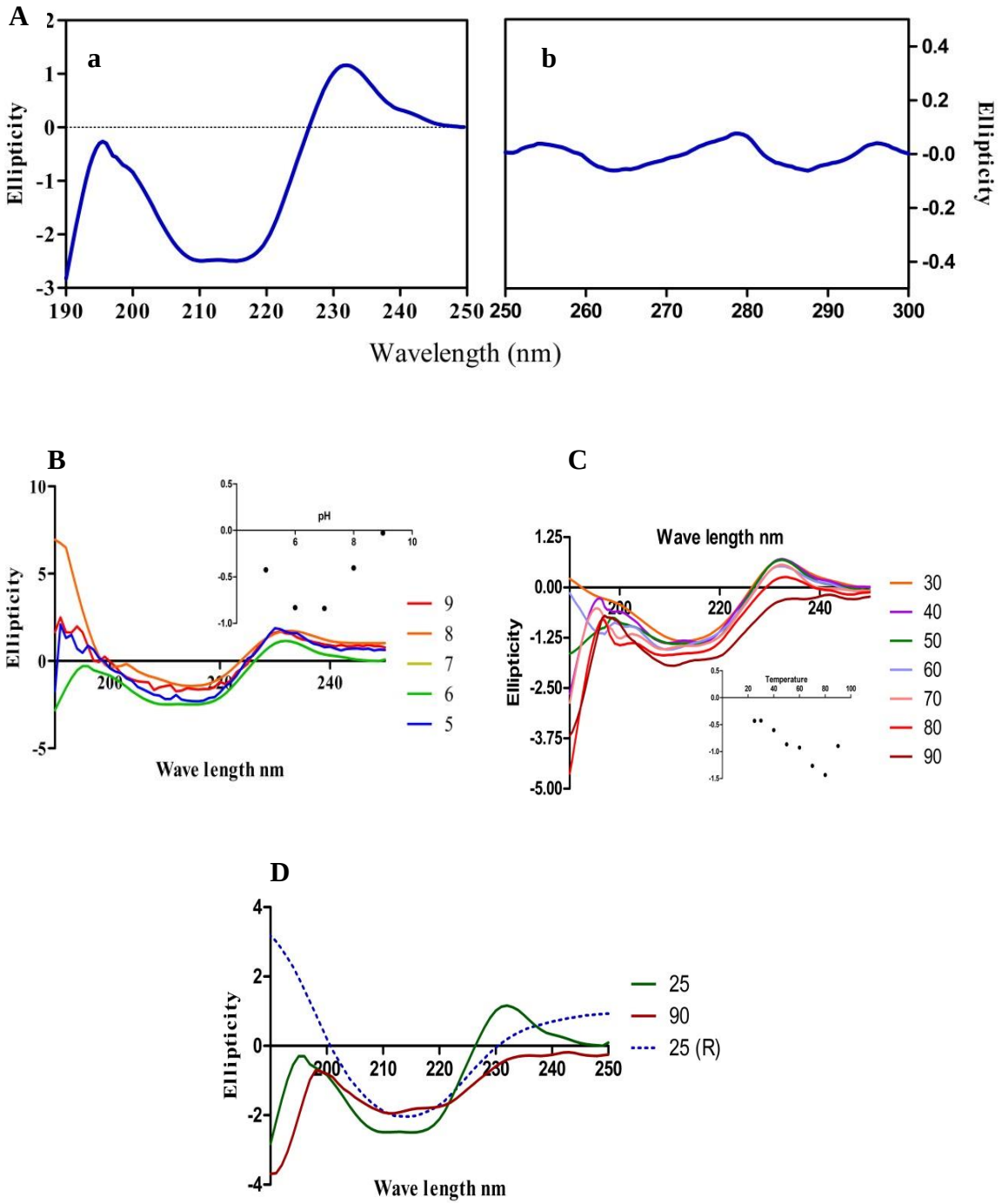


Fig. 4.3. A) Secondary confirmation of the lectin by CD spectrometry, a) Far UV, b) Near UV; Influence of B) pH and C) Temperature on structure, D) Spectrum of cooled lectin after incubated at 90°C.

Binding studies: Galactose specific lectin showed more affinity towards lactose than galactose. Thus, the binding affinity of lectin with lactose was studied with various spectrometers like UV, CD and Fluorescence. The decrease in the absorption of lectin

with gradient addition of lactose shows the binding efficacy of lectin (Fig. 4.4A). Change in the secondary structure of the lectin with the addition of the lactose reveals the influence on binding site of the lectin. Binding of lactose to lectin changed it confirmations to more β configuration than native form (Fig. 4.4B). Further, supports the binding efficacy of lectin by fluorescence quenching studies. The native form of tryptophan, phenylalanine, and tyrosine of the protein naturally emits fluorescence as it exited at 285 nm called intrinsic fluorescence. This change in the intrinsic fluorescence was observed with lactose (Fig. 4.4C). Quenching of intrinsic fluorescence was studied using the stern-volmer equation and found the stern volmer binding constant to be $1.17\times10^{-3}M^{-1}$ with 0.96 regression.

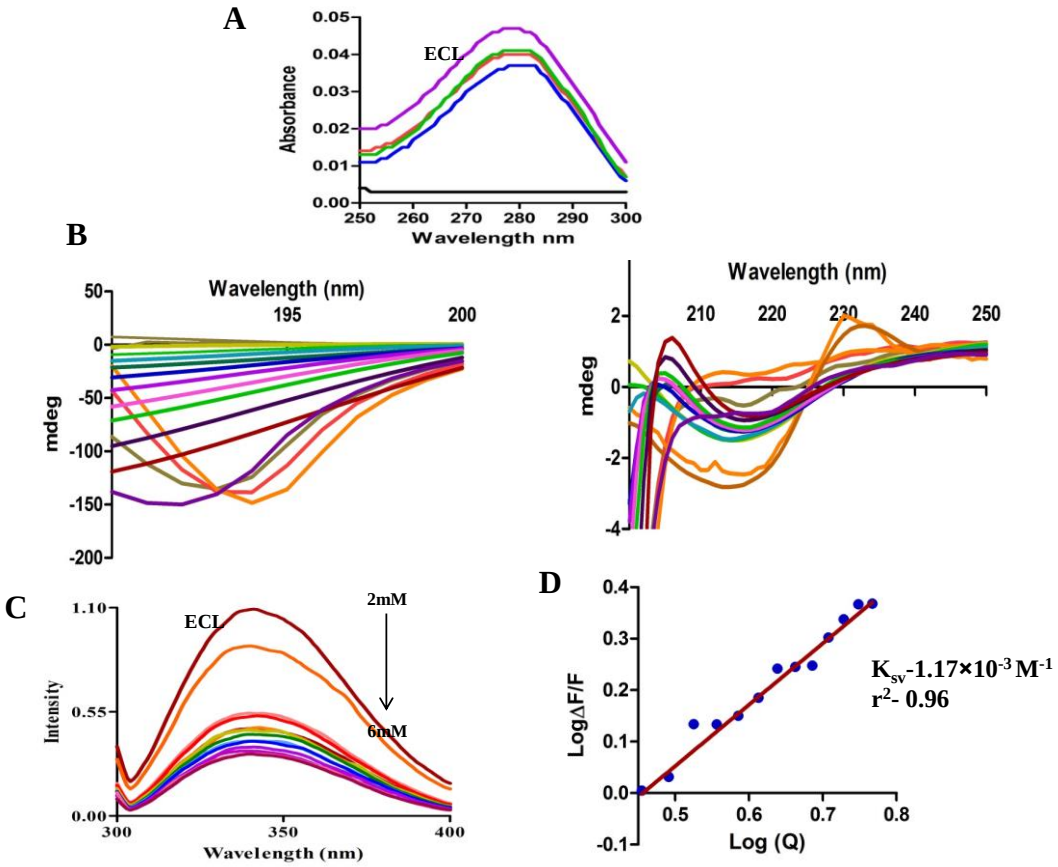


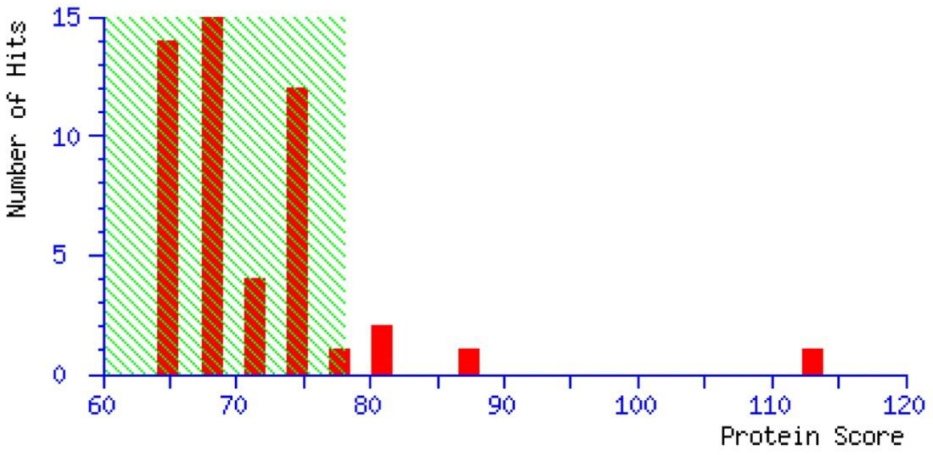
Fig. 4.4. Binding studies, A) UV spectrum, B) CD spectrum studies, C) Fluorescence quenching, D) Stern-volmer plot

Partial peptide sequencing: Purified lectin was subjected to peptide sequence to discover the fragments or domains of the protein. Peptides gained many attentions towards medical and biotechnology aspects, and over 7000 peptides have been identified as they play important roles in human physiology (Fosgerau & Hoffmann, 2015). Much more, peptide research on therapeutic experiencing a commercial renaissance. 3 Major and significant peptides were identified from a total of 8 obtained peptides from *Euphorbia caducifolia* lectin (Fig. 4.5). 3 peptides were sequenced for amino acids by MS/MS analysis and blasted the sequences for identification through NCBI protein database. Not many reports have been found on Euphorbia latex or seed lectin protein sequence or peptide sequence. Previously, only peptide sequence reported from E. characias and E. marginata lectin (Stirpe et al., 1993) and total protein sequence of a mannose-binding lectin from E. tirucalli has been reported recently (Kitajima et al., 2016). The peptide sequence of Euphorbia lectins was not identical and didn't show any conservative domains of the family (Fig. 4.5).

Hemolytic activity: Aside of agglutination, prolong incubation of RBC with latex serum exhibits the hemolytic activity. *Euphorbia caducifolia* lectin did not show any hemolytic activity with RBC used in haemagglutination assay. Some of the seed lectins like *Sterculia foetida L* and *Clitoria fairchildiana* were reported for their hemolytic activity as most of the lectins in particular latex lectin does not display such activity. Absence of haemolytic activity of the drugs or molecules imparts therapeutic attention as they does not harm the biological system. Lectins are using in various therapeutic application due to non-haemolytic nature as like ECL.

Cell proliferation and Anti-tumour activity: Lectins gained attention because of their proliferative activity on different cell types due to cell surface carbohydrate recognition variabilities (Ashraf & Khan, 2003). Euphorbia latex lectins can act as mitogens which

A



B

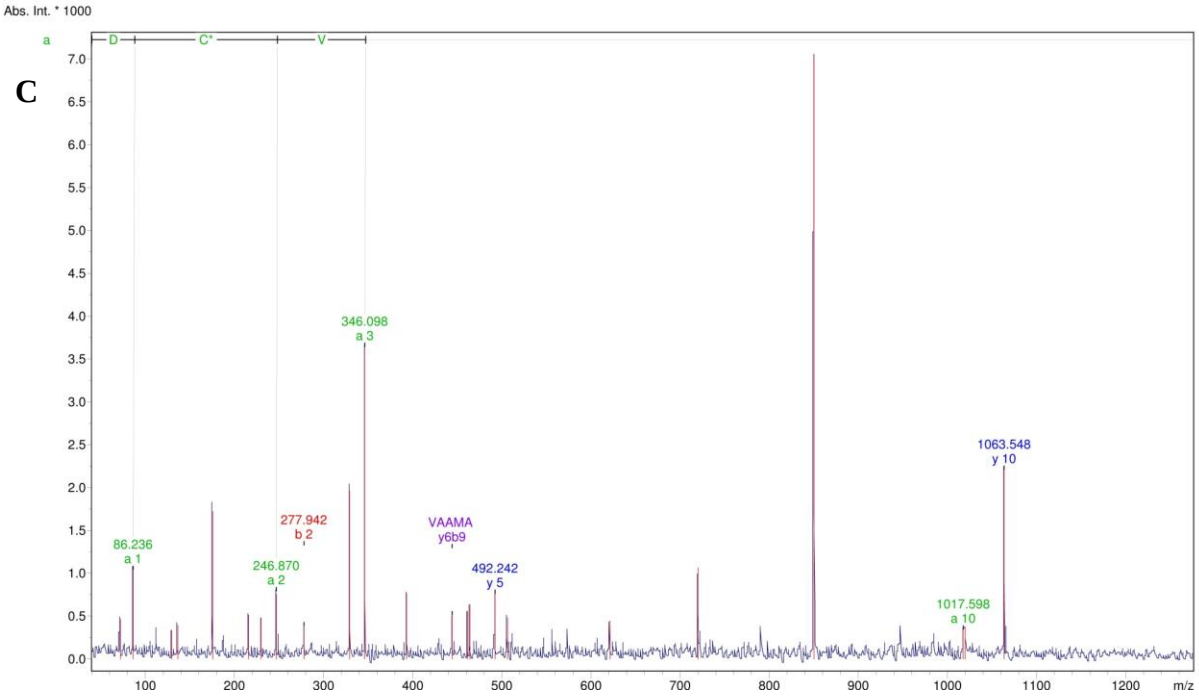
E. characias
E. marginata
E. tirucalli

```
  1 sesytpisgp ngyxvdvk
  1 aypgshisgp ngfxmdvk
  1 mhkpiilsls lffsaisfia ealvppsetf qyvnagdfgd yiveyeanyr vldpfaqpfq
 61 lcfynttpne ytlalrmgtv rseslmrwvw eanrakpvre natvtfsedg nlvladadgt
121 iawqtntsnk gvvgfkllpn gnmvlydsng gfvwqsfdyp tdtllvgqsl klgaatklvs
181 rasreenvng pyslemeett lslyykspns pnpliyfsfr dllsvsegpl kfvtlgpels
241 leydrgtlil rkpkynttlt ylrleidgnv rlhtyednad wsawevtytl fdrnswetec
301 qlpqrcgnfg lceddqcvac pspegllgws ksceaekvss cgvndfryye lkgvdhfssk
361 ysdgegpmki ddcgnkcted ckclgyfyht ksstcwivyd lntltkvdns thlafikapi
421 kqvlsdi
```

E. caducifolia

```
    1dcvvvaamak
    eaptwrledp aghgvnmhr
    iianaldvnm glnvlglrhr
```

C



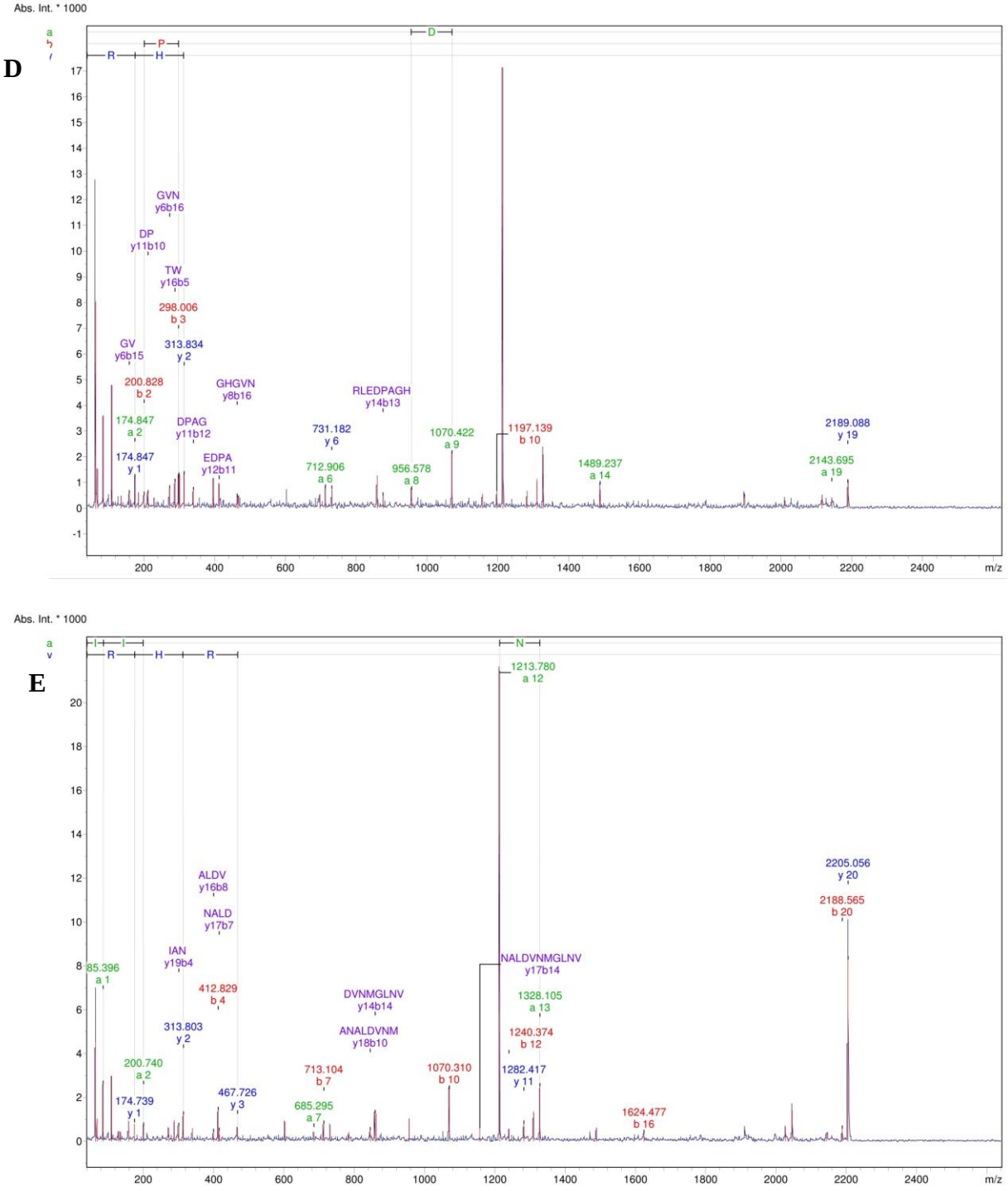


Fig. 4.5. Partial peptide sequencing of Lectin, A) Protein score histogram (78*), B) Euphorbia lectins peptide sequences; Peptide mass spectrum of masses C) 1063, D) 2189, E) 2205.

* Protein score greater than 78 are significant ($p<0.5$)

induces the mitosis of the cells. Latex lectins from *E. nerifiolia* and *E.marginata* were reported for their mitogenic activity (Stirpe et al., 1993). In similar *E. caducifolia* also exhibited a mitogenic activity which was studies by cell proliferation. Mitogens induce the mitotic division of the cell which ultimately leads to the cell proliferation. *E. caducifolia* lectin treated cells were showed an increase in the cell number at all time points and at all concentrations (Fig. 4.6). Cell number increased with increasing concentration of the lectin and this might be due to the mitogen activity of lectin. Maximum activity showed at 3 and 24 h of incubation but it slightly decreased at 100 µg/ml concentration in relative to the other time points after 24 h of treatment. Proliferation ranged in between 0-50% more as compared to the untreated cells (control) and it considered as ECL has the ability to act as a Mitogen. The diverse role of lectins in cell proliferation and differentiation is still undelivered.

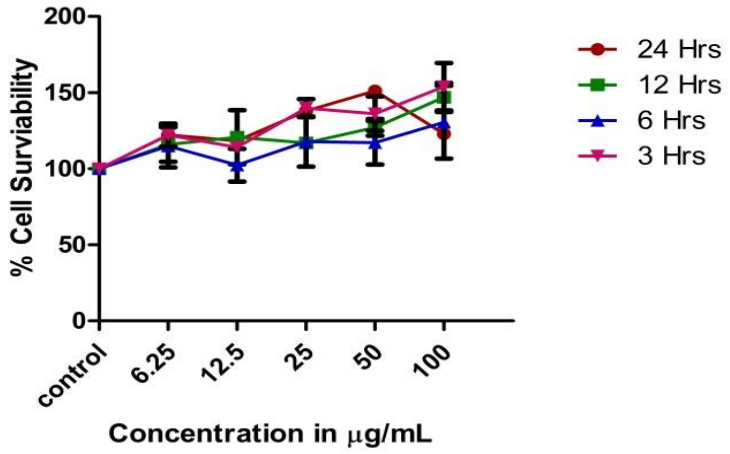


Fig. 4.6. Cell Proliferation assay on RAW 264.7 cell lines at different time points

Various chemicals and metabolites established their role in inhibiting tumour growth and its metastasis. Likewise, many lectins were reported as anti-tumour agents in treating different cancers. They are playing an important role in inhibiting tumour growth and metastasis by involving or influencing the autophagy and apoptosis (Yau et al., 2015).

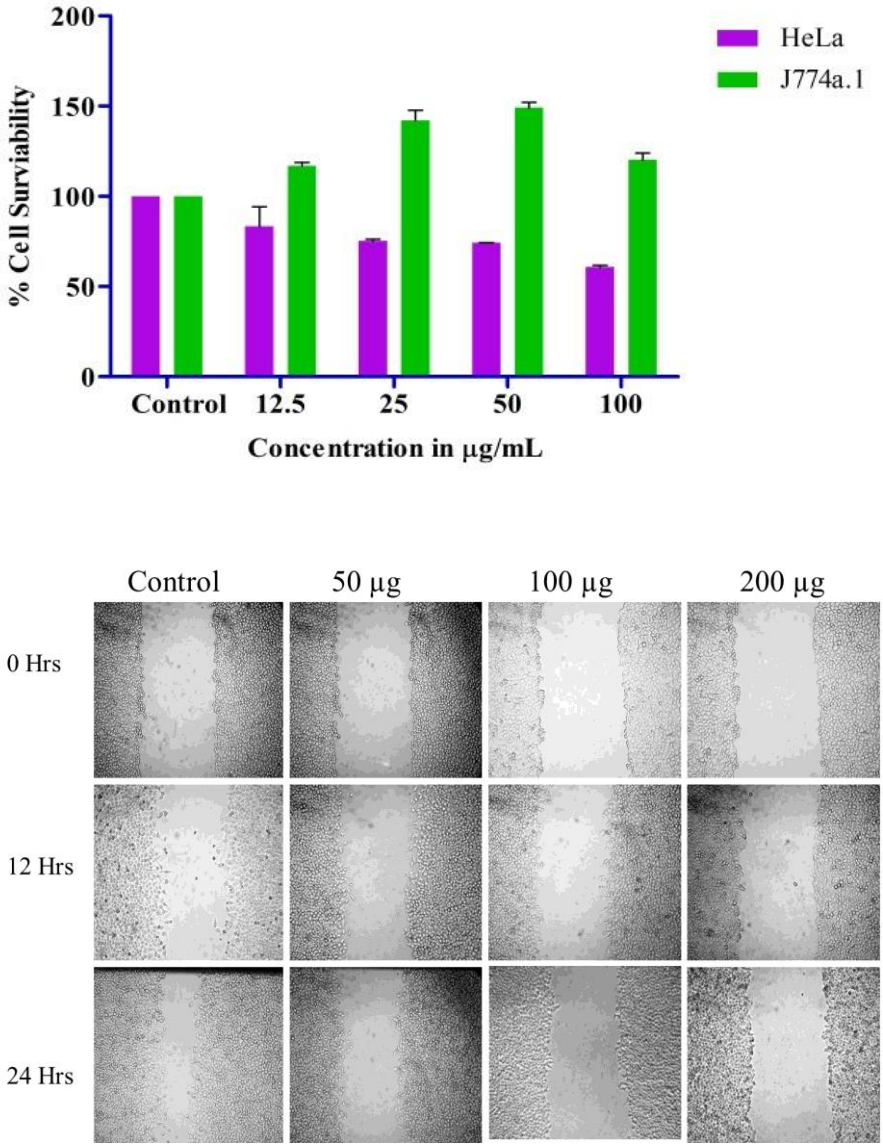


Fig. 4.7. Anti-tumour activity on Hela cells after 24 h of treatment and Inhibition of HeLa cells migration

Eutirucallin, a latex lectin from *Euphorbia tirucalli* exhibited potential anti-tumour activity against various cancer cell lines (Santiago et al., 2017). ECL also exhibited anti-tumour activity against HeLa cell lines and inhibited cell migration (Fig. 4.7). Cell proliferation was observed with non-tumour cells (J774A.1) whereas cell death was noticed in tumour cells (HeLa). Anti-tumour activity was observed more as increasing the concentration where the inverse effect was shown with non-tumour cells.

The ability of cell migration was also ceased by lectin with all concentrations and exhibited maximum anti-tumour activity at 200 µg/ml. These results promise the anti-tumour activity of the lectin from *E. caducifolia* latex.

Adherent capacity Assay, Cell Aggregation Assay: Lectin shows an affinity towards carbohydrates. Thus agglutinate the RBC by recognizing surface carbohydrates. In the similar ability of lectin aggregation was evaluated with different cell lines (Fig. 4.8). More percentage of aggregation was observed with J774a.1 and MCF-7 cells in comparison to HeLa cells. This might be due to over expression of mucin in J774a.1 and MCF-7. In addition adherent capacity of the cells was also seen similar in both J774a.1 and MCF-7. But it shows more adherent capacity with HeLa cells 2 fold with control.

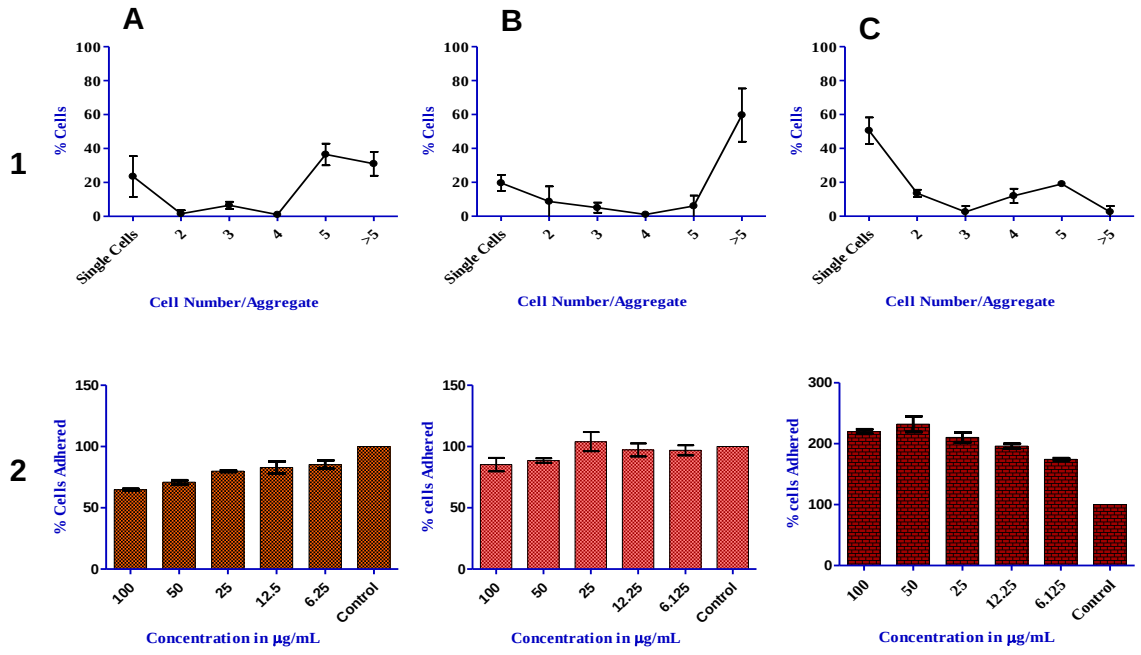


Fig. 4.8. 1) Cell Aggregation Capacity and 2) Adherent capacity of lectin on A) J774a.1, B) MCF 7, C) Hela cells

Conclusions

E. caducifolia lectin is Galactose specific lectin with mitogenic nature and anti-tumor activity. Lectin is predominant in β conformation and active at pH 6-8 and 60-70°C.

CHAPTER V

ISOLATION
&
CHARACTERIZATION OF STARCH GRANULES FROM LATEX OF *E.caducifolia*

Starch is a major carbohydrate reserve in plants. Starch is stored in tubers and seeds of the plants as granules. It is made up of amylose and branched amylopectin. The granules are water insoluble, dense and quasicrystalline in nature. Physical parameters of the starch depend on the contents of amylose and amylopectin present. Granule structure of the starch under genetic control, therefore the starch granules from different parts of the same plant may differ in their structure. Czaja has reported structure and classification of the starch granules in vascular plants in 1969 and 1978 (Czaja, 1969, 1978). Characteristic shapes of Euphorbiaceae latex starch granules were reported by Rafn (1798) , Groom (1889), Molisch (1901), Solereder (1908), P. Mahlberg (1973 & 1975), Seshagirirao and Prasad (1988).

Materials and Methods

Isolation and Purification: The latex of *Euphorbia caducifolia* was collected and frozen in liquid nitrogen, frozen latex were thawed and centrifuged at 20,000 x g for 1 hour at 4°C. The supernatant was removed and sediments were dissolved in hexane to remove the fatty acids, oils and etc. The sediments remained were dried at room temperature. The

dried powder was washed with citrate buffer pH 6.0 for 2-3 times and suspended in water. From the suspension, the starch grains were isolated by centrifugation in 2M sucrose at 2000 X g for 30 min. the sediment starch grains were washed with 0.2 M phosphate buffer pH 7.2 and distilled water for 3 times (K. Seshagirirao & Prasad, 1988). The prepared starch grains were used for microscopic studies and Physiochemical properties.

Microscopic studies: Latex starch grains were studies with a light microscope. Tincture iodine was used as a stain. Scanning Electron Microscope studies were done by transferring the grains to metallic stubs, dried in a desiccator. The starch grains were coated with Palladium-gold (Pd/Au) and observed under SEM at 15-20 kV(P. Mahlberg, 1973) . The confocal laser scanning microscope was used to analyze structural architecture. A Leica 60x/1.4NA/oil immersion was used to study the grain structure. Ar^{+} laser was used as the excitation source and by using a set of PMTs, photoluminescence images were recorded (Manca et al., 2015). 2% Starch grains were prepared in distilled water containing 0.02% sodium azide as a preservative. Further, observed thinly sectioned starch granules under Transmission Electronic Microscope (Mamoru, Keiji, & Dexter, 1979). For sectioning, Starch granules were prefixed in fixative composed with glutaraldehyde (3%) along with sucrose (0.1 M) in 50 mM sodium cacodylate buffer at pH 7.5 and incubated in osmium tetraoxide (2.5%) for 2.5 h. After the post fixation, granules were dehydrated with successive percentages of ethanol (50%, 70%, 80% and finally 100%). Dehydrated specimens were washed with acetone 3 times and embedded in resin and sectioned using the ultratome. Sectioned specimens were observed under TEM.

Composition

Blue value (BV) and amylose content: The blue value of the complex (Iodine-starch) was measured at 680 nm. In brief, starch was dissolved in acetate buffer pH 4.8 and

iodine and potassium iodide added to it and measured the blue value (BV) (Takeda, Takeda, & Hizukuri, 1986).

Infra Red spectroscopy analysis: The starch granules for analyzed for the functional groups referred to the standard potato starch. FT-IR analysis was performed by using Platinum –Alpha, a single reflection Attenuated Total Reflection(ATR) module. The spectrum of the starch granules was measured from 4000 cm^{-1} to 500 cm^{-1}.

X-ray diffraction Analysis: Starch granules are semi-crystalline in nature. The crystalline behavior of the granules was studied using X-ray powder diffractometer. The diffractogram of the starch was measured in 2Θ between 5 and 40^0 with 1^0 /min scan rate. The crystallinity of the starch was calculated as follows,

$$DC\ (\%) = A_c/(A_c + A_a)$$

DC- Degree of crystallinity, A_c- Total area under crystalline peaks, A_a- Area under amorphous peaks.

Thermal and retrogradation properties: Differential scanning calorimeter (DSC) used to study the thermal characteristics of the starch. Gelatinization of the starch was investigated under the natural condition and different water proportions. The pan was sealed after addition of water and equilibrated at room temperature for 2 hrs. The experiment was performed from 30 to 120°C with heating rate 5°C/min and the empty sealed pan was used as a reference. After cooled down, the pan was kept at 4 °C and -20°C for 1 week and rescanned for retrogradation properties. The retrogradation percentage was calculated by:

$$R\% = \Delta H_r/\Delta H_g \times 100$$

Solubility, Swelling Index and Turbidity: Starch granules were analyzed for its solubility in water along with swelling index according to a method of Li & Yeh (2001). The starch granules (db) was added to the water I a screw-capped tube and vortexed for 10 s. Incubated for 30 min at temperatures 50, 60, 70, 80 and 90°C with additional mixing at 2 min interval. Then, cooled to room temperature and centrifuged for 30 min at 2000 g. The cloudy layer and sediments were separated and dried to constant weight. The weight of supernatant (W_L) and sediments (W_S) was used to calculate the water solubility Index (WSI) and Swelling Index (SI) as follows (Kong et al., 2016):

$WSI = W_L/0.1 \text{ X } 100\%$

$SI = W_S/[0.1 \text{ X } (100\% - WSI)]$ (gg^{-1})

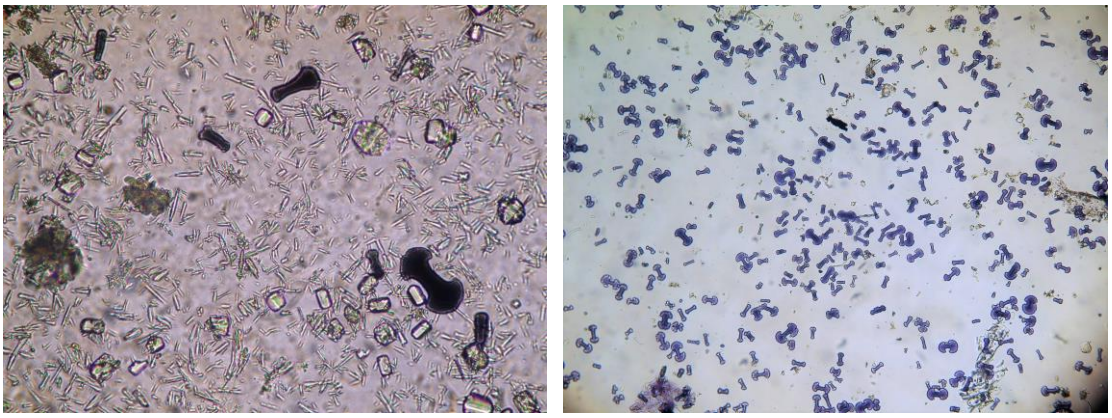
Turbidity was measured as mentioned by Lan et al. (2008). The water suspension of starch was boiled for 1 hrs with constant stirring and cooled to 30°C. The turbidity of the solution was determined with Chemito UV2100 spectrophotometer at 620 nm against blank (distilled water) (Reis, Cristiana, & Beirão-da-costa, 2012)

Lintnerization: The starch granules were subjected to mild acid hydrolysis with 2.5 N HCl for 5 days at 30°C and aliquots of the treated samples at different time points was diluted and centrifuged. The supernatants were analyzed for sugars released at various temperatures and time points (Bertoft, 2004).

Results and Discussions

Microscopic analysis: Starch granules from the Euphorbia latex is diverge in shape and structure in comparison to other starch grains like rice, maize, corn and other. The characteristic starch grains were isolated from latex and analyzed under various microscopes. Dumb bell shaped starch was noticed with a light microscope and

confirmed in iodine staining (Fig. 5.1). Scanning electron microscope revealed the structure with the size of 40-60 µm where maximum size of starch grains 110 µm with potato (Fig. 5.2). Similar shape starches have been reported from *Euphorbia cattimandoo, E. milli, E. nerifolia, E. tirucalli* and also in its family members like *Pedilanthus tithymaloides* and *Synadenium grantii* (K. Seshagirirao & Prasad, 1988). Apposition growth was reported in starch granules. At early stages, starch granules are round or ovoid but on mature they stretch to characteristic shapes like discoid, rod or dumb bell (French, 1984) (Fig. 5.3). Molecular orientation of the starch architecture gives the birefringent pattern with polarized light. Dark Maltese cross was reported with avocado starch in 1987 by Kahn (Kahn, 1987) and in potato starch (French, 1984).



A **B**

Fig. 5.1 Iodine starch granules from *Euphorbia caducifolia* latex
A) Starch granules in the latex along with calcium oxalate crystals, B) Purified starch

Confocal laser microscopy divulges the internal structure of the starch granules. Generally, starch granules are made up of amylose and amylopectin from the midpoint of the granule termed as “Hilum”. At lower levels of the structure, the alternate arrangement of amorphous and crystalline regions are made due to the higher level arrangement by clustering of side chains branching of radially arranged amylopectin. This appearance of the periodicity of starch granules is a universal feature and irrespective of botanical

sources(Daniel J Gallant, Bouchet, & Baldwin, 1997). The almost identical molecular architecture was noticed with *E. caducifolia* starch (Fig. 5.4). Alternative arrangements or growth rings formed were noted and the same was confirmed by TEM studies. TEM studies spotted the growth rings with 100-400 nm thick and smooth edges of starch (Fig. 5.5). It can observe central cavity of the starch granule which is universal.

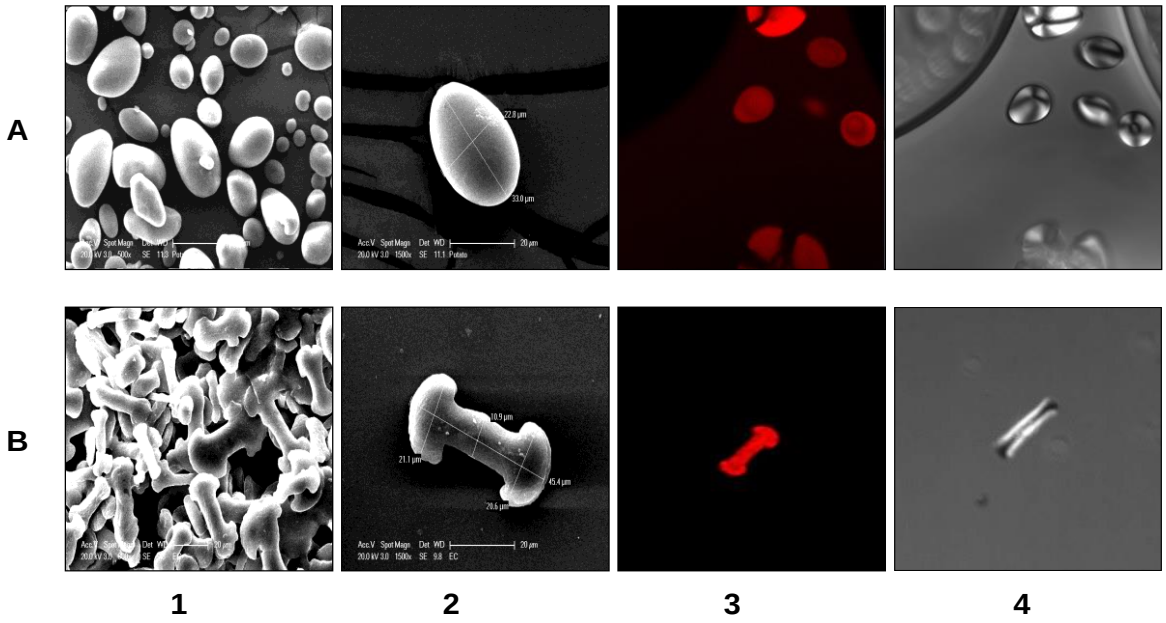


Fig. 5.2 Microscopic view of Starch granules.

A) Potato Starch, B) *Euphorbia caducifolia* Starch;
1,2-Scanning Electron microscope; 3, Confocal Laser microscopy, 4, Polarized light

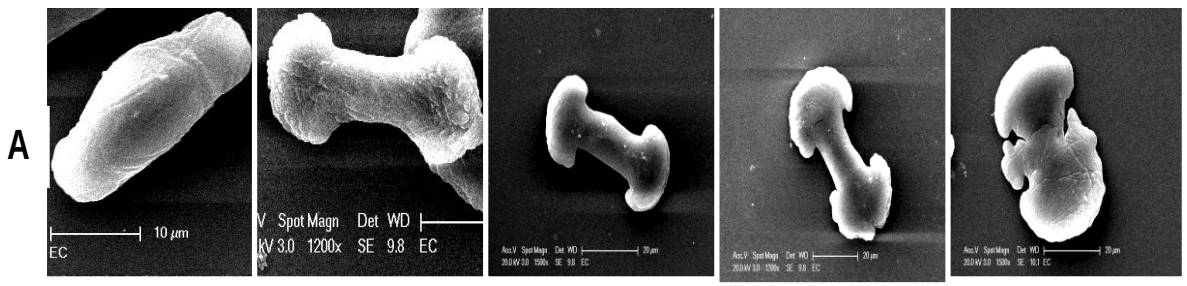


Fig. 5.3 Structural View of starch granules.

A) Growth process of starch granules (Left to Right) young to mature stages

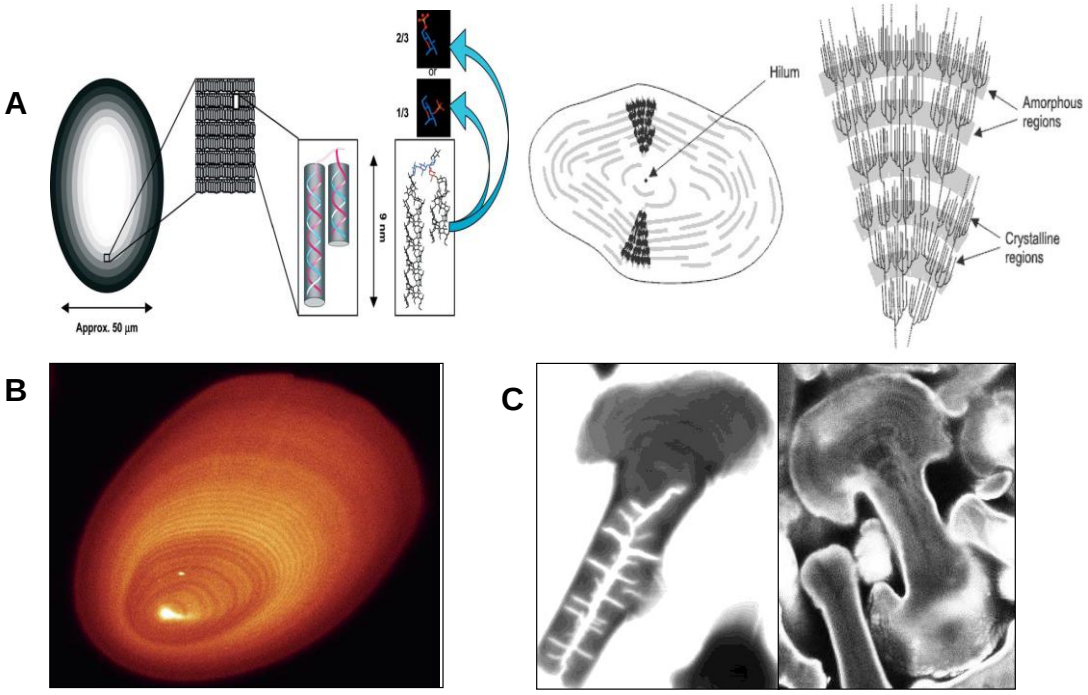


Fig. 5.4 Starch granule Architecture

A) Starch granule architecture model; B) Potato starch; C) *Euphorbia caducifolia* starch

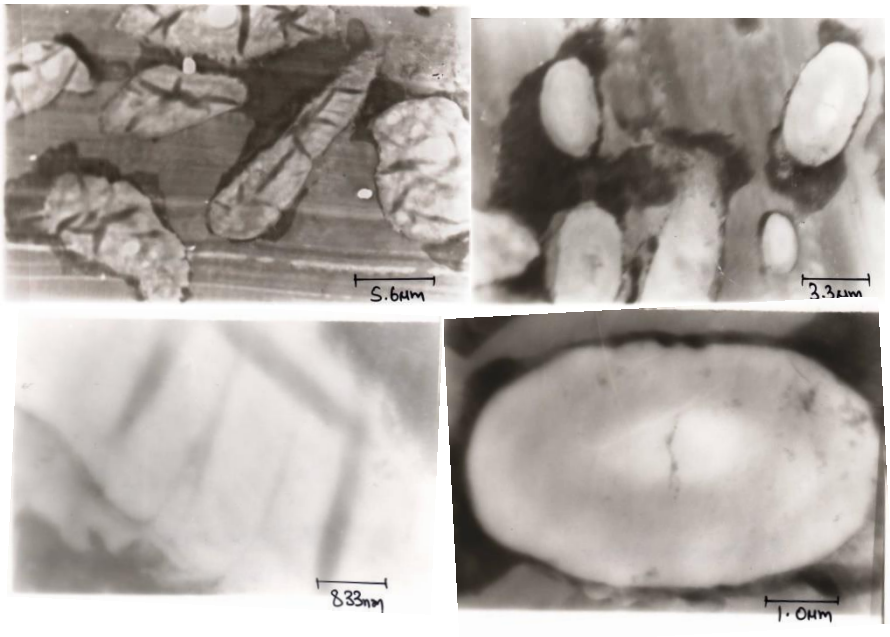


Fig. 5.5 Transmission Electron Microscopy of sectioned starch granule

Fragmentation: Fragmentation of the starch granules reveals the sub microscopic structure of the starch granule. Starch granules were fragmented with high impact pressure and High-speed trituration and observed its structure under scanning electron microscope. It observed that the radial arrangement of rod like micro fibrillary structure embedded in an amorphous matrix of starch granules. The appearance of fragmented starch granules is characteristic of the structure (Fig. 5.6). These observations were supported by previous studies of corn starch where similar arrangements of microfibrils in the amorphous region (Sterling & Spit, 1958).

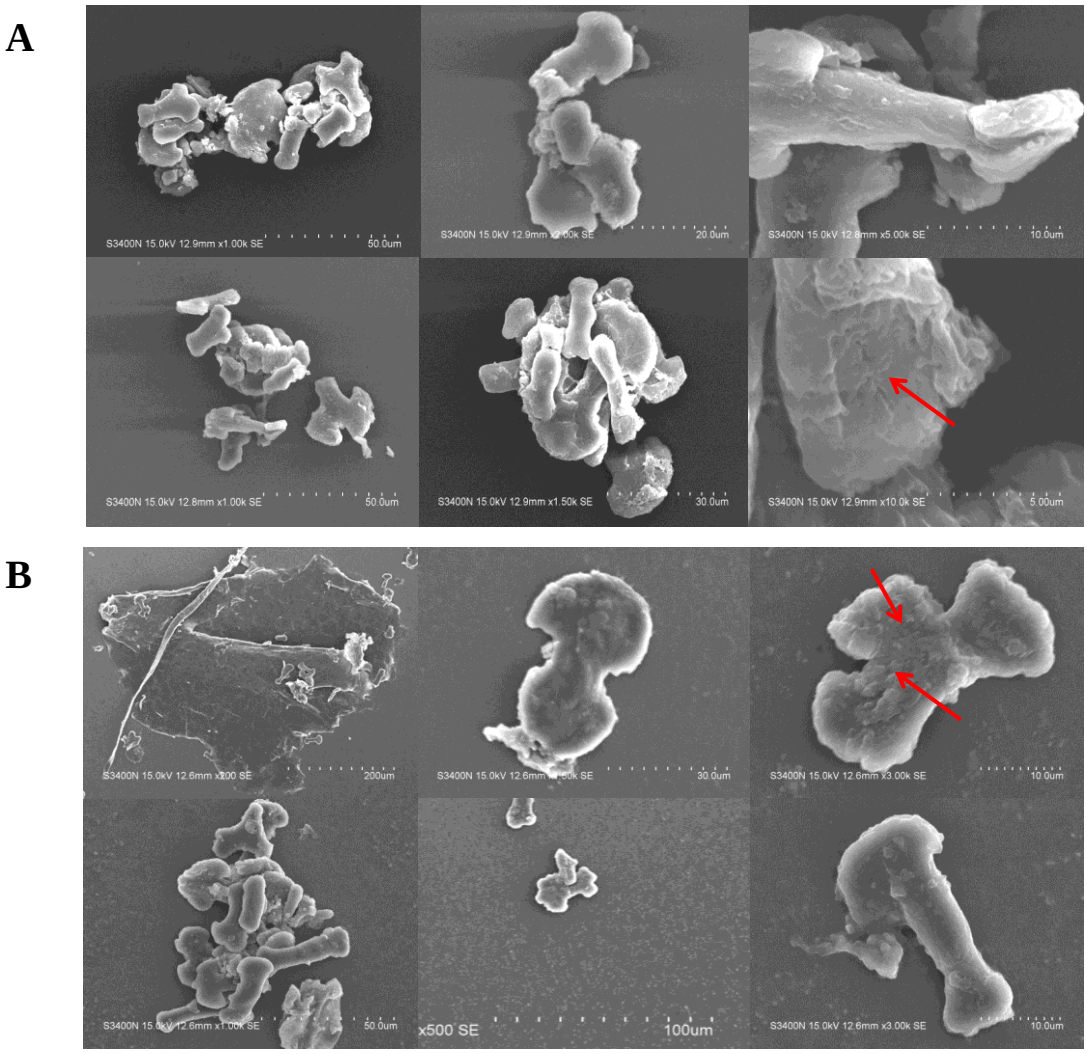
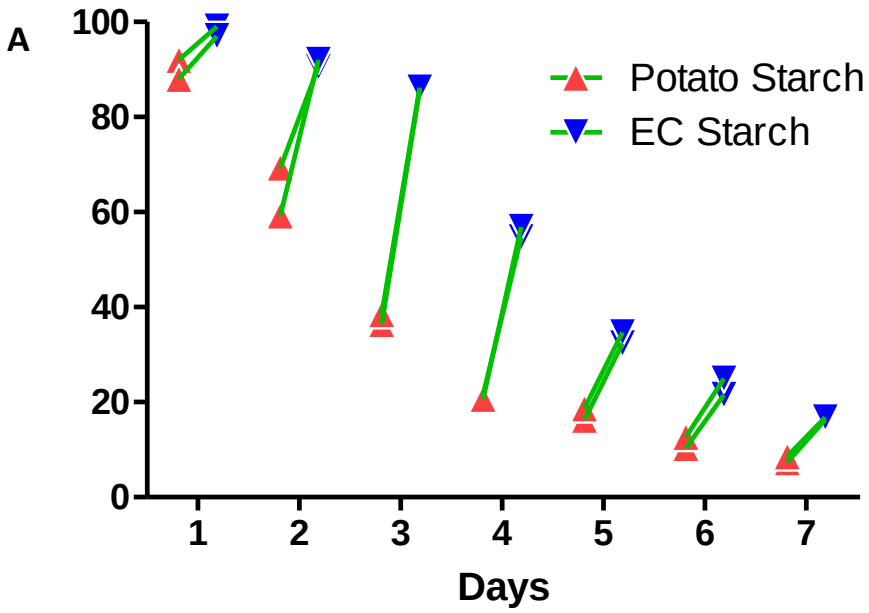


Fig. 5.6. Fragmentation of the starch granules under SEM

A) Fragmented by Rotation, B) Fragmented by High pressure

Lintnerization: Lintnerization is the process of acid treating starch. Acid catalyzes the starch was reported by Nageli in 1874 (Nageli, 1874). Acid digests the amorphous regions of the starch granules and left crystalline regions are called lintners.This has been first reported by Lintner in 1886 (Lintner, 1886). Lintnerization reduces the molecular weight of the starch . Lintnerization was observed with potato as well as with EC starch.The rate of lintnerization is high for the EC starch in comparison to potato starch. EC starch lintners are constant after 10 days with 20% of lintners where as potato starch shown after 7 days with 5 %. Later, the lintners at every stage were noted under Scanning Electron Microscope (Fig. 5.7). Effect of lintnerization on various starch granules shows the complexity in the starches (Jayakody & Hoover, 2002).



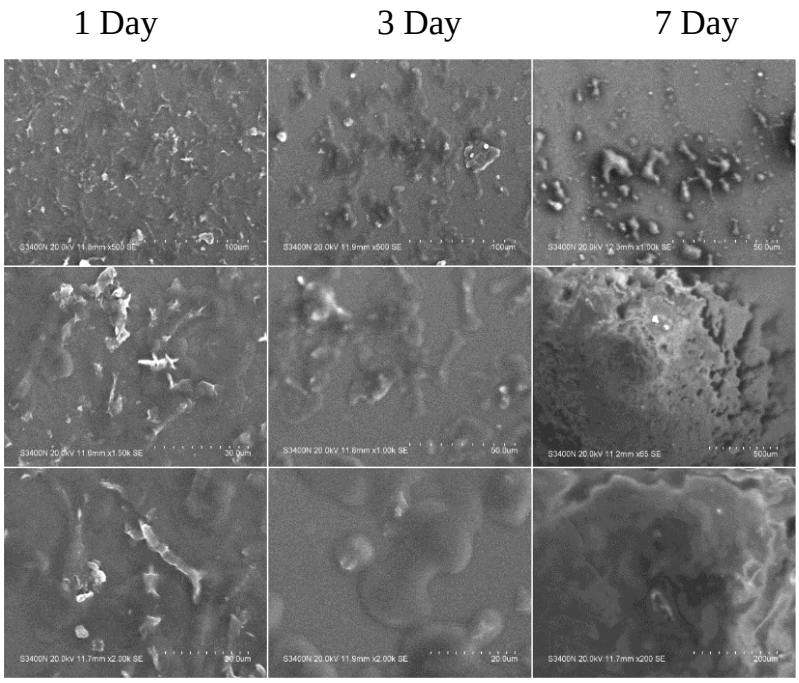


Fig .5.7 Lintnerization of starch

A) Percentage of lintners, B) Lintner images on SEM

Physicochemical properties and Amylose contents: Amylose contents were found to be 45% where normal starch grains from rice, wheat and maize were found to be 20-33%. This implies the high content of the amylose in EC starch. This high content of the amylose was supported by high blue values in comparison to potato (Fig. 5.8). Overall indicates the presence of high amylose content to the potato. Iodine forms complex with amylose of the starch granules give a blue colour to the molecule it can also form red to purple colour complex with amylopectin (Colin & Claubry, 1814; Foster et al., 1943; Stromeyer, 1815). These results suggest the structures possessing intermediate between amylopectin and amylose.

Physicochemical properties like solubility, swelling index and turbidity were analyzed in comparison to reference potato starch (Fig.5.9). The swelling index decreased and not completely solubilized even after 100°C with EC starch whereas potato starch

completely solubilized above 80°C. This results in the complexity of the molecular structure of EC starch granule. The starch granules with high amylose contents swell to a low degree since starch granules are more rigid and resistant to digestibility (Sandhya Rani & Bhattacharaya, 1989). EC starch shows lower turbidity value than potato starch. Lower turbidity due to reluctant nature settled starch granules (Lan et al., 2008).

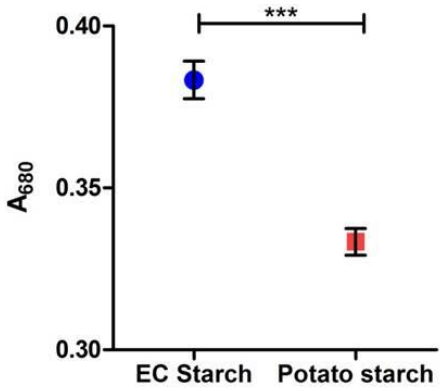


Fig. 5.8. Blue value of Iodine starch complex

Crystallinity and functional group analysis: Starch granules isolated were analyzed for crystallinity and variations in the functional group present. Crystal structure released the presence of the intermediate composition of Amylose and amylopectin. EC starch shows the C-type crystallinity whereas potato with A-type. Starch have 15-45% of crystallinity varies botanical sources. Thus, starch granules displays “Maltese cross” under polarized light (D J Gallant et al., 1992; Zobel, 1988). It has been known that starch granules from the EC and other Euphorbiaceae are devoid of “Maltese cross” (Spilatro & Mehlberg, 1985). Therefore, the crystallinity of the starch granules exhibits intermediate i.e C-type crystallinity. XRD patterns suggest the C-type crystallinity of the EC starch (Fig. 5.10) with peaks at 13°, 15°, 23° and 25°. Infra-Red spectra of EC starch and potato starch are look similar which suggest the absence of impurities (Fig. 5.10).

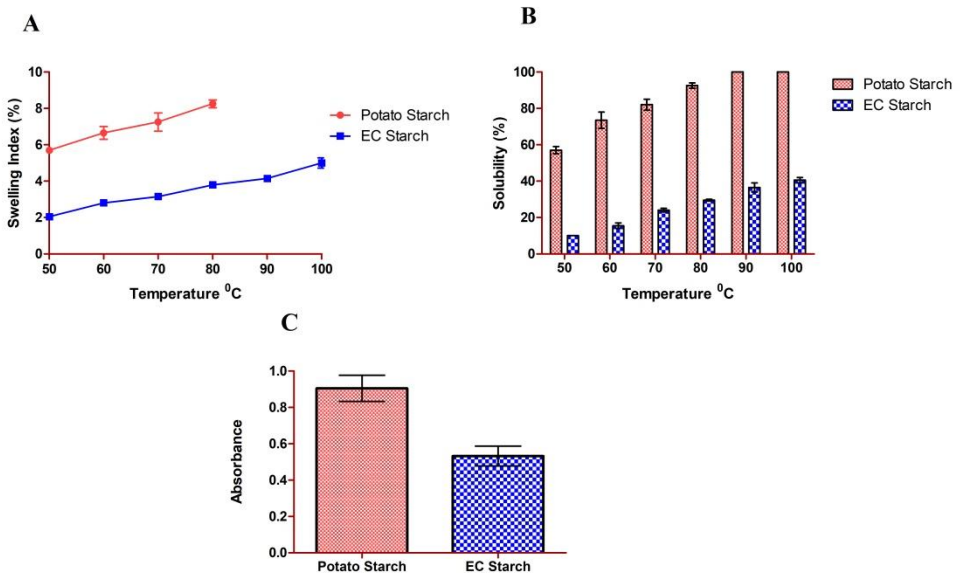


Fig. 5.9 Physicochemical properties of starches
A) Swelling index, B) Solubility, C) Turbidity

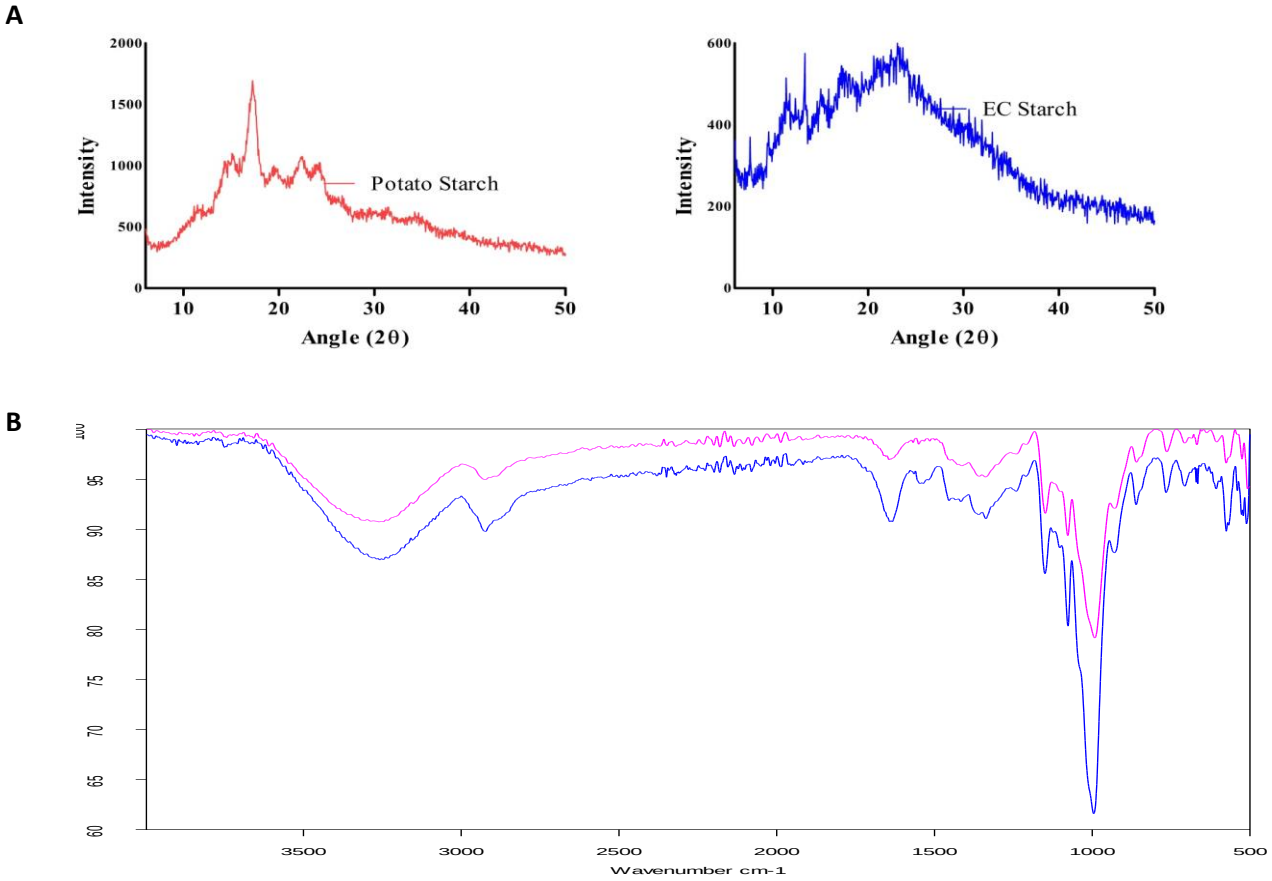


Fig. 5.10. A) X-ray diffractions of potato and EC starch;
B) IR spectrum of the starches potato (pink), EC starch (blue)

Thermal and retrogradation properties: Differential Scanning Calorimetry was used to study the gelatinization properties of starch granules (Table 5.1). When a molecule heated, thus results in the loss of weight and absorption or release of the energy occurs as a result of phase transition. Starch granules with 5% water and 5% NaCl result in the endothermic peaks at 58.89°C and 61.22°C respectively. Change in the enthalpy was 24 and 21 J. Gelatinization temperatures and change in the enthalpy closely resembles the corn starch (Jane et al., 1999). The apparent amylose content of starch correlated to onset and end set of temperatures. High amylose content lowers the onset temperature. EC starch granules do not exhibit any retrogradation properties with 5% water. Realignment of heat disrupted amylose and amylopectin called retrogradation (Wang et al., 2015).

Table 5.1 Thermal properties of EC starch

Sample	Thermal			
	Onset	End set	Peak	ΔH
EC Starch	**0**	**0**	**0**	**0**
EC Starch + 5% Water	**50.47**	**69.41**	**58.89**	**24.176**
EC Starch + 5% NaCl	**52.77**	**70.54**	**61.22**	**21.593**

Conclusions

Starch granules from *E. caducifolia* latex are flat dumb bell shape. Laser Confocal microscope revealed the birefractive nature and growth rings of the starch granules as like potato starch. Granules grow from round or ovoid (young) to full mature dumb bell or discoid shapes. Blue values of the starch disclose the high contents (45%) of amylose with C-type crystallinity. High amylose presence decreased the solubility, swelling ability, turbidity, and lintnerization. Typical starch spectrum was identified with IR spectra with devoid of retrogradation properties.

Summary

Euphorbia caducifolia Haines, a folk medicinal plant was selected to evaluate potentiality of the plant scientifically. Established pharmacognosy of the plant with phytochemical studies including traditional morphological characters. *Euphorbia caducifolia* is an arid semi-arid plant with paracytic stomata, vein islets- 5-7, vein termination-7-10. Phytochemical screening reveals the presence of acidic polysaccharides, terpenoids, phenolics, and flavonoids. Phylloclade extract can be exhibited strong free radical scavenging activity and latex of the plant cause irritation due to terpenoids and Haemolytic in nature.

An acidic polysaccharide was isolated and found to be brown yellow colour. ECP exhibited the free radical-scavenging activity (DPPH), Nitric oxide scavenging activity and Reducing power as antioxidant agent and also act as Emulsifying agent for pharmaceutical purpose. I*nVitro* studies of the Polysaccharide on cell line RAW 267.4, showed the effective production of inflammatory cytokines IL-6 and TNF-α, and cytotoxicity assay showed the significant Cell proliferation. Production of Reactive Oxygen Species by ECP may be responsible for the cell proliferation by inducing the inflammatory cytokines (IL-6 & TNF-α).

Euphorbia caducifolia latex contains Galactose binding lectin which was isolated using two matrices, where CLLSG matrix recovered more lectin than common CLGG. Lectin is thermally stable and active at pH 6-8, and predominant in β structure. *Euphorbia caducifolia* lectins are mitogenic and show Anti-tumour activity.

Euphorbia caducifolia latex contains Flat dumbbell shaped starch granules. Starch granules showed Birefringence with polarized light as a characteristic feature of the starch. EC starches are intermediate of amylose and amylopectin contents. Thus results in C-type crystallinity in XRD patterns. *Euphorbia caducifolia* starch granules contain rodlike microfibrils arranged in radial orientation and do not exhibit any retrogradation properties.

This study suggests that *Euphorbia caducifolia* is proven to be a medicinally potential plant with strong antioxidant extracts, immunomodulatory polysaccharides, mitogenic and antitumor lectin, and novel structured starch granules for the preparation of biocomposites.

REFERENCES

Afza, N., Khan, A. Q., Malik, A., & Badar, Y. (1989). Cyclocaducinol, a cycloartane type triterpene from Euphorbia caducifolia. *PhytochePmistry*, *28*(7), 1982–1984.

Aksoy, L., Kolay, E., Aĝilönü, Y., Aslan, Z., & Kargioĝlu, M. (2013). Free radical scavenging activity, total phenolic content, total antioxidant status, and total oxidant status of endemic Thermopsis turcica. *Saudi Journal of Biological Sciences*, *20*(3), 235–239.

Alam, M. N., Bristi, N. J., Rafiquzzaman, M., Alam, N., & Bristi, N. J. (2013). Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal*, *21*(2), 143–152.

Annapurna, J., Chowdary, I. P., Lalitha, G., Ramakrishna, S. V., & Iyengar, D. S. (2004). Antimicrobial Activity of *Euphorbia nivulia* Leaf Extract. *Pharmaceutical Biology*, *42*(2), 91–93.

Ashraf, M. T., & Khan, R. H. (2003). Mitogenic lectins. *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research*, *9*(11), RA265-9.

Baloch, I. B., & Baloch, M. K. (2010). Irritant and co-carcinogenic diterpene esters from the latex of Euphorbia cauducifolia L . *Journal of Asian Natural Products Research*, *12*(7), 600–613.

Baloch, B., Kaleem, M., & Najam, Q. (2005). Tumor-Promoting Diterpene Esters from Latex of Euphorbia cauducifolia L . *Helvetica Chimica Acta*, *88*, 3145–3150.

Bano, S., Siddiqui, B. S., Farooq, A. D., Begum, S., Siddiqui, F., Kashif, M., & Azhar, M. (2017). *In vitro* growth inhibition and cytotoxicity of *Euphorbia caducifolia* against four human cancer cell lines and its phytochemical characterisation. *Natural Product Research*, (June), 1–5.

Barbieri, L., Falasca, A., Franceschi, C., Licastro, F., Rossi, C. A., & Stirpe, F. (1983). Purification and properties of two lectins from the latex of the euphorbiaceous plants Hura crepitans L. (sand-box tree) and Euphorbia characias L. (Mediterranean spurge). *The Biochemical Journal*, *215*(3), 433–9.

Basma, A. A., Zakaria, Z., Latha, L. Y., & Sasidharan, S. (2011). Antioxidant activity and phytochemical screening of the methanol extracts of Euphorbia hirta L. *Asian Pacific Journal of Tropical Medicine*, *4*(5), 386–390.

Bedoya, L. M., Márquez, N., Martínez, N., Gutiérrez-Eisman, S., Álvarez, A., Calzado, M. A., … Alcamí, J. (2009). SJ23B, a jatrophane diterpene activates classical PKCs and displays strong activity against HIV in vitro. *Biochemical Pharmacology*, *77*(6), 965–978.

Benhura, M. A. N., & Chidewe, C. K. (2004). The emulsifying properties of a polysaccharide isolated from the fruit of Cordia abyssinica. *International Journal of Food Science and Technology*, *39*(5), 579–583.

Bertoft, E. (2004). Lintnerization of Two Amylose-free Starches of A- and B-Crystalline Types , Respectively, *Starch*, *56*, 167–180.

Betancur-Galvis, L., Morales, G., Forero, J., & Roldan, J. (2002). Cytotoxic and Antiviral Activities of Colombian Medicinal Plant Extracts of the Euphorbia genus. *Memórias Do Instituto Oswaldo Cruz*, *97*(4), 541–546.

Bhattacharyya, S. K., Kar, S., & Chakraborty, S. (2013). Technical Analysis of Euphorbia caducifolia Haines Latex of South-East Asian Origin - Part I, *4*(3), 133–151

Bian, C., Xie, N., & Chen, F. (2010). Preparation of bioactive water-soluble pachyman hydrolyzed from sclerotial polysaccharides of Poria cocos by hydrolase. *Polymer Journal*, *42*(3), 256–260.

Bisht, A., Zaman, K., Singh, M., Gupta, R., & Singh, V. (2011). Pharmacognostical studies on Oroxylum indicum (Linn.)Vent. stem bark. *Indian Journal of Natural Products and Resources*, *2*(4), 472–478.

Boora, F., Chirisa, E., & Mukanganyama, S. (2014). Evaluation of Nitrite Radical Scavenging Properties of Selected Zimbabwean Plant Extracts and Their Phytoconstituents. *Journal of Food Processing*, *Article ID*, 1–7.

Butler, M. S. (2004). The Role of Natural Product Chemistry in Drug Discovery †. *Journal of Natural Products*, *67*(12), 2141–2153.

Cerqueira, M. A., Souza, B. W. S., Simões, J., Teixeira, J. A., Domingues, M. R. M.,

Coimbra, M. A., & Vicente, A. A. (2011). Structural and thermal characterization of galactomannans from non-conventional sources. *Carbohydrate Polymers*, *83*(1), 179–185.

Chaiklahan, R., Chirasuwan, N., Triratana, P., Loha, V., Tia, S., & Bunnag, B. (2013). Polysaccharide extraction from Spirulina sp. and its antioxidant capacity. *International Journal of Biological Macromolecules*, *58*, 73–78.

Chavre, B. . (2013). Botanicals used on the treatment of snakebite in some parts of Maharashtra. *Indian Journal of Plant Sciences*, *2*(1), 52–54.

Chika C. Ogueke, Jude N. Ogbulie, Ifeanyi C. Okoli, B. N. A. (2007). Antibacterial Activities And Toxicological Potentials Of Crude Ethanolic Extracts of Euphorbia hirta. *Journal of American Science*, *3*(3), 11–16.

Colin, J.-J., & Claubry, H.-F. G. d. (1814). Sur les combinaisons de l'iode avec les substances végétales et animales. *Annales de Chimie*, *90*, 87–100.

Cragg, G. M., & Newman, D. J. (2013). Natural products: A continuing source of novel drug leads. *Biochimica et Biophysica Acta - General Subjects*, *1830*(6), 3670–3695.

Czaja, A. T. (1969). Die Mikroskopie der Starkekorner, 16–17.

Czaja, A. T. (1978). Structure of Starch Grains and the Classification of Vascular Plant Families. *Taxon*, *27*(5), 463–470.

Damme, E. J. M., & Peumans, W. J. (1990). Isolectins in Narcissus: complexity, inter- and intraspecies differences and developmental control. *Physiologia Plantarum*, *79*(1), 1–6.

Damme, E. J. M. Van, Allen, A. K., & Peumans, W. J. (1988). Related mannose-specific lectins from different species of the family Amaryllidaceae. *Physiologia Plantarum*, *73*(1), 52–57.

De, J. L., Ruiz-bermejo, M., Menor-salván, C., & Osuna-esteban, S. (2011). Thermal characterization of HCN polymers by TG-MS , TG , DTA and DSC methods. *Polymer Degradation and Stability*, *96*(5), 943–948.

De Oliveira Dias, R., Dos Santos Machado, L., Migliolo, L., & Franco, O. L. (2015).

Insights into animal and plant lectins with antimicrobial activities. *Molecules*, *20*(1), 519–541.

Dev, S. (1999). Ancient-modern concordance in ayurvedic plants: Some examples. *Environmental Health Perspectives*, *107*(10), 783–789.

Devi, L., & Pamila, G. (2000). Evaluation of some plant latices against Heterodera cajani on cowpea (Vigna sinensis). *National Academy Science Letters*, *23*(5/6), 65–67.

Dias-Baruffi, M., Sakamoto, M., Rossetto, S., Vozári-Hampe, M. M., & Roque-Barreira, M. C. (2000). Neutrophil migration and aggregation induced by euphorbin, a lectin from the latex of Euphorbia milii, var. milii. *Inflammation Research*, *49*(12), 732–736.

DuBois, M., Gilles, K. a., Hamilton, J. K., Rebers, P. a., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356.

Duk, M., & Lisowska, E. (1984). Effect of pH on the binding of Ecia graminea lectin to erythrocytes Dependence on the chemical character of red-cell receptors, *78*, 73–78.

Falodun, A., Ali, S., Quadir, I. M., & Choudhary, I. M. I. (2008). Phytochemical and biological investigation of chloroform and ethylacetate fractions of Euphorbia heterophylla leaf (Euphorbiaceae). *Journal of Medicinal Plant Research*, *2*(12), 365-369.

Fonseca, K. C., Morais, N. C. G., Queiroz, M. R., Silva, M. C., Gomes, M. S., Costa, J. O., Oliveira, F. (2010). Purification and biochemical characterization of Eumiliin from Euphorbia milii var. hislopii latex. *Phytochemistry*, *71*(7), 708–715.

Fosgerau, K., & Hoffmann, T. (2015). Peptide therapeutics: Current status and future directions. *Drug Discovery Today*, *20*(1), 122–128.

Foster, J. F., Baldwin, R. R., Foster, J. F., & Baldwin, R. R. (1943). the Nature of the Starch-Iodine Complex', *66*(5), 2116–2120.

French, D. (1984). Organization of starch granule. In J. . BeMillel & C. . Paschall (Eds.), *Starch chemistry and technology* (pp. 183–247). New York: Academic press.

Gallant, D. J., Bouchet, B., & Baldwin, P. M. (1997). Microscopy of starch : evidence of a new level of granule organization, *8617*(97), 177–191.

Gallant, D. J., Bouchet, B., Buléon, A., & Pérez, S. (1992). Physical characteristics of starch granules and susceptibility to enzymatic degradation. *European Journal of Clinical Nutrition*, *46, Suppl 2*, S3-16.

Gietl, C., Kauss, H., & Ziegler, H. (1979). Affinity Chromatography of a Lectin from Robinia pseudoacacia L. and Demonstration of Lectins in Sieve-Tube Sap from Other Tree Species. *Planta*, *144*, 367–371.

GOLDSTEIN, I.J., HUGHES, R.C., MONSIGNY, M., OSAWA, T., & SHARON, N. (1980). What should be called a lectin? *Nature*, *285*(5760), 66–66.

Govardhan, C., PrasadReddy, R., & Sundararamaiah, T. (1984). 3 Epi cyclolaudenol and known triterpenes from Euphorbia caducifolia. *Phytochemistry*, *23*(2), 411–413.

Govt. of India (online). FRLHT's Indian Medicinal Plants Database. Retrieved May 12, 2017, from http://www.medicinalplants.in/aboutfrlhtdb

Goyal, M., Nagori, B. P., & Sasmal, D. (2012). Wound healing activity of latex of Euphorbia caducifolia. *Journal of Ethnopharmacology*, *144*(3), 786–790.

Goyal, M., Sasmal, D., & Nagori, B. P. (2012). GCMS analysis and antimicrobial action of latex of Euphorbia caducifolia. *Journal of Intercultural Ethnopharmacology*, *1*(2), 119–123.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., & Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Analytical Biochemistry*, *126*(1), 131–138.

Groom, P. (1889). On the function of Laticiferous Tubes. *Annals of Botany*, *3*, 157–169.

Gupta, S., & Acharya, R. (2017). An appraisal on ethno-medicinal claims of Euphorbia caducifolia Haines. – A source drug of ayurvedic medicinal plant"Rakta-snuhi." *Global J Res. Med. Plants & Indigen. Med.*, *6*(3), 54–63.

Haefner, B. (2003). Drugs from the deep: Marine natural products as drug candidates. *Drug Discovery Today*, *8*(12), 536–544.

Hamid, R., Masood, A., Wani, I. H., & Rafiq, S. (2013). Lectins: Proteins with diverse applications. *Journal of Applied Pharmaceutical Science*, *3*(4suppl.1), S93–S103.

Harvey, A. L. (2008). Natural products in drug discovery. *Drug Discovery Today*, *13*(19–20), 894–901.

Horejsi, V., Haskovec, C., & Kocourek, J. (1978). Studies on lectins XXXVIII: Isolation and characterization of lectin from locust bak (Robinia pseudacacia L.). *Biochimica et Biophysica Acta*, *532*, 98–104.

Huang, S. Q., Li, J. W., Wang, Z., Pan, H. X., Che, J. X., & Ning, Z. X. (2010). Optimization of alkaline extraction of polysaccharides from ganoderma lucidum and their effect on immune function in mice. *Molecules*, *15*(5), 3694–3708.

Irshad, M., Zafaryab, M., Singh, M., & Rizvi, M. M. a. (2012). Comparative Analysis of the Antioxidant Activity of Cassia fistula Extracts. *International Journal of Medicinal Chemistry*, *2012*, 157125.

Janarthanan, P., Zin Wan Yunus, W. M., & Ahmad, M. Bin. (2003). Thermal Behavior and Surface Morphology Studies on Polystyrene Grafted Sago Starch. *Journal of Applied Polymer Science*, *90*(8), 2053–2058.

Jane, J., Chen, Y. Y., Lee, L. F., McPherson, A. E., Wong, K. S., & Radosavljevic., M. (1999). Effects of amylopectin branch chain length and amylose content on the gelatinization and pasting properties of starch. *Cereal Chemistry*, *76*, 629–637.

Jawade, A. A., Pingle, S. K., Tumane, R. G., Sharma, A. S., Ramteke, A. S., & Jain, R. K. (2016). Isolation and characterization of lectin from the leaves of Euphorbia tithymaloides (L.). *Tropical Plant Research*, *3*(3), 634–641.

Jayakody, L., & Hoover, R. (2002). The effect of lintnerization on cereal starch granules. *Food Research International*, *35*(7), 665–680.

Jermyn, M. ., & Yeow, Y. . (1975). A class of lectins present in the tissues of seed plants. *Aust. J. Plant Physiol.*, *2*, 501–531.

Jermyn, M. A. (1962). Chromatography of Acidic Polysaccharides on Deae-Cellulose. *Australian Journal of Biological Sciences*, *15*(4), 787–792.

Kahn, V. (1987). Characterization of starch isolated from avacado seeds. *J. Food Sci*, *52*, 1646–1648.

Kapoor, B., Mishra, R., Acharya, S., Lakhera, S., & Purohit, V. (2013). Antimicrobial screening of some herbal plants of the Rajasthan Desert: An Overview. *Unique Journal of Engineering and Advanced Sciences*, *1*(1), 38–40.

Kapoor, L. D. (2001). *Handbook of ayurvedic medicinal plants*. CRC Press.

Kato, M. J., & Pezzuto, J. M. (online). Phytochemistry and pharmacognosy. In *Phytochemistry and Pharmacognosy*. Encyclopedia of Life Support Systems (EOLSS).

Khan, N., Rahim, S. S., Boddupalli, C. S., Ghousunnissa, S., Padma, S., Pathak, N., Mukhopadhyay, S. (2006). Hydrogen peroxide inhibits IL-12 p40 induction in macrophages by inhibiting c-rel translocation to the nucleus through activation of calmodulin protein. *Blood*, *107*(4), 1513–1520.

Kitajima, S., Miura, K., Aoki, W., Yamato, K. T., Taira, T., Murakami, R., & Aburaya, S. (2016). Transcriptome and proteome analyses provide insight into laticifer's defense of Euphorbia tirucalli against pests. *Plant Physiology and Biochemistry*, *108*, 434–446.

Kong, X., Kasapis, S., Zhu, P., Sui, Z., Bao, J., & Corke, H. (2016). Physicochemical and structural characteristics of starches from Chinese hull-less barley cultivars, *International Journal of Food Science and Technology*, 51, 509–518.

Kumar, R. S., Sivakumar, T., Sunderam, R. S., Gupta, M., Mazumdar, U. K., Gomathi, P., Kumar, K. A. (2005). Antioxidant and antimicrobial activities of Bauhinia racemosa L. stem bark. *Brazilian Journal of Medical and Biological Research*, *38*(7), 1015–1024.

Lamb, J. E., Shibata, S., & Goldstein, I. J. (1983). Purification and Characterization of Griffonia simplicifolia Leaf Lectins. *Plant Physiology*, *71*(4), 879–87.

Lan, W., Zhihua, Y., Yun, Z., Bijun, X., & Zhida, S. (2008). Morphological, physicochemical and textural properties of starch separated from Chinese water chestnut. *Starch/Staerke*, *60*(3–4), 181–191.

Lapasin, R., & Pricl, S. (1995). Industrial applications of polysaccharides. In *Rheology of Industrial Polysaccharides: Theory and Applications* (pp. 134–161). Boston, MA: Springer US.

Laurienzo, P. (2010). Marine polysaccharides in pharmaceutical applications: An overview. *Marine Drugs*, *8*(9), 2435–2465.

Lewis, K. (2012). Antibiotics: Recover the lost art of drug discovery. *Nature*, *485*(7399), 439–440.

Lin, C.-C., Cheng, H.-Y., Yang, C.-M., & Lin, T.-C. (n.d.). Antioxidant and antiviral activities of Euphorbia thymifolia L. *Journal of Biomedical Science*, *9*(6 Pt 2), 656–64.

Lintner, C. J. (1886). Studien uber diastase. *J. Prakt Chem*, *34*, 378–386.

Lis, H., & Sharon, N. (1972). Soy bean (Glycine max) agglutinin. *Method. Enzymol.*, *28B*, 360–365.

Liu, J., Sun, Y., Liu, L., & Yu, C. (2011). The extraction process optimization and physicochemical properties of polysaccharides from the roots of Euphorbia fischeriana. *International Journal of Biological Macromolecules*, *49*(3), 416–421.

Liu, J., Sun, Y., Liu, L., & Yu, C. (2012). A water-soluble polysaccharide (EFP-AW1) from the alkaline extract of the roots of a traditional Chinese medicine, Euphorbia fischeriana: Fraction and characterization. *Carbohydrate Polymers*, *88*(4), 1299–1303.

Liu, Y., Murakami, N., Ji, H., Abreu, P., & Zhang, S. (2007). Antimalarial Flavonol Glycosides from *Euphorbia hirta* . *Pharmaceutical Biology*, *45*(4), 278–281.

Luyen, B. T. T., Tai, B. H., Thao, N. P., Eun, K. J., Cha, J. Y., Xin, M. J., Kim, Y. H. (2014). Anti-inflammatory components of Euphorbia humifusa Willd. *Bioorganic and Medicinal Chemistry Letters*, *24*(8), 1895–1900.

Lynn, K. R., & Clevette-Radford, N. A. (1986a). Isolation and characterization of proteases from Euphorbia lactea and Euphorbia lactea cristata. *Phytochemistry*, *25*(4), 807–810.

Lynn, K. R., & Clevette-Radford, N. A. (1986b). Lectins from latices of Euphorbia and Elaeophorbia species. *Phytochemistry*, *25*(7), 1553–1557.

Mahlberg, P. (1973). Scanning electron microscopy of starch grains from latex of Euphorbia terracina and E. tirucalli. *Planta*, *110*(1), 77–80.

Mahlberg, P. G. (1975). Evolution of the Laticifer in Euphorbia as Interpreted from Starch Grain Morphology. *American Journal of Botany*, *62*(6), 577–583.

Mamoru, Y., Keiji, K., & Dexter, F. (1979). Electron Microscopic Observations of Waxy Maize Starch. *Journal of Ultrastructure Research*, *69*, 249–261.

Manca, M., Woortman, A. J. J., Loos, K., & Loi, M. A. (2015). Imaging inclusion complex formation in starch granules using confocal laser scanning microscopy, 132–138.

Marris, E. (2006). Drugs from the deep. *Nature*, *443*(7114), 904–906.

Maqbool, M. ., Hashmi, S., & Ghaffar, A. (1987). Effect of latex extracts from Euphorbia caducifolia and Calotropis procera on root-knot nematode meloidogyne incognita infesting tomato and egg plant. *Pak. J. Nematol*, *5*(1), 1987.

Mauricio Carvalho de Vasconcellos, A. de A. (2003). Activity of Euphorbia splendens var. hislopii N.E.B. (Euphorbiaceae) Latex against Lymnaea columella (Say, 1817) (Pulmonata: Lymnaeidae), Intermediate Host of Fasciola hepatica, Linnaeus, 1758 (Trematoda: Fasciolidae). 2: Limited Field-testing. *Mem Inst Oswaldo Cruz Rio de Janeiro*, *98*(7), 981–985.

Mayer, A. M. S., Glaser, K. B., Cuevas, C., Jacobs, R. S., Kem, W., Little, R. D., … Shuster, D. E. (2010). The odyssey of marine pharmaceuticals: a current pipeline perspective. *Trends in Pharmacological Sciences*, *31*(6), 255–265.

Meena, K. L., & Yadav, B. L. (2010). Some traditional ethnomedicinal plants of southern Rajasthan. *Indian Jornal of Traditional Knowledge*, *9*(3), 471–474.

Mellor, R.B., Gadd, G.M., Rowell, P., & Stewart, W.D.P. (1981). A phytohaemagglutinin from the Azolla-anabaena symbiosis. *Biochemical and Biophysical Research Communications*, *99*(4), 1348–1353.

Mishra, B. B., & Tiwari, V. K. (2011). Natural products: An evolving role in future drug discovery. *European Journal of Medicinal Chemistry*, *46*(10), 4769–4807.

Mohammed, S., Kasera, P. K., & Shukla, J. K. (2004). Unexploited plants of potential medicinal value from the Indian Thar desert. *Natural Product Radiance*, *3*(2), 69–74.

Molisch, H. (1901). *Studien über den milchsaft und schleimsaft der pflanzen.* Jena: G. Fischer.

Molyneux, P. (2004). The use of the stable free radical diphenylpicryl- hydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin J. Sci. Technol.*, *26*(2), 211–219.

Mwine, T. J., & Van Damme, P. (2011). Why do Euphorbiaceae tick as medicinal plants?: a review of Euphorbiaceae family and its medicinal features. *Journal of Medicinal Plants Research*, *5*(5), 652–662.

Nageli, C. W. (1874). Beitage zur na¨ heren kenntniss der starke grupe. *Annalen Der Chemie*, *173*, 218–227.

Nemethy, E. K., Skrukrud, C., Piazza, G. J., & Calvin, M. (1983). Terpenoid biosynthesis in Euphorbia latex. *BBA - General Subjects*, *760*(3), 343–349.

Newman, D. J., & Cragg, G. M. (2016). Natural Products as Sources of New Drugs from 1981 to 2014. *Journal of Natural Products*, *79*(3), 629–661.

Nsimba-Lubaki, M., Allen, A.K., & Peumans, W.J. (1986). Isolation and partial characterization of latex lectins from three species of the genus Euphorbia (Euphorbiaceae). *Physiologia Plantarum*, *67*(2), 193–198.

Nsimba-lubaki, M., Peumans, W.J., & Carlier, A.R. (1983). Isolation and partial characterization of a lectin from Euphorbia heterophylla seeds. *Biochem. J.*, *215*, 141–145.

Oda, Y., & Minami, K. (1986). Isolation and characterization of a lectin from tulip bulbs, Tulipa gesneriana. *European Journal of Biochemistry*, *159*(2), 239–245.

Ogata, a, Chauhan, D., Teoh, G., Treon, S. P., Urashima, M., Schlossman, R. L., & Anderson, K. C. (1997). IL-6 triggers cell growth via the Ras-dependent mitogen-activated protein kinase cascade. *Journal of Immunology*, *159*, 2212–2221.

Ohtani, K., Okai, K., Yamashita, U., Yuasa, I., & Misaki, A. (1995). Characterization of an Acidic Polysaccharide Isolated from the Leaves of Corchorus olitorius (Moroheiya). *Bioscience, Biotechnology, and Biochemistry*, *59*(3), 378–381.

Okeniyi, S. ., Adedoyin, B. ., & Garba, S. (2012). Phytochemical Screening, Cytotoxicity, Antioxidant and Antimicrobial Activities of Stem and Leave Extracts of Euphorbia Heterophylla. *Journal of Biology and Life Science*, *4*(1), 87–91.

Parikh, A., & Madamwar, D. (2006). Partial characterization of extracellular polysaccharides from cyanobacteria. *Bioresource Technology*, *97*(15), 1822–1827.

Pettitt, J., (1977). Detention in primitive gymnosperms of protein and glycoprotein of possible significance in reproduction. *Nature*, *266*, 530–532.

Peumans, W. J., Nsimba-Lubaki, M., Carlier, A. R., & Van Driessche, E. (1984). A lectin from Bryonia dioica root stocks. *Planta*, *160*(3), 222–228.

Pitthard, V., & Finch, P. (2001). GC-MS Analysis of Monosaccharide Mixtures as their Diethyldithioacetal Derivatives : Application to Plant Gums Used in Art Works. *Chromatographia Supplement*, *53*, 317–321.

Procházková, D., Boušová, I., & Wilhelmová, N. (2011). Antioxidant and prooxidant properties of flavonoids. *Fitoterapia*, *82*(4), 513–523.

Rafiq, S., Qadir, S., Wani, I. H., Ganie, S. A., Masood, A., & Hamid, R. (2014). Purification and partial characterization of a fructose-binding lectin from the leaves of Euphorbia helioscopia. *Pakistan Journal of Pharmaceutical Sciences*, *27*(6), 1805–1810.

Rafn. (1798). Entwurf einer pflanzenphysiologie, auf die neuen theorien der physik und chemie gegründet. - leipzig. *Leipzig*.

Rajeswari, B., Kumar, S. P., Rao, A. P., Shaik, P., & Valli, S. (2014). A Distribution and Ultrastructure of Laticifers in the Phylloclade of Euphorbia caducifolia Haines , a Potential Hydrocarbon Yielding CAM Plant, *2014*(January), 70–79.

Rajeswari, B., Pradeep Kumar, S., Sharma, A., Sahoo, P. K., & Sha Valli Khan, P. . (2013). Effect of C-5 and C-10 fuel blends of Euphorbia caducifolia Haines on IC diesel engine emissions. *International Journal of Advances in Engineering &*

Technology, *6*(3), 1177–1186.

Reis, P., Cristiana, M., & Beirão-da-costa, M. L. (2012). Food Hydrocolloids The effect of starch isolation method on physical and functional properties of Portuguese nuts starches . I . Chestnuts (Castanea sativa Mill . var . Martainha and Longal) fruits, *27*, 256–263.

Rice-Evans, C., Miller, N., & Paganga, G. (1997). Antioxidant properties of phenolic compounds. *Trends in Plant Science*, *2*(4), 152–159.

Ros, J.M., Laencina, J., Hellín, P., Jordán, M. J., Vila, R., & Rumpunen, K. (2004). Characterization of juice in fruits of different Chaenomeles species. *LWT - Food Science and Technology*, *37*(3), 301–307.

Sandhya Rani, M. R., & Bhattacharaya, K. R. (1989). Rheology of rice-flour paste: effect of variety, concentration, and temperature and time of cooking. *Journal of Texture Studies*, *20*, 127–137.

Santana, S.S., Gennari-Cardoso, M.L., Carvalho, F.C., Roque-Barreira, M.C., Santiago, A.D.S., Alvim, F.C., & Pirovani, C.P. (2014). Eutirucallin, a RIP-2 type lectin from the latex of Euphorbia tirucalli L. presents proinflammatory properties. *PLoS ONE*, *9*(2).

Santhi, K., & Sengottuvel, R. (2016). Qualitative and Quantitative Phytochemical analysis of Moringa concanensis Nimmo, *Int.J.Curr.Microbiol.App.Sci ,5*(1), 633–640.

Santiago, F.M., Palharini, J.G., Richter, A.C., Silva,M. F., Ferreira, F.B., Pirovani, C.P., Silva, M.J.B. (2017). Eutirucallin: A Lectin with Antitumor and Antimicrobial Properties. *Frontiers in Cellular and Infection Microbiology*, *7*(April), 1–13.

Schepetkin, I.A., & Quinn, M.T. (2006). Botanical polysaccharides: Macrophage immunomodulation and therapeutic potential. *International Immunopharmacology*, *6*(3), 317–333.

Schepetkin, I.A., Xie, G., Kirpotina, L.N., Klein, R.A., Jutila, M.A., & Quinn, M.T. (2008). Macrophage immunomodulatory activity of polysaccharides isolated from Opuntia polyacantha. *International Immunopharmacology*, *8*(10), 1455–1466.

Sen, D. N. (1968). Leafless Euphorbia on Rajasthan rocks, India I. Ecological life-history. *Folia Geobotanica et Phytotaxonomica*, *3*(1), 1–15.

Seshagirirao, K., Leelavathi, C., & Sasidhar, V. (2005). Cross-linked leucaena seed gum matrix: an affinity chromatography tool for galactose-specific lectins. *Journal of Biochemistry and Molecular Biology*, *38*(3), 370–2.

Seshagirirao, K., & Prasad, M. N. V. (1995). Purification and partial characterization of a lectin from uphorbia neriifolia latex. *Biochem. Mol. Biol. Int.*, *35*, 1199–1204.

Seshagirirao, K., Sasidhar, V., & Fatima.A. (1999). Purification of a lectin from Euphorbia milli latex. *Biochemical Archives*, *15*(1), 19–23.

Seshagirirao, K., & Prasad, M. N. V. (2001). Latex Biochemistry of Certain Euphorbiaceous Taxa: Medical Importance. In P. . Saxena (Ed.), *Development of Plant-Based Medicines: Conservation, Efficacy and Saftey* (pp. 199–210). Netherlands: Kluwer Academic Publishers.

Seshagirirao, K., & Prasad, M. N. V. (1988). Typology of latex starch grains of certain Euphorbiaceae and their possible significance in systematics. *Plant Systematics and Evolution*, *160*, 189–193.

Shaik, P., Valli, S., Rajeswari, B., & Kumar, S. P. (2013). Potential of Euphorbia caducifolia Haines as a renewable source for biofuel. *Indian Journal of Energy*, *2*(2), 99–107.

Shibuyas, N., Goldstein$, I. J., Broekaertg, W. F., Nsimba-Lubakiq, M., Peetersll, B., & Peumansg, W. J. (1987). The Elderberry (Sambucus nigra L.) Bark Lectin Recognizes the Neu5Ac(a2-6)Gal/GalNAc Sequence*. *Journal of Biological Chemistry*, *262*(4), 1596–1601.

Singh, G. (2008). *Facilitative effects of Euphorbia caducifolia and rehabilitation of degraded hills in dry areas*. (online report).

Solereder, H. (1908). *Systematic anatomy of the dicotyledons*.

Spilatro, S., & Mehlberg, P. (1985). Composition and structure of laticifer starch grains of Euphorbia pulcherrima Willd. *Bot.Gaz.*, *146*, 26–31.

Sterling, C., & Spit, B. J. (1958). Submicroscopic Structure of the Cereal Starch Grain, *9*(35), 75–81.

Stillmark. (1888). Uber rizin, ein giftiges ferment aus dem samen von Ricinus communis L. und einigen anderen Euphorbiaxeen. *inaug.Diss. Dorpat*.

Stirpe, F., Licastro, F., Morini, M. C., Parente, A., Savino, G., Abbondanza, A., Rossi, C. A. (1993). Purification and partial characterization of a mitogenic lectin from the latex of Euphorbia marginata. *BBA - General Subjects*, *1158*(1), 33–39.

Stromeyer. (1815). Ein sehr empfindliches Reagens für Jodine aufgefunden in der Stärke (Amidon). *Annalen Der Physik, Series 1 (*(49), 146–153.

Sutherland, I. W. (1998). Novel and established applications of microbial polysaccharides. *TIBTECH*, *16*, 41–46.

Suzuki, I., Satto, H., Inoue, S., Migita, S., & Takahashi, T. (1979). Purification and Characterization of Two Lectins from Aloe arborescens Mill. *Biochem*, *85*, 163–171.

Tadesse, S., Ganesan, K., Nair, S. K. P., Letha, N., Gani, S. B., (2016). Preliminary Phytochemical Screening of Euphorbia Cotinifolia L . (Family : Euphorbiacae) by using different solvent extracts, *World journal of pharmacy and pharmaceutical sciences*, *5*(3), 1176–1183.

Takeda, C., Takeda, Y., & Hizukuri, S. (1986). Physicochemical properties of lily starch. *Cereal Chemistry*, 60 (3), 212-216.

Taviad, K., Vekariya, S., Bhati, H., & Patgiri, B. J. (2017). Exposure to Snuhi Ksheera (Euphorbia caducifolia Haines . latex) resulting in ocular injury : A case report, *3*(1), 1–4.

Torky, Z. A. (2016). Antivirals & Antiretrovirals Antiviral Activity of Euphorbia Lectin Against Herpes Simplex Virus 1 and its Antiproliferative Activity Against Human Cancer Cell-Line. *Journal of Antivirals & Antiretrovirals*, *8*(4), 107–116.

Umar, D. ., Yakoub, K. ., & Menon, A. (1990). Screening of some pakistani plants for milk clotting activity. *Journal of Islamic Academy of Sciences*, *3*(4), 284–286.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free

radicals and antioxidants in normal physiological functions and human disease. *Int.J Biochem Cell Biol.*, *39*(1), 44–84.

Van Damme, E. J. M., Allen, A. K., & Peumans, W. J. (1987). Isolation and characterization of a lectin with exclusive specificity towards mannose from snowdrop (Galanthus nivalis) bulbs, *FEBS Letters*, *215*(1), 140–144.

Van Hung, P., & Morita, N. (2005). Physicochemical properties of hydroxypropylated and cross-linked starches from A-type and B-type wheat starch granules. *Carbohydrate Polymers*, *59*(2), 239–246.

Veeru, P., Kishor, M. P., & Meenakshi, M. (2009). Screening of medicinal plant extracts for antioxidant activity. *Journal of Medicinal Plants Research*, *3*(8), 608–612.

Verpoorte, R. (2000). Pharmacognosy in the New Millennium: Leadfinding and Biotechnology. *Journal of Pharmacy and Pharmacology*, *52*(3), 253–262.

Villanueva, J., Quiros, L. M., & Castanon, S. (2015). Purification and partial characterization of a ribosome-inactivating protein from the latex of Euphorbia trigona Miller with cytotoxic activity toward human cancer cell lines. *Phytomedicine*, *22*(7–8), 689–695.

W G Carter, & M E Etzler. (1975). Isolation, characterization, and subunit structures of multiple forms of Dolichos biflorus lectin. *Journal of Biological Chemistry*, *250*, 2756–2762.

Wang, S., Li, C., Copeland, L., Niu, Q., & Wang, S. (2015). Starch Retrogradation : A Comprehensive Review, *Comprehensive Reviews in Food Science and Food Safety*,14, 569-585.

Wang, Z., Luo, D., & Ena, C. (2007). Optimization of polysaccharides extraction from Gynostemma pentaphyllum Makino using Uniform Design. *Carbohydrate Polymers*, *69*(2), 311–317.

Webster, G. L. (1994). Classification of the Euphorbiaceae. *Ann. Missouri Bot. Gard*, *81*, 3–32.

Xu, Q., Yajima, T., Li, W., Saito, K., Ohshima, Y., & Yoshikai, Y. (2006). Levan (beta-2, 6-fructan), a major fraction of fermented soybean mucilage, displays

immunostimulating properties via Toll-like receptor 4 signalling: induction of interleukin-12 production and suppression of T-helper type 2 response and immunoglobulin E production. *Clinical and Experimental Allergy : Journal of the British Society for Allergy and Clinical Immunology*, *36*(1), 94–101.

Yau, T., Dan, X., Ng, C.C.W., & Ng, T. B. (2015). Lectins with potential for anti-cancer therapy. *Molecules*, *20*(3), 3791–3810.

Ye, C.Lin., & Lai, Y.Feng. (2015). Optimization of Extraction Process and Antioxidant Activity of Polysaccharides from Leaves of Artemisia argyi Levl. et Vant. *Journal of Food Processing and Preservation*, *39*(6), 1309–1317.

Ye, D., Jiang, Z., Zheng, F., Wang, H., Zhang, Y., Gao, F., Shi, G. (2015). Optimized extraction of polysaccharides from Grateloupia livida (Harv.) yamada and biological activities. *Molecules*, *20*(9), 16817–16832.

Ye, H., Wang, K., Zhou, C., Liu, J., & Zeng, X. (2008). Purification , antitumor and antioxidant activities in vitro of polysaccharides from the brown seaweed Sargassum pallidum. *Food Chemistry*, *111*, 428–432.

Yeung, H.W., Wong, D.M., & Li, W.W. (1980). A galactose - binding lectin from the Chinese herb, Tien - Hua - Fen, Trichosanthes kirilowii. In *4 th Asian symp. medicinal plants and species* (p. 94). Bankok, Thailand: Mahidol. Unive.

Yildirim, A., Oktay, M., & Bilaloglu, V. (2001). The Antioxidant Activity of the Leaves of Cydonia vulgaris. *Journal of Medicine (Cincinnati)*, *31*, 23–27.

Yu, F., Lu, S., Yu, F., Feng, S., McGuire, P. M., Li, R., & Wang, R. (2006). Protective effects of polysaccharide from Euphorbia kansui (Euphorbiaceae) on the swimming exercise-induced oxidative stress in mice. *Canadian Journal of Physiology and Pharmacology*, *84*(10), 1071–1079.

Yuan, X., Zeng, Y., Nie, K., Luo, D., & Wang, Z. (2015). Extraction Optimization, Characterization and Bioactivities of a Major Polysaccharide from Sargassum thunbergii. *PLoS ONE*, *10*(12), 1–11.

Zheng, W., Cui, Z., & Zhu, Q. (1998). Cytotoxicity and Antiviral Activity of the Compounds from *Euphorbia kansui*. *Planta Medica*, *64*(8), 754–756.

Zhou, P., Zie, M., & Fu, B. (2000). A review of the studies on the polysaccharide structure. *Journal of Nanchang University (Natural Science)*, *25*(2), 197–204.

Zobel, H. F. (1988). Molecules to Granules: A Comprehensive Starch Review. *Starch - Stärke*, *40*(2), 44–50.

Zohuriaan, M. J., & Shokrolahi, F. (2004). Thermal studies on natural and modified gums. *Polymer Testing*, *23*(5), 575–579.

Zou, Y., Jiang, A., & Tian, M. (2015). Extraction optimization of antioxidant polysaccharides from Auricularia auricula fruiting bodies. *Food Science and Technology*, *35*(3), 1–6.

Isolation and Characterization of Certain Biomolecules from Euphorbia caducifolia Haines

ORIGINALITY REPORT

%11	%7	%9	%2
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Seshagirirao, Kottapalli, Chaganti Leelavathi, and Vemula Sasidhar. "Cross-linked Leucaena Seed Gum Matrix: An Affinity Chromatography Tool for Galactose-specific Lectins", Journal of Biochemistry and molecular biology, 2005. Publication	%1
2	Submitted to Higher Education Commission Pakistan Student Paper	<%1
3	www.ncbi.nlm.nih.gov Internet Source	<%1
4	mdpi.com Internet Source	<%1
5	Xiumei Yuan, Yawei Zeng, Kaiying Nie, Dianhui Luo, Zhaojing Wang. "Extraction Optimization, Characterization and Bioactivities of a Major Polysaccharide from Sargassum thunbergii", PLOS ONE, 2015 Publication	<%1

Phytochemical screening and Antioxidant activity of *Euphorbia caducifolia* extracts

Kusuma Venumadhav* and Kottapalli Seshagirirao

Department of Plant Sciences, School of Life Sciences, University of Hyderabad,
Hyderabad-500046 Telangana, India

Abstract

Purpose: This study was conducted to screen the phytochemical compounds present in the phylloclade of *Euphorbia caducifolia* Haines and to measure the ability of free radical scavenging activity as an antioxidant. **Methods:** The collected plant phylloclades were properly dried, powdered and extracted with hexane, methanol, and water. Extracts were tested for phytochemicals, total phenolic and flavonoid content, and antioxidant activities. **Results:** Carbohydrates, phenols, flavonoids, tannins, glycosides were present in water extract and terpenoids along with phenols and flavonoids in methanol. Hexane contains a maximum of terpenoids and reducing sugars. Water extract showed maximum DPPH˙ free radical (86±1.5%) and nitric oxide scavenging activities (39.23±4.7%) followed by methanol and hexane due to the presence of phenolic (68.740 ± 0.025 mg of GAE/g) and flavonoid contents (50.04±0.125 mg of RE/g). **Conclusion:** This study suggests that *Euphorbia caducifolia* is a promising plant for the therapeutic purposes due to its antioxidant activity of water extract and high contents of terpenoids in methanol and water extracts. A further study of isolation and characterization of active compounds from the plant is necessary to treat various diseases and for multiple applications.

Keywords: *Euphorbia caducifolia*, Free radical scavenging activity, Phylloclade, Phenolic content, Flavonoids.

Introduction

Plants are a rich source of useful drugs since ancient times. In India, plants have been used for many therapeutic applications by our traditional system of medicines such as Ayurveda, Unani, Homeopathy, and Siddha. Traditional medicine system gave birth to many active compounds in early years of natural chemistry, but reports of natural products from different sources has been decreased rapidly in last decade and looking for new active compounds [1][2]. According to National Medicinal Plant Board (NMPB), a maximum number of plants using in the folk medicine compared to other documented medicine systems [3]. Now it needs to explore new natural compounds from potential folk medicinal plants and ethnomedicinal plants for better therapeutic applications.

All living organisms contain an antioxidant defensive mechanism to counter the free radicals, reactive oxygen species (ROS), reactive nitrogen species (RNS) and other oxidants produced as byproducts of the metabolism. Most of the diseases caused by overproduction and reactive mechanism of free radicals [4][5]. Many medicinal plants, spices, aromatic plants using as food supplements and medicines are natural antioxidants [6]. It has been an upsurge of interest for potential antioxidants from plants sources.

Euphorbia caducifolia Haines (leafless milk hedge), a folk medicinal plant belongs to Euphorbiaceae (Castor family). It is a latex-producing plant and found in rocky areas of the tropical region [7]. The plant latex is used for wound healing, leucoderma, and skin eruptions [8]. Latex and root of the plant used to treat cancer [9]. Root also used for snakebite in Maharashtra [10]. Leaf extracts of the plant were reported for its antimicrobial activity [11]. Apart, there are not many reports on phylloclade of the plant which are a rich source of biofuel and other compounds [12]. Therefore, the present study was conducted to reveal the phytochemical compounds exist in the phylloclade and to explore the ability of free radical scavenging activity as an antioxidant.

Materials and Methods

Collection of Plant material

The aboveground parts of *Euphorbia caducifolia* plant were collected from Bhongiri rocky areas, Yadadri district of Telangana (Fig. 1) and shade dried until the material obtains a constant weight.

Preparation of Extracts

The dried plant material was made into powder and used for extraction. The powder was extracted with hexane, methanol, and water in successive order. The extracts were concentrated at temperature 40°C under vacuum and used for further analysis.

Phytochemical screening
Extracts were analyzed for alkaloids, carbohydrates, glycosides, flavonoids, phenolic compounds, quinones, tannins, saponins, and terpenoids [13].

Estimation of Total Phenolic Content (TPC)
The TPC of the extracts was determined using the Folin-Ciocalteu colorimetric method. 1 ml of extract was mixed with 1 ml of 1 N Folins reagents and well shaken. After 5 min, 1 ml of 10% sodium carbonate was added and incubated for 1 h at room temperature. The absorbance was taken after incubation at 760 nm against blank. Total phenolic content was given as Gallic acid equivalents (GAE) [14].

Estimation of Flavonoids
0.1 ml of extract was added to 0.3 ml 5% sodium nitrite and mixed with 3 ml of 1% aluminum chloride. After 5 min incubation, 2 ml of 1 M NaOH was added and made up the volume to 10 ml with water. The solution was mixed well and measured absorbance at 510 nm against blank. The flavonoid content was calculated using rutin as standard [14].

Antioxidant Activity

DPPH Scavenging Assay
DPPH (2,2-diphenyl 1-picrylhydrazyl) free radical used to determine the scavenging activity of extracts [15]. DPPH (1 ml, 0.1mM) added to various concentrations of extracts and BHT (3 ml) and 0.5 h incubated in the dark and measured absorbance at 517 nm against the blank (n=3). The activity was calculated by the following equation [16]:

Percentage inhibition = [(Abs control – Abs sample)/Abs control] × 100

Nitric oxide radical scavenging Activity
Nitric oxide (NO) produced by Sodium Nitroprusside at pH 7.2-7.4 was determined by Griess reagent (Griess diazotization reaction). The reaction mixture (3 ml) was incubated at 25°C for 2 hr containing Sodium Nitroprusside (10 mM) and extracts with different concentrations. An Aliquot (0.5 ml) was added to Griess reagent (0.5 ml) and measured at 546nm (n=3). The activity was calculated by comparing control and test sample. Ascorbic acid used as reference compound [17].

Results and Discussion

Phytochemical analysis
Phytochemical studies of extracts showed the presence of various classes of metabolites. *E. caducifolia* showed the high quantity of terpenoids in methanol and hexane extracts as *Euphorbia* species are predominant with cyclic and acyclic terpenoids [18]. Test for the presence of alkaloids shown no significant colouration. Water extract was rich in carbohydrates, glycosides, tannins, phenols, flavonoids and saponins. Further experiments revealed that the presence of acidic polysaccharides and saponin glycosides. Methanol extract contained reducing sugars, glycosides and some phenolic compounds whereas in hexane extract showed positive for reducing sugars and glycosides along with terpenoids (Table 1).

Estimation of total phenolic and flavonoid content
Phenolic compounds such as flavonoids and tannins are excellent free radical scavengers and protect the plant from different types of oxidative damages [19]. Water extract showed the high phenolic and flavonoid contents whereas no significant amounts observed in hexane extract. Methanol extract showed good amounts of flavonoids compared to phenolics (Table 2). Antioxidant activity of the extracts might be due to the presence of phenols and flavonoids present [20][21].

Antioxidant activity
Free radicals are the most reactive molecules and damage the cells. Aerobic organisms protect themselves from free radical by the defensive antioxidant mechanism, and it is necessary to provide external antioxidants in failure defensive system [22]. The extracts were analyzed for their antioxidant capacity to use as a free radical scavenger. Most of the members of the castor family are a good source of natural antioxidants such as *Euphorbia hirta* [14], *E. heterophylla* [23]. *E. caducifolia* extracts also exhibited antioxidant activity. The potentiality of the extract to scavenge the free radical produced was considered for antioxidant activity (Fig. 2). DPPH and NO are the two radicals used for the experiments. All extracts were showed activity on concentration dependency. Water extract exhibited more scavenging activity towards DPPH (86±1.5%) as well NO (39.23±4.7%) to hexane extract. More free scavenging activity of water extract might be due to the presence of the high phenolic and

flavonoid contents. Methanol extract also exhibited similar (DPPH-77.81±1.3% and NOSA-36.03±2.71%) to water but dropped activity frequency at high concentration of the extract. Higher antioxidant activity was observed at higher concentrations (Fig. 3). Extracts showed less ability to scavenge the NO free radical as compared to DPPH.

Conclusion

Euphorbia caducifolia is a promising plant for the various therapeutic applications with rich contents of terpenoids, polysaccharides, tannins, flavonoids and phenolic compounds. The presence of significant amount of total phenolics and flavonoids contents in water extract might be responsible for strong antioxidant activity as compared with methanol and hexane extract. Hexane and methanol extract can be used for the terpenoid sources and water extract for phenolics and flavonoids. High contents of terpenoids in methanol and hexane extracts are useful in various applications. A further study of isolation and characterization of bioactive molecules from the plant is required for therapeutic and multiple applications.

Acknowledgements

Kusuma Venumadhav is grateful to Rajiv Gandhi National Fellowship (RGNF), UGC, New Delhi, India, for providing fellowship. Authors also acknowledge Ms. G.V.S. Tulasi, for technical support.

References

[1] G. M. Cragg and D. J. Newman, "Natural products: A continuing source of novel drug leads," *Biochim. Biophys. Acta - Gen. Subj.*, 2013, vol. 1830, no. 6, pp. 3670–3695.

[2] J. P. Bégué and D. Bonnet-Delpon, "Recent advances (1995-2005) in fluorinated pharmaceuticals based on natural products," *J. Fluor. Chem.*, 2006, vol. 127, no. 8, pp. 992–1012.

[3] G. O. I. National Medicinal Plant Board, Ministry of AYUSH, "FRLHT's Indian Medicinal Plants Database." [Online]. Available: http://www.medicinalplants.in/aboutfrlhtdb.

[4] M. N. Alam, N. J. Bristi, M. Rafiquzzaman, N. Alam, and N. J. Bristi, "Review on in vivo and in vitro methods evaluation of antioxidant activity," *Saudi Pharm. J.*, 2013, vol. 21, no. 2, pp. 143–152.

[5] M. Valko, D. Leibfritz, J. Moncol, M. T. Cronin, M. Mazur, and J. Telser, "Free radicals and antioxidants in normal physiological functions and human disease," *Int.J Biochem Cell Biol.*, 2007, vol. 39, no. 1, pp. 44–84.

[6] P. Veeru, M. P. Kishor, and M. Meenakshi, "Screening of medicinal plant extracts for antioxidant activity," *J. Med. Plants Res.*, 2009, vol. 3, no. 8, pp. 608–612.

[7] G. Singh, "Facilitative effects of *Euphorbia caducifolia* and rehabilitation of degraded hills in dry areas. Division of Forest Ecology, Arid Forest Research Institute, New Pali Road, Jodhpur. "http://dlc.dlib.indiana.edu/dlc/bitstream/handle/10535/3828/Degradedhills.pdf?sequence=1 .

[8] M. Goyal, D. Sasmal, and B. P. Nagori, "Ethnopharmacology GCMS analysis and antimicrobial action of latex of *Euphorbia caducifolia*," *J. Intercult. Ethnopharmacol*, 2012, vol. 1, no. 2, pp. 119–123.

[9] I. B. Baloch, M. K. Baloch, and Q. Najam us saqib, "Tumor-Promoting Diterpene Esters from Latex of Euphorbia cauducifolia L ." *Helv. Chim. Acta,* 2005, vol. 88, pp. 13–18.

[10] B. W. Chavre, "Botanicals used on the treatment of snakebite in some parts of Maharashtra," *Ind J Plant Sci*, 2013, vol. 2, no. 1, pp. 52–54.

[11] K. Bbs, M. Raksha, A. Sanjay, L. Swati, and P. Veena, "Antimicrobial screening of some herbal plants of the Rajasthan desert: An overview," *U. J. Eng and Adv. Scie*, 2013, vol. 1, no. 1, pp. 38–40.

[12] B. Rajeswari, P. K. S, A. Sharma, P. K. Sahoo, and S. V. K. P. S, "Effect of C-5 and C-10 fuel blends of *Euphorbia caducifolia* Haines on IC diesel engine emissions," *Int. J adv. Eng. Tech,* 2013, vol. 6, no. 3, pp. 1177–1186.

[13] K. Santhi and R. Sengottuvel, "Qualitative and Quantitative Phytochemical analysis of Moringa concanensis Nimmo," *Int J Current Microbiol & App Sci,* 2016, vol. 5, no. 1, pp. 633–640.

[14] A. A. Basma, Z. Zakaria, L. Y. Latha, and S. Sasidharan, "Antioxidant activity and phytochemical screening of the methanol extracts of *Euphorbia hirta L*," *Asian Pac. J. Trop. Med.*, 2011, vol. 4, no. 5, pp. 386–390.

[15] P. Molyneux, "The use of the stable free radical diphenyl picryl- hydrazyl (DPPH) for estimating antioxidant activity," *Songklanakarin J. Sci. Technol.,* 2003, vol. 50, no. 2, pp. 211-219.

[16] R. S. Kumar *et al.*, "Antioxidant and antimicrobial activities of *Bauhinia racemosa L.* stem bark," *Brazilian J. Med. Biol. Res.*, 2005, vol. 38, no. 7, pp. 1015–1024.

[17] F. Boora, E. Chirisa, and S. Mukanganyama, "Evaluation of Nitrite Radical Scavenging Properties of Selected Zimbabwean Plant Extracts and Their Phytoconstituents," *J. Food Process.*, 2014, pp. 1–7.

[18] E. K. Nemethy, C. Skrukrud, G. J. Piazza, and M. Calvin, "Terpenoid biosynthesis in Euphorbia latex," *BBA - Gen. Subj.*, 1983, vol. 760, no. 3, pp. 343–349.

[19] S. Tadesse *et al.*, "Preliminary Phytochemical Screening of *Euphorbia Cotinifolia L* . (Family : Euphorbiaceae) By Using," 2016, vol. 5, no. 3, pp. 1176–1183.

[20] C. Rice-Evans, N. Miller, and G. Paganga, "Antioxidant properties of phenolic compounds," *Trends Plant Sci.*, 1997, vol. 2, no. 4, pp. 152–159.

[21] D. Procházková, I. Boušová, and N. Wilhelmová, "Antioxidant and prooxidant properties of flavonoids," *Fitoterapia*, 2011, vol. 82, no. 4, pp. 513–523.

[22] L. Aksoy, E. Kolay, Y. Ağilönü, Z. Aslan, and M. Kargioğlu, "Free radical scavenging activity, total phenolic content, total antioxidant status, and total oxidant status of endemic Thermopsis turcica," *Saudi J. Biol. Sci.*, 2013, vol. 20, no. 3, pp. 235–239.

[23] S.O. Okeniyi, B. J. Adedoyin, and S. Garba, "Phytochemical Screening, Cytotoxicity, Antioxidant and Antimicrobial Activities of Stem and Leave Extracts of Euphorbia Heterophylla.," *J. Biol. Life Sci.*, 2012, vol. 4, no. 1, pp. 87–91.

Table 1: Phytochemical Screening of *E. caducifolia* extracts

Fraction	Water	Methanol	Hexane
Alkaloids	--	--	--
Carbohydrates	++	++	++
Glycosides	++	++	++
Flavanoids	++	+	--
Phenolics	++	+	--
Tannins	++	--	--
Saponins	++	--	--
Quinones	--	++	++
Terpenoids	--	++	++

Table 2: Total Phenolic and Flavonoid contents in *E. caducifolia* plant extracts

Fraction	Phenolics	Flavonoids
	mg of GAE/g of Extract	mg of RE/g of Extract
Water	68.740 ± 0.025	50.04 ± 0.125
Methanol	00.926 ± 0.06	13.534 ± 0.754
Hexane	00.080 ± 0.014	00.004 ± 0.0002

(n=3, Mean ± SD)

Figure 1: Distribution of *Euphorbia caducifolia* in the wild.

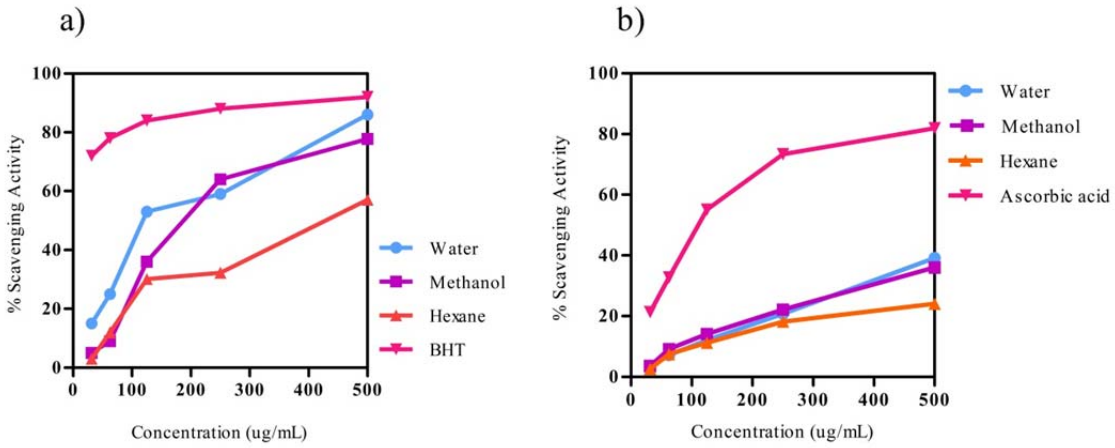


Figure 2: Antioxidant activity of *E. caducifolia* plant extracts a) DPPH Scavenging activity, b) Nitric Oxide Scavenging activity

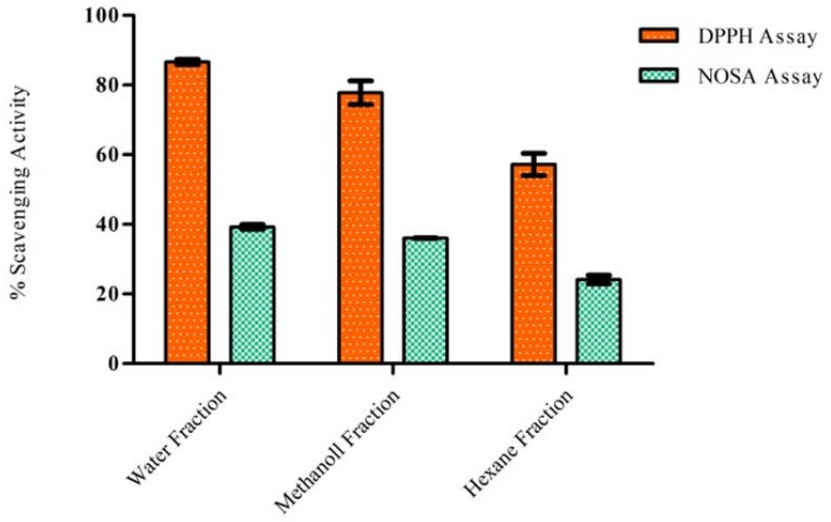
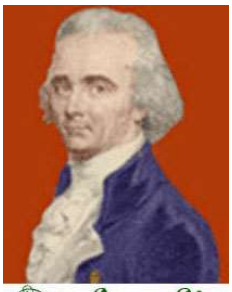


Figure 3: Antioxidant activity at maximum concentration (500 µg/ml) of *E. caducifolia* plant extracts

Roxburghia 6(1-4): 111-119, 2016 **Education Article**



Roxburghia
ISSN: 0973-4244 (Online)
0973-4252 (Print)

PREPARATION OF HERBARIUM SPECIMEN FOR PLANT IDENTIFICATION AND VOUCHER NUMBER

Kottapalli Seshagirirao*, Lavudi Harikrishnanaik, Kusuma Venumadhav, Badithi Nanibabu, Kizukala Jamir, Basimalla Kasi Ratnamma, Rutuparna Jena and Dhokne Kunal Babarao

Department of Plant Sciences, School of Life Sciences
University of Hyderabad, Hyderabad – 500 046, Telangana, India

***Corresponding author: ks@uohyd.ac.in Tel: +91-(0)40-23134504**

Received on 25th July, 2016 Accepted on 5th August, 2016

Abstract: A herbarium is a storehouse of plant specimens which are collected, dried and mounted on handmade paper sheets. They will be arranged in plant families recognized system of classification and kept in pigeon holes of steel or wooden cup boards and maintained carefully for current and future studies. It is a reference material for naming, identification and classification of the plants. The herbaria are indexed with unique codes in the "*Index Herbariorum*" presently assigned and maintained by New York Botanical Garden herbarium. Also the non-vascular plant specimens (algae, certain fungi, lichens, bryophytes and certain pteridophyptes) can be fixed in FAA fixative and bring to the herbarium for identification and voucher specimen number. The article also informed the merits of the "Plant Specimen Preparation Kit" from Nihon Vogue-Sha, Japan for use of specimens' preparation both vascular and non-vascular plants where there is no involvement of chemicals and harsh environmental conditions. Since the identification and voucher specimen number is essential in these days for research as well as for publications, here we are presenting useful information.

Key words: Herbarium; Plant identification; Voucher specimen number; Voucher material number; Plant Specimen Preparation Kit - Nihon Vogue-Sha

Introduction

The "Herbarium" has two meanings; one is a repository or storehouse of collected plant specimens and second is a plant specimen according to accepted international standards. If the term "Herbarium" followed by a code assigned by of "*Index Herbariorum* (IH)*"* authorities in the parenthesis (Thiers, 2016), it shall be consider as repository and the plural is "herbaria". If the term "Herbarium" is not followed by the code in parenthesis we shall be consider it as a "plant specimen" and plural is "herbarium specimens". It is believed that the first established in 1570 in Bologna, Italy, by Luca Ghini. It is now estimated that there are nearly 350,000,000 specimens that are documented from the world's vegetation. These herbarium specimens are available at approximately 3,000 herbaria in the world, with approximately 12,000 associated curators and plant specialists (Thiers, 2016). It shall be noted that those collections that are permanent scientific repositories are included in IH and the new registrants must demonstrate that their collection is accessible to scientists, and is actively managed. Each institution is assigned a permanent unique identifier in the form of a one to eight letter code, a practice that dates from the founding of IH in 1935. We shall note that the first six editions of IH were print copies published by the International Association for Plant Taxonomy in the Netherlands (1952–1974), subsequently Dr. Patricia Holmgren, then Director of the New York Botanical Garden, served as co-editor of edition 6, and subsequently became the senior editor of IH. She oversaw the compilation of print copies volumes 7 and 8, and Dr. Noel Holmgren, scientist on the NYBG staff, oversaw the development of the IH database, which became available from 1997 in on-line. From September 2008 on words Dr. Barbara M. Thiers, Director of the NYBG Herbarium, became the editor of IH.

IH is a detailed directory of public herbaria of the world and the staff and plant specialists associated with them. They are authorized to identifying the plant and provide "Voucher Specimen

Number". Each herbarium in the Index is assigned an official code that is used as a standard reference for citation. It consists of the herbarium code, institution, city, state, staff member, correspondent, and research specialty. The data is maintained and available at New York Botanical Garden Herbarium at http://sweetgum.nybg.org/science/ih/ (Thiers, 2016)

With the advent of Information Technology, new techniques are adopted now for the herbaria include the micro-herbaria in the form of photographs and the electronic herbaria in the form of digitized databases. The Voucher herbarium specimen is pressed plant sample deposited for future reference and it will be verify the identity of the specific plant used in a study. It should be deposited in a recognized herbarium indexed in "*Index Herbariorum*". The herbaria also keep the voucher materials such as seeds, wood sections, pollen, micro slides, fluid preserved flowers, fruits etc. Herbaria keep all categories of plants specified by International Code of Nomenclature of algae, fungi and plants (ICN) (McNeill et al, 2012; Anonymous, 1996). Although the herbarium and specimen preparations are mostly confines to seed plants, there are specific techniques practicing to algae, fungi, and plants (Anonymous, 1996). These specimens are in liquid preservation for non-vascular plants, bryophytes etc. (Anonymous, 1996). As we may aware that now we are extracting bioactive compounds from these organisms and several of the international journals and periodicals insisting for the voucher specimen number for the specimens.

In the recent days it is practicing that a voucher specimen must be deposited in a recognized herbarium committed to long-term maintenance. It was started from knowledge exchange through ethnic groups by oral tradition and then in the documented form. Herbaria conserving identified and authenticated plants for future correspondence play an important role in this regard and it declared different auxiliary aspects of herbaria for phytomedicinal research (Funk, 2003; Neisbitt, 2014; Ahmed and Hasan, 2016). Also the vouchers are crucial in authenticating the taxonomy of an organism, as a tool for identifying localities of the taxon, and for additional taxonomic, genetic, ecological, and/or environmental research (Eisenman et al., 2012). Although collaboration between botanists and chemists is extremely important for the initial collection and identification of plant materials, a need also exists for documentation at later stages of research, such preparation for publications. Botanists collaborating with chemists need to inform and educate colleagues about proper vouchering methods and providing detailed voucher information in all publications. Making vouchers is extremely important, but sharing the information about where the vouchers are stored is also critical. Voucher information is being increasingly required by many journals publishing articles on plant-based chemistry research.

Since the identification and voucher specimen number is essential in these days for research as well as for publications such as plant natural products, pharmaceutical sciences, medicinal botany and allied subjects (WHO, 1998; Hildreth et al, 2007), the information on the herbarium specimens preparation for the plant identification and voucher specimen number we would be presenting useful information on these aspects.

Preparation of herbarium specimens:

The preparation of the specimens was followed by methods stated in Lawrence (1967), Jones and Luchinger (1987), Anonymous (1996) and Manilal and Kumar (1998)

i) Collection: The specimen material (plants) which you are interested in should be collected as whole (if they are herbs) or part of plant along with flowers and fruits/carpels (Figures 1 to 6). Before putting your specimens in the collection bag you should carefully remove all the insects, spider-webs and foreign bodies attached to your specimens. Then the specimens mounted in 42 cm x 29 cm (16 ½" x 11 ½") size blotting paper. If the plant specimen larger than the specimen blotting paper they can mounted in V or N or M shape to accommodate the entire plant material in above size. Also the leaves mounted in dorsiventral position. The half-size of the regular newspaper can fit this size and economical! Put the mounted specimens in between cardboards/wooden-frames (Figures 7 to 9).

A well-designed field notebook has numbered sheets in printed form with standard pro-forma for entering field data. Usually a field note book has 100 leaves and the pages are serially numbered (called field numbers) to be suffixed to the collector's name, when cited (Figure 10).

ii) Poisoning and drying the specimen: Poisoning kills the plants and prevents the formation of abscission layer and thereby the leaves, flowers and fruits will be intact with the specimen (twig) will not be getting detached from the plant. The poisoning is generally done by dipping the whole plant in a saturated solution of mercuric chloride in ethyl alcohol (usually 20 gm in a litre of alcohol). The plant is again put between the blotters in the presser till it gets completely dried. Mercuric chloride is corrosive for metals, and hence enamel trays and disposable gloves are used. Lauryl Pentachloro-phenate (LPCP) is also used (3.75% in white spirit) for poisoning the specimens. It is safer than mercuric chloride and leaves the plant features more intact. The solution can also be applied to mounted specimens by spraying. Then, the specimens are spread out for pressing and drying. It is important that the plants are put under sufficient pressure; otherwise more time will be required to achieve a good desiccation, besides they could be damaged by dampness and moulds. Every specimen in the press must be linked with the data in the field note book (Figure 10). Detailed notes should be entered in the field note book at the time of collection in the field itself. The best one can do is to use a tag for each specimen. Bulky plant parts can directly be placed in contact with corrugated material to speed up drying. Instead of blotting paper we can use newspapers, which are cheap and readily available. Once a specimen has become dry and stiff, it is ready for mounting.

Cacti, succulent and tuberous/bulbose plants can be cut their stems, and leaves into transverse and longitudinal sections to observe their morphology. These plants should be poisoned longer-time to completely kill the specimens. Also they can also the softening of the cactus can be aided by the immersion in boiling water for half a minute, taking care of avoiding the immersion of the flowers. Instead of boiling, one can employ dilute acetic acid or strong alcohol or formalin (1.5 parts formalin, 1 part water). Some plants have tubers or bulbs and they must be treated before drying.

iii) Mounting and labelling: The dried plant specimens are now ready for mounting on herbarium sheets. Fixing the processed plant specimen on herbarium sheet is called *mounting*. A standard herbarium sheet is 28 cm (breadth) x 42 cm (length) and usually made up of heavy long-lasting white handmade paper or thick sheet (Figure 11). The sheet is usually stiff and flexible so as to prevent damage during the handling of mounted specimens. The common technique is pasting specimens to sheet with natural glue (usually Gum Arabic). Small quantity of copper sulphate or thymol crystals or may be added to the glue as insect repellent. It is advised to have a paper bag/pouch attach to the herbarium sheet to keep any seed/fragments detached from the specimens (Figure 11). Now the herbarium specimen sheet pasted with a label usually at the right side bottom corner.

The herbarium specimen sheet is now ready for deposition/reposition in the one of the indexed herbaria for the plant identification as well as to acquire a "Voucher Specimen" number.

The non-vascular plant specimens (algae, certain fungi, lichens, bryophytes and certain pteridophyptes) either dried or can be fixed in FAA fixative [Formalin-Acetic Acid–Alcohol in the ratio of 50% ~ 70% Ethanol (90 ml), Glacial Acetic Acid (99.6%) 5 ml and Formaldehyde (38%) 5 ml] and bring to the herbarium (Lawrence, 1967; Anonymous, 1996). Further if you have any inquiry can contact the Curator or Prof-in-charge of the herbarium.

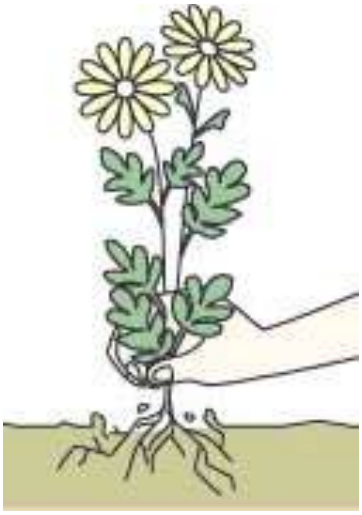


Figure 1*

Figure 2

Figure 3

Figure 4

Figure 5

Figures 1-5: Collect the whole plant with the roots (if possible) or branches with flower & fruits

Figure 6*: Insert the plant between the newspaper sheets

Figure 7*: Write the information about the plant (e.g., location, date, collector, habitat, etc.)

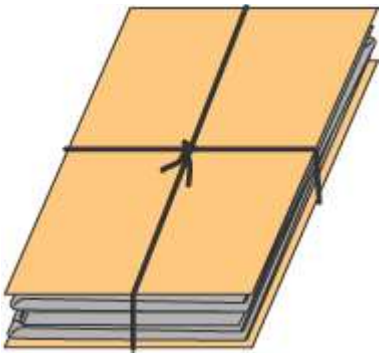
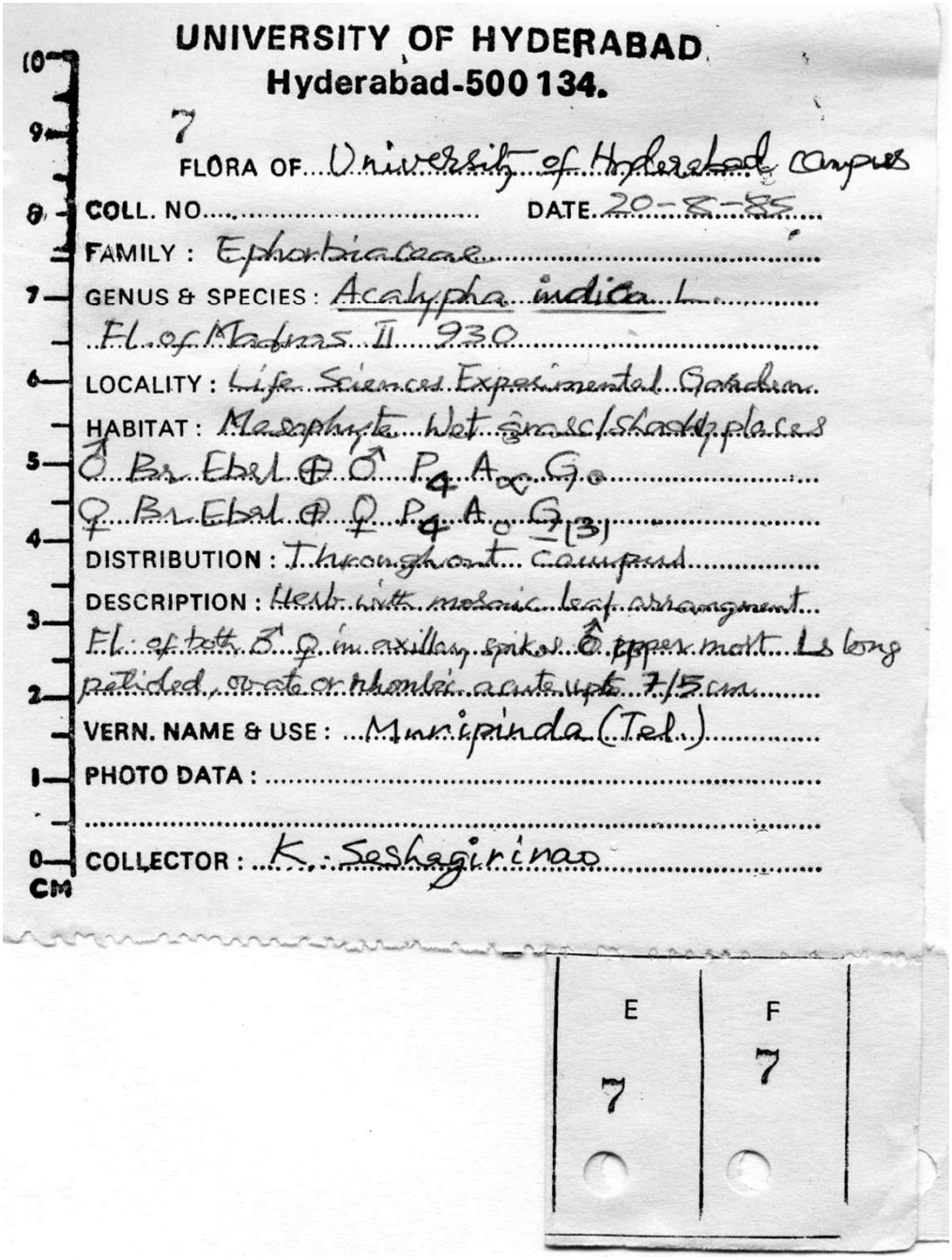


Figure 8*

Figure 9

Figures 8* & 9: Bundle the plants (in newspapers) with the field boards/wooden-frame



UNIVERSITY OF HYDERABAD
Hyderabad-500 134.

7

FLORA OF... University of Hyderabad campus

COLL. NO..... DATE. 20-8-85

FAMILY : Euphorbiaceae

GENUS & SPECIES : Acalypha indica L.

Fl. of Madras II 930

LOCALITY : Life Sciences Experimental Garden.

HABITAT : Mesophyte Wet areas/shaddy places

♂ Br. Ebl. @ ♂ P4 A∞ G0

♀ Br. Ebl. @ ♀ P4 A0 G(3)

DISTRIBUTION : Throughout campus

DESCRIPTION : Herb with mosaic leaf arrangment.

Fl. of both ♂ ♀ in axillary spikes ♂ upper most 1.5 long

pilided, ovate or rhombic acute upto 7/5 cm.

VERN. NAME & USE : Munipinda (Tel.)

PHOTO DATA :

COLLECTOR : K. Seshagirirao

E 7 F 7

Figure 10: A page from field note book

Figure 11: Mounted herbarium specimen with number tag and seed/fragment pouch

Recently the Nihon Vogue-sha, Japanese company is offering a new approach/technique to botanical plant preservation for herbarium specimens. The "Plant Specimen Preparation Kit" utilizes a simple and reliable vacuum pump and a unique air press to ensure that plant specimens are beautifully preserved with their natural colors retained (Anonymous, 2016).

This method also offers an improvement in preserving plant specimens by using special "stock" bags that create an airtight condition for long periods. These stock bags are the optimal way to display natural looking plant specimens and are ideal to protect specimens loaned to other institutions or individual researchers. These bags are essential for long lasting specimen preservation (Figures 12 to 14). You watch the drying process in a video at https://www.oshibana.com/herbarium/en/index.php/making-a-specimen/drying-the-plant . The Plant Specimen Preparation Kit makes it easy to solve the problems that have traditionally been part of creating conventional pressed flowers and plants.

- It's easy to do and takes less time
- The beautiful plant colors are saved/preserved
- A special bag prevents damage so you'll feel a great satisfaction with the beautiful results of your efforts.

Figure 12*: Nihon Vogue-sha, Japanese company's "Plant Specimen Preparation Kit"

Figure 13*: Peanut plant herbarium specimen

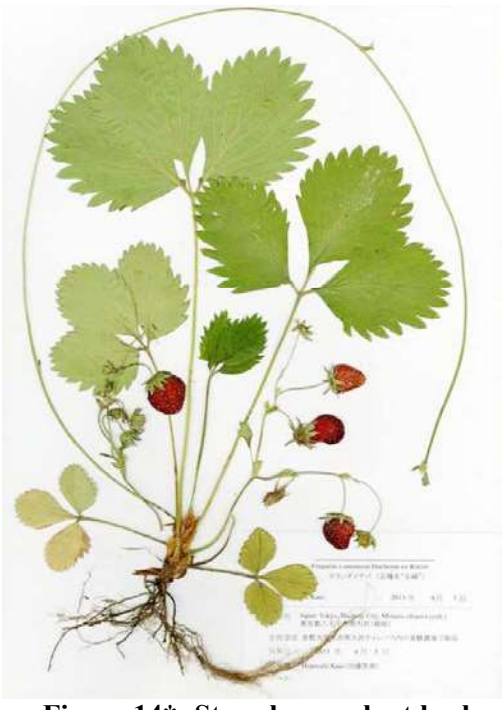


Figure 14*: Strawberry plant herbarium specimen

*The figures & information are curtsey of Nihon-Vogue-sha, Tokyo, Japan (https://www.oshibana.com/herbarium/en/index.php)

Conclusions

Voucher specimens provide a permanent, physical record and form the foundation on which all natural product research stands (Funk, 2003; Culley, 2013). The annotations and recommendations in this regard are noteworthy (Perkins, 2016). An additional voucher specimen is advisable, and can serve back-ups in case damage to or loss of the main voucher specimen. The preparation and proper storage of vouchers can also include provide plant tissue for DNA analysis. In the recent the research journals now require that DNA and amino acid sequences intended for publication be submitted to a sequence database, such as GenBank before being published, but many journals do not yet require that vouchers be made for the plants from which these sequences were isolated (Pleijel et al., 2008). It is now indeed very much critical for dioecious plants where the male and female plants analysis followed by separate voucher specimen number for each sex plant. The additional conclusion on the usage of the "Plant Specimen Preparation Kit" from Nihon Vogue-Sha, Japan would be more promising for specimens' preparation both vascular and non-vascular plants where there is no involvement of chemicals and harsh environmental conditions. These specimens can be recommending using in the analysis of chemical, biochemical and molecular biology.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

The authors are acknowledging the University of Hyderabad for providing facilities. Thanks are due to DST-SERB (Ref: SB/EMEQ-294/2013), IRTG–MCGS (DFG, Germany), IRTG-UGC, DST-FIST, UGC-SAP and UGC-DRS projects/programs for their support funding research facilities. Thanks are also due to Dr. Boyd Lugenbehl, Executive Director, TechTrans, Inc. (Representing Nihon-Vogue-sha), Tokyo, Japan for permitting to use their photos and other information from their web source at http://www.oshibana.com/herbarium/en

References

Ahmed, S. and Hasan, M.M., 2016: Importance of herbaria in herbal drug discovery, *World J Pharm Sci.,* **4:** 127-129.

Anonymous, 1996: Techniques and procedures for collecting, preserving, processing, and storing botanical specimens, British Columbia Ministry of Forests Victoria, B.C. Work. Pap.

Anonymous, 2016: Plant Specimen Preparation Kit (http://www.oshibana.com/herbarium/en) (Accessed on July 24, 2016)

Culley, T.M., 2013: Why vouchers matter in botanical research, *Appln. Pl. Sci*., **1:** 1-5.

Eisenman, S.W., Tucker, A.O., and Struwe, L., 2012: Voucher Specimens are Essential for Documenting Source Material Used in Medicinal Plant Investigations, *Journal of Medicinally Active Plants*, **1:** 30-43.

Funk, V., 2003: The importance of herbaria, *Pl. Sci. Bull.* **49:** 94-95.

Hildreth, J., Hrabeta-Robinson, E., Applequist, W., Betz, J. and Miller., J., 2007: Standard operating procedure for the collection and preparation of voucher plant specimens for use in the nutraceutical industry. *Anal. Bioanal. Chem*., **389:**13-17

Jones, Jr. S.B. and Luchsinger, A.E. 1987: Plant Systematics and Evolution, McGraw-Hill International Editions, New Delhi, India

Lawrence, G.H.M., 1967: Taxonomy of Vascular Plants, Oxford & IBH Publishing Co., New Delhi, India.

Manilal, K.S. and Kumar, M.S.M, 1998: A Handbook on Taxonomy Training, Department of Sciences and Technology, Govt. of India, New Delhi, India

McNeill, J., Barrie, F.R., Buck, W.R., Demoulin, V., Greuter, W., Harksworth, D.L., Herendeen, P.S., Knapp, S., Marhold, K., Prado, J., Prud'Homme van Reine, W.F., Smith, G.F., and Wiersema, J.H., 2012: International Code of Nomenclature for algae, fungi and plants (Melbourne Code) adopted by the Eighteenth International Botanical Congress Melbourne, Australia, July 2011. Publ. (Regnum Vegetabile, 154). XXX, 240 p. (ISBN 978-3-87429-425-6); The web copy also available at http://www.iapt-taxon.org/nomen/main.php (Accessioned on 24th July, 2016)

Nesbitt, M., 2014: Use of herbarium specimens in ethnobotany: In Curating Biocultural Collections - A Handboo, Chapter 22, Salick, J., Konchar, K. and Nesbitt, M. (Eds.), p. 250, Royal Botanical Garden, Kew, UK., E-book: ISBN: 9781842465097 (Available at http://www.ancientgrains.org/Curating_Biocultural_Collections_22.pdf

Perkins K.D., 2016: Annotation of herbarium specimens: Recommendations, https://www.flmnh.ufl.edu/herbarium/anno/ (Accessioned on 14th July, 2016)

Pleijel, F., Jondelius, U., Norlinder, E., Nygren, A., Oxelman, B., Schander, C., Sundberg, P., Thollesson, M., 2008. Phylogenies without roots? A plea for the use of vouchers in molecular phylogenetic studies. Mol. Phylogenet. Evol. 48, 369–371.

Thiers, B., 2016 [continuously updated], : Index Herbariorum: A global directory of public herbaria and associated staff, New York Botanical Garden's Virtual Herbarium, http://sweetgum.nybg.org/science/ih/ (Accessioned on Accessioned on 24th July, 2016)

World Health Organization (WHO), 1998: *Quality control methods for medicinal plant materials*, World Health Organization, Geneva.