IN VITRO PROPAGATION AND *IN SILICO* STUDIES OF IMPORTANT MEDICINAL PLANTS

THESIS

Submitted to the Delhi Technological University
for the award of the degree of

DOCTOR OF PHILOSOPHY

in

BIOTECHNOLOGY

By

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JULY 2018

Dedicated to My Parents

DECLARATION

I, Nupur Jauhari, certify that the work embodied in this Ph.D. thesis is my own bonafide work carried out under the supervision of Dr. Navneeta Bharadvaja (Assistant Professor, Department of Biotechnology, Delhi Technological University, Delhi, India) and Dr. Neelam Sharma (Principal scientist, ICAR-National Bureau of Plant Genetic Resources, Delhi, India) for a period of November, 2011 to May, 2018. The matter embodied in this Ph.D. thesis has not been submitted for the award of any other degree/diploma.

I declare that I have devotedly acknowledged, given credit and refereed to the research workers wherever their works have been cited in the text and the body of thesis. I further certify that I have not willfully lifted up some other's work, para, text, data, results etc. reported in the journal, books, reports, dissertations, thesis etc. or available at websites and included them in Ph,D thesis and cited as my work.

Date:
Place:

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(Reg. No: 2K11/PhD/BT/06)

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CERTIFICATE

This is to certify that the Ph.D. thesis entitled **"*In vitro* propagation and *in silico* studies of important medicinal plants"** submitted by **Nupur Jauhari** (Reg. No: 2K11/PhD/BT/06) to the Delhi Technological University, Delhi for the award of the degree of **Doctor of Philosophy** is based on the original work carried out under our supervision. It is further certified that the work embodied in this thesis has neither partially nor fully submitted to any other University or Institution for the award of any degree or diploma.

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ACKNOWLEDGEMENT

I am indebted to God under whose power I pursued this Ph. D. Thanks to Almighty for granting me wisdom health and strength to undertake this research task and enabling me to its completion. I pray that I can always serve him in whatever he wants me to; and for which I need his blessings too.

My parents are my life-coach, especially my loving, encouraging and adorable my ***(late) mother Vibha Saxena*** because I owe it all to her. I deeply miss my mother who is not with me to share this joy. I feel proud of dedicating this Ph. D. onto her lotus feet. I am grateful to my father ***Dr. R.P. Saxena*** being an inspiration for me from the childhood. He always taught me not to fear in any adverse situation and fight all odds with courage and patience. His journey of life is an excellent example for me in achieving this doctoral degree. The learning's delivered by my parents will always enlighten my path of life. Many Thanks! To my parents.

I am grateful towards my supervisor, ***Dr. Navneeta Bharadvaja***, without whom this achievement would not have been realized. It has been an honor to be her first Ph.D. student. It was her valuable guidance and consistent encouragement all through my research period, which helped me to overcome the challenges that came in the way. This feat was possible only because of the unconditional support provided by her. A person with an amicable and positive temperament, she has always made herself available to clarify my doubts despite her busy schedules and I consider it as a great opportunity to do my doctoral programme under her guidance and to learn from her research experience.

I would like to express my sincere gratitude to my co-supervisor ***Dr. Neelam Sharma*** for her insightful comments, encouragement and the hard questions, which incented me to widen my research from various perspectives. Her critical assessment of my research work is worth appreciable, as it made me learnt various critical aspects of scientific writing, importantly how to put the ideas going on in the mind into words, which are self descriptive and specific, thus providing a smooth reading. I am heartily thankful to both of them for the precious qualities I have

received from them, which will always stay with me as their reminiscences.

I am thankful to ***National Bureau of Plant Genetic Research***, New Delhi for providing plant material for my research work. I express my regards to ***Dr. Rakesh Singh***, Principal Scientist, National Bureau of Plant Genetic Resources, India who developed CBDP markers and suggested me to use these markers on my plants.

My sincere regards to ***Prof. Yogesh Singh, Vice Chancellor, Delhi Technological University*** for providing me a platform for pursuing my Ph.D. work. I express my gratitude to ***Prof. Jai Gopal Sharma, Head, Department of Biotechnology*** *and* ***Prof. D. Kumar, Former Head, Department of Biotechnology*** for their kind support and providing necessary facilities to undertake this research. I take this opportunity to thankfully acknowledge ***Prof. B.D Malhotra, Dr. Asmita Das, Dr. Yasha Hasija, Prof. Pravir Kumar*****,** all my teachers and rest of my research committee.

I would also like to thank the senior lab technicians and staff members ***Mr. C. B. Singh, Mr. Jitendra Singh Mr. Sandeep, Mr. Rajesh*** for providing me the chemicals and helping me in running the instruments as per my requirement during the research work.

I do not have words to thank my fellow labmates and friends of the department of biotechnology and applied chemistry that helped me whenever I needed. It is my privilege to thank ***Nidhi, Richa, Aditya, Isha, Himani, Abhishek, Saumya, Mr. Satish*** and many others who offered me their time, help and support whenever needed. I am thankful to ***Harshita*** for her timely help and support when I needed the most.

"Kind words can be short and easy to speak, but their echoes are endless." Special mention goes to my husband ***"Mr. Anurag Jauhari"***. He is the real power behind my energy and completion of Ph. D. Whenever I feared of hard times, I felt sheltered through his care and belief on me. I thank not only for his tremendous academic support but also for motivating me whenever I feel upset. He had always inspired me to overcome all the hurdles and failure faced during my research work and will keep inspiring me in future too. ***Thanks for being my life partner***. Extra special thanks to my two little kids' son ***Vijyant*** and

daughter ***Jaazlyn*** who always supported me to achieve my goal with their love and affection. Thank You!

The constant support of my mother-in-law and father-in-law was great impetus for me. My deep sincere regards goes to my brother-in-law ***Lt Col. Ashish Jauhari*** and his wife ***Monika Jauhari*** for continuous moral support and help. I also thank to other family members for understanding my situation during Ph.D.

Very sincere and endless thanks to my sister ***Parul Saxena*** and brother ***Ankur Saxena*** who always supported me at every stages of my life. They strengthen me by providing moral and emotional support. I am confident to be energized with their care, affection and brilliant advises in rest of my life. I express my gratitude to my other loving family members who always showed confidence on me in hard times.

During the amazing journey of my Ph.D. I would also like to thank all those who tried to bring me down, but remained unable to do so. In fact, their efforts prompted me to work hard, even harder to work out my abilities.

Nupur Jauhari

ABSTRACT

Blooming and acceptability of traditional system of medicine has drastically increased the demand of medicinal plants. Demand of therapeutically important medicinal species is continuously increasing in national and international markets. Exploring elite accessions among several accessions, collected from different agro-climatic zones, can be a suitable approach to meet the increasing demand of medicinal plants. Biotechnological approaches such as plant tissue culture and molecular marker-based techniques are reliable for producing elite accessions and offer long-term utilization of the herbs.

Two pharmaceutically important medicinal plants, *Bacopa monnieri*(L.) Wettst and *Chlorophytum borivilianum* Sant and Fern. have been explored in the present study.

In vitro conservation protocol was established for 13 accessions of *B. monnieri*, a medicinal plant with legendry reputation as brain energizer. Sixteen media combinations (MS with varying concentrations of auxins and cytokines) were tested for 13 accessions of *B. monnieri* to find the optimum media based on growth parameters for *in vitro* propagation. The accession with maximum number of shoots, shoot length and number of propagules was selected for further study. Furthermore, the concentration of therapeutically important secondary metabolite such as total bacosides, total phenol content and antioxidant potential of 13 accessions of *B. monnieri* were evaluated. The concentration of total bacosides in 13 accession ranged from 216.93 - 515.47 µg/g dry weight bases. Amount of total bacosides and total phenol content in wild grown plants is generally low. Use of elicitors in cell culture technology not only increased the yield of total bacosides (2.7-3.9 fold) but the production of total phenols and antioxidant potential has also been increased to 5-18 fold and 7.9 fold respectively. Principal Component Analysis (PCA) using correlation matrix was carried out to evaluate the relative contribution of moisture content, antioxidant potential, total bacoside and total phenols to the total variability in the *B. monnieri* accessions. Moreover, accessions of different agro-climatic zones were assessed for genetic diversity analysis with gene targeted molecular markers SCoT (Start Codon Targeted Polymorphism) and CBDP (CAAT box Derived Polymorphism). Genetically diverse

accessions can be utilized by plant breeders to produce elite accessions having high amount of therapeutically important secondary metabolites. Accession-based study of *in vitro* plant cultures based on growth parameters, presence of therapeutically important secondary metabolites and antioxidant potential has identified accessions IC 554588, IC 344312 and IC 554585 of *B. monnieri* as elite accession.

The present study also explored the effect of bacoside on age-related neurodegenerative diseases through *in silico* studies as well as through *in vitro* assay. AD is associated with depletion in amount of acetylcholine, a neurotransmitter. The results generated by molecular docking and molecular dynamics simulation programs revealed that the AChE complex with bound Bacoside is very stable throughout the simulations period of 30 ns.

The second important medicinal plant is *C. borivilianum*, known to possess stigmasterol, a phytosterol. Ten media combinations have been used for *in vitro* propagation and root induction. The optimum selected media based on growth parameters such as maximum number of shoots, shoot length, number of roots and root length can be used for rapid proliferation and conservation. Biochemical analyses of pharmaceutically active metabolite, stigmasterol and total phenols along with antioxidant potential have been assessed for the three accessions. Variable amount of stigmasterol (1.18-1.58 mg/g DW), phenol content (89.0 - 140.0 µg/g) and antioxidant potential has been observed in three accessions. The accession with maximum biochemical activity was selected for elicitation study. Elicitation has increased stigmaterol 3.2 fold, 4.3 fold and 17.7 fold. Principal Component Analysis showed antioxidant potential as major contributor for variability. Accession IC 558379 has been identified as promising accession with higher stigmasterol production, phenol content and antioxidant potential.

The present study also explored the anticancer activity of stigmasterol through computational approach. The ligand stigmasterol was docked with the target tumor necrosis factor alpha converting enzyme (TACE) to explore its effect on colon cancer. It can be suggested that stigmasterol, a green material may be considered as a possible therapeutic agent in the treatment of colon cancer and the same can certainly be confirmed through *in vivo* studies and clinical trials to confirm their effectiveness in patients.

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LIST OF ABBREVIATIONS

2,4-D	:	2,4- dichlorophenoxy acetic acid
AC	:	Activated Carbon
AChE	:	Acetylcholinesterase
AD	:	Alzheimer's disease
BA	:	Benzyl Aminopurine
CBDP	:	CAAT box-derived polymorphism
CUPRAC	:	Cupric ion reducing antioxidant capacity
DMEM	:	International Union for Conservation of Nature and Natural Resources
DMRT	:	Duncan's Multiple Range Test
DPPH	:	2,2-diphenyl-1-picrylhydrazyle
ELISA	:	Enzyme-linked immunosorbent assay
FTIR	:	Fourier Transform Infrared Spectroscopy
HPLC	:	High Performance Liquid Chrmatography
HPTLC	:	High Performance Thin Layer Chromatography
IAA	:	Indole Acetic Acid
IBA	:	Indole Butyric Acid
JA	:	Jasmonic Acid
JSC	:	Jaccard's similarity coefficient
Kn	:	Kinetin
LOD	:	Limit of Detection
LOQ	:	Limit of Quantification
MD	:	Molecular Dynamics

ME	:	Malt Extract
MLR	:	Multilinear regression
MS	:	Murashige and Skoog
NAA	:	Naphthalic Acetic Acid
NMPB	:	National Medicinal Plant Board
PCA	:	Principal Component Analysis
RMSD	:	Root Mean Square Deviation
SA	:	Salicylic Acid
SCoT	:	Start Codon Targeted Polymorphism
SH	:	Schenk and Hildebrandt
TACE	:	Tumour Necrosis Factor Alpha Converting Enzyme
TPC	:	Total Phenol Content
UPGMA	:	Unweighed Pair-Group Method with Arithmetic Means

LIST OF PUBLICATIONS

1. **Nupur Jauhari**, Navneeta Bharadvaja, Neelam Sharma (2017) *Swertia chirata*: A comprehensive review with recent advances. Current Pharmaceutical Biotechnology 18:730-739. doi: 10.2174/1389201018666171026153515

2. **Nupur Jauhari**, Sanjay Menon, Neelam Sharma and Navneeta Bharadvaja (2017) Uptake of heavy metals from industrial wastewater using in vitro plant cultures. Bulletin of Environmental Contamination and Toxicology 99:614-618. doi: 10.1007/s00128-017-183-6

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6. **Nupur Jauhari**, K Lavanya, Navneeta Bharadvaja, Neelam Sharma (2014) *In vitro* propagation of endangered medicinal herb *Chlorophytum borivilianum* Sant and Fern. Proceedings of National Symposium on Crop Improvement for Inclusive Sustainable Development. Indian Society of Genetics and Plant Breeding, Nov 07-09, 933-935. ISSN No (Online): 0975-6906

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1. **Nupur Jauhari**, Navneeta Bharadvaja, Neelam Sharma (**2016**) Phytoremediation of heavy metals from polluted water using *Bacopa monnieri* (L.). 1st International Agrobiodiversity Congress, November 06-09, Pusa Campus, New Delhi, India.

2. **Nupur Jauhari**, Sanjay Menon, Navneeta Bharadvaja, Neelam Sharma (**2016**) Plant derived bio-active compound to remediate Chromium and Cadmium from industrial waste water to save environment, ECO-BIO, March 06-09, Beurs World Trade Centre, Rotterdam, The Netherlands.

3. **Nupur Jauhari**, Navneeta Bharadvaja, Neelam Sharma (**2016**) Anticancer property of green material through computational approach, International Conference on Materials Science & Technology (ICMTech), March 01-04, University of Delhi, Delhi, India.

4. **Nupur Jauhari**, Navneeta Bharadvaja, Neelam Sharma (**2013**). *In Silico* studies on phytocompound as lead molecule against colon cancer. Asian Congress on Biotechnology (Acb) December 15-19, Indian Institute of Technology, Delhi, India.

5. **Nupur Jauhari**, Navneeta Bharadvaja, Neelam Sharma (**2013**) Rapid *in vitro* multiplication via axillary meristems of *Bacopa monnieri* [Brahmi]. India-Japan Workshop on "Biomolecular Electronics and Organic Nanotechnology for Environment Preservation" (IJWBME), December 13-15, Delhi Technological University, Delhi, India.

6. **Nupur Jauhari**, Navneeta Bharadvaja, Neelam Sharma (**2013**) Accumulation of bacosides in an endangered medicinal herb *Bacopa monnieri*, Indraprastha International Conference on Biotechnology (IICB), October 22-25, Guru Gobind Singh Indraprastha University, New Delhi, India.

7. **Nupur Jauhari**, Navneeta Bharadvaja, Neelam Sharma (**2013**) High frequency multiple shoots induction in *Bacopa monnieri*: An important medicinal plant. National Conference On Recent Advances In Biotechnology & Nanobiotechnology (BIONANO), October 29-30, Amity University Gwalior, India.

CHAPTER 1

INTRODUCTION AND OBJECTIVES

1.1. Introduction

Conservation of medicinally important germplasm is of global concern. Economically important medicinal plants can be conserved through *in vitro* and cryopreservation techniques. Conservation can be done in their natural habitat (*in situ*) and apart from their natural habitat (*ex situ*). Conservation of medicinal plants leads to management and sustainable use of indigenous and exotic plant genetic resources for herbal drug preparation and carry out related research.

Diversity of medicinal plants is being lost at an unprecedented rate. Furthermore, inadequate availability of useful germplasm to breeders is the major cause for limited use of germplasm in breeding programs presently. Exploring different accessions in providing valuable traits to species is demanded. Simultaneously, studying different accessions for revealing genetic diversity from which selection can be made for improvement is justifiable. Genetic diversity can be assessed with the use of molecular markers, which are very reliable in genetic diversity analysis because of their high power of resolution. Crosses between species having desirable trait contribute to crop improvement. Genetically diverse and improved accessions can be safely conserved in *in vitro* repository, which can be utilized by plant breeders. Reproductive and vegetative propagating material can be conserved as plant genetic resources. Plant genetic resources are the only source of plant genetic diversity that provides valuable traits required to combat the challenges of adapting plant species.

Herbal plants are the priceless resource of new drugs. Over-exploitation, unorganized collection of the plants from their natural habitat by medicinal plant-based industries, urbanization, industrialization, invasion of exotic species that compete with native species and inadequate availability in gene bank has resulted in population depletion from wild. Hence, it is essential to strike a balance between conservation and utilization of important herbal plants.

Medicinal plants contain high concentrations of bioactive compounds. Bioactive compounds or phytochemicals are secondary metabolites produced by the medicinal plants. These phytocompounds can act as lead molecule, drug precursors and pharmacological probe. *Bacopa monnieri* (L.) Wettst. and *Chlorophytum borivilianum* Sant and Fern. are the two important medicinal plants that produce pharmaceutically important secondary metabolites. *B. monnieri* contains bacosides, which is used to relive mental stress, improve memory function, anxiety neurosis (Tiwari et al. 2001; Kumar et al. 2016) and neuropharmacological disorders (Russo and Borrelli, 2005) like Alzheimer's and Parkinson's diseases. Important secondary metabolite of *C. borivilianum* is stigmasterol, which acts as rejuvenator, aphrodisiac and as anticancer agent for colon cancer and prostate cancer. Because of these properties, pharmaceutical companies collect these plants from their natural habitat in order to extract these bioactive compounds. However, wild collection of the plants can be saved by utilization of *in vitro* plant cultures. Use of *in vitro* plant cultures has several advantages over landracers besides conserving the valuable plant species. *In vitro* cultures does not depend on season, grown on microbial free environment, can be manipulated to differentiate the outcome and metabolic potential of *in vitro* maintained plants from the wild grown plants as they encountered with microorganisms. Plant tissue culture

approaches can be applied to understand the enzymatic process involved metabolic pathways including phytoremediation. Production bioactive compounds can be enhanced with the incorporation of elicitors in *in vitro* plant cultures.

Apart from that, presence of bioactive compounds, their yield and composition is strongly affected by environmental conditions. Different accessions belonging to different agro-climatic zones contain different concentrations of bioactive compounds. Environmental conditions or stressors may decrease the total yield but the overall therapeutic potential may be significantly increased by increasing the bioactive compounds. When a plant is encountered to stressors, also known as elicitors, enzymatic pathways are encouraged that enhance the yield of biologically active secondary metabolites (Ebel and Cosio, 1994).

The overall information by accession-based study can be utilized by plant breeders to produce superior accessions and help in identifying the elite accession with maximum concentration of bioactive compounds. The superior accession or elite accession can be supplied to farmers for commercial production and to pharmaceutical industries to produce superior quality drugs.

Although, therapeutic effects of *B. monnieri* and *C. borivilianum* are well documented with their respective secondary metabolites bacosides and stigmasterol, the detailed specific action mechanism on the specific disease-associated targets is still unknown. A new trend in the preparation and marketing of plant-derived products has come up with the use of bioinformatics tools/computational tools, which helps in identifying specific target of the particular disease in less time and reduced cost. Plant secondary metabolite acts as ligand against the target of corresponding disease. The specific bioinformatics

tools analyze stability of the complex (ligand and target), which also helps to identifying lead molecule for the particular disease. To make this perception clear, we have used computational tools to elucidate the mode of action of bacosides and stigmasterol on their respective targets using *in silico* experiments. Acetylcholinesterase (AChE), a potential target for Alzheimer's disease (AD) (Tsolaki et al. 2001) was selected for *in silico* elucidation of bacosides. Whereas, tumor necrosis factor alpha converting enzyme (TACE) has been selected as probable target for colon cancer with stigmasterol as ligand based on various studies associate TACE with colon cancer (Ramirez et al. 2007).

Currently, the role of computational tools has become remarkably indispensible in journey of drug discovery and design processes. Techniques from computational chemistry are now used regularly to explore the drug-receptor complexes in atomic detail and to calculate characteristics of small-molecule drug candidate. Techniques like molecular docking and molecular docking simulations can be used to elucidate the interaction between ligands (bacosides and stigmasterol) and their respective target molecules (AChE and TACE) to identify the stability of the interactions. Molecular docking studies have been used to identify the binding modes. The interactions between ligands and their macromolecular receptors can be simulated through molecular dynamics studies consequently describing the flexibility of targets and of the ligands.

1.2. Objectives

Present work was therefore undertaken to study *in vitro* propagation techniques for the production of secondary metabolites, phytoremediation potential and genetic diversity analysis of tissue culture plants to obtain the elite accession. Mode of action of bacosides from *B. monnieri* and stigmasterol from *C. borivilianum* has also been studied.

i. *In vitro* propagation of medicinal plants
    i. *In vitro* multiplication and proliferation of medicinal plants
    ii. Biochemical Analysis
    iii. Phtyoremediation
    iv. Genetic Diversity Analysis

ii. Elucidation of pharmacological mode of actions of important secondary metabolites
    i. *In silico* insight into potential anti-Alzheimer's disease activity of *Bacopa monnieri* (L.) Wettst.
    ii. *In silico* insight into potential anticancer activity of *Chlorophytum borivilianum* Sant and Fern.

CHAPTER 2
LITERATURE REVIEW

2.1. Plant tissue culture

Plant tissue culture is the science of growing plant cell, tissue or organs on artificial nutrient media under controlled environmental conditions. Most of the produced plants are true-to type of selected genotype. The controlled conditions for growth and multiplication involve adequate supply of nutrients, suitable pH medium, optimum temperature and appropriate liquid and gaseous environment. Plant tissue culture is a valuable technique for mass multiplication and conservation of endangered medicinal plants. Therapeutic effects of medicinal plants are attributed to their bioactive compounds or secondary metabolites because of which wild plants are exploited by pharmaceutical industries. At present herbal drugs are in great demand in national and international market.

Loss of habitat, over-exploitation and inadequate availability in gene bank are the few major factors responsible for plants becoming endangered. It is undoubtedly necessary to conserve these priceless resources. *In vitro* propagation is a promising alternative in increasing demand of medicinal plants with various advantages over traditional breeding such as rapid multiplication of desired plants, all time availability of planting material i.e. independent to season, production of disease free planting material, reduction in land use, independent of environmental factors such as climate, pests, geographical and seasonal constrains, use of improved strategies for better yield and

cost benefit ratios by growth medium and microenvironment manipulation, possibility of obtaining continuous production of metabolites that usually transiently occur in the life cycle of wild plant, understanding of complex metabolic pathways involved in the synthesis of secondary metabolites production and identification of key enzymes, use of metabolic engineering at cellular level for enhanced production. Moreover, tissue cultured plants have potential to produce higher amount of secondary metabolites (Sharma et al, 2012) and more potent of remediating toxic heavy metals from polluted wastewater (Jauhari et al..2017).

B. monnieri and *C. borivilianum* are the medicinal plants that hold important place in '*Ayurveda*' largely due to high therapeutic value. These therapeutically and economically important medicinal plants are harvested by pharmaceutical industries from their natural habitat, which leads to habitat destruction and causes depletion of plant material. The annual trade of *B. monnieri* was around 1000-2000 MT per year. The plant is categorized as prioritized medicinal plant with 30% subsidy at the cost of Rs. 23425.6 per acre for 2016-17 (National Medicinal Plant Board, 2017), which is expected to increase in the upcoming years due to its multifarious remedial properties. NMPB and Technology Information Forecasting and Assessment Council (TIFAC) listed *B. monnieri* as one of the seven important medicinal plants that require immediate attention (http://www.nmpb.nic.in/prioritisedmedicinalplants.htm). IUCN has categorized *B. monnieri* as endangered. Dried roots of *C. borivilianum* are available in Indian market at a price of Rs. 1000-2300/kg. It was ranked as prioritized medicinal plant with a cost of Rs 1,83,012.5 per acre in 2016-17 with 30% subsidy (NMPB, 2017). Demand of phytosterols including stigmasterol is increasing worldwide. Global market value of

phytosterol is expected to increase USD 7216 Million by 2020 (Markets and Market, 2015 https://www.marketsandmarkets.com/). According to IUCN, status of *C. borivilianum* is critically endangered.

2.2. About the plants

2.2.1. *Bacopa monnieri*

B. monnieri has long history of ethanopharmacological relevance in Indian *Ayurveda* as '*Medhya Rasayana*' (relive mental stress), commonly known as Brahmi (family - Scrophulareaceae). The plant is grown in wetland and muddy shores. It grows faster in 30°C to 40°C and 65-80% humidity. Whole plant of *B. monnieri* is medicinally useful (Bone, 1996). Historically, *B. monnieri* has esteemed reputation as memory vitalizer. According to *Ayurveda*, it is categorized as 'Medhya Rasayana', which is used to relive mental stress, improves memory function and anxiety neurosis (Tiwari et al. 2001; Kumar et al. 2016).

i. *In vitro* conservation

High pharmaceutical demand generated the need to conserve the pant. *In vitro* propagation is the plant tissue culture technique to conserve therapeutically important medicinal plant. Many researchers have reported *in vitro* propagation for *B. monnieri* (Tiwari et al. 2001; George et al. 2004; Sharma et al., 2007; Verma et al. 2012; Jauhari et al. 2016).

ii. Pharmacological significance

Apart from vitalizing memory functions, Brahmi plays a significant role in antidepressant (Dar and Channa, 1997), antiinflammatory, immunomodulatory, antioxidant

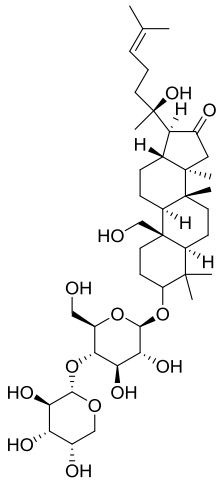
(Bhattacharya and Ghosal 1998), cardiac diseases (Roodernrys et al., 2002), cell stabilization, hepatoprotactive (Sairam et al., 2002) and neuropharmacological disorders (Russo and Borrelli, 2005). *B. monnieri* is effective against age-related neurodegenerative diseases (Singh et al., 2008; Stough et al., 2008) such as Alzheimer's disease (AD), Parkinson's disease and schizophrenia. AD is characterized with reduced concentration of neurotransmitter acetylecholine. Acetylecholine plays vital role in memory and cognitive function (Matthews 1996). Acetylecholine is hydrolyzed by acetylcholine esterase (AChE) to choline and acetyl group. Reduction in the concentration of acetylecholine is associated with decreased cognitive activities (Francis et al 1999).

Studies revealed that *B. monnieri* contributes in increasing the level of acetylcholine (Bhattacharya et al. 2000). According to Rastogi et al. (2012) bacoside is effective against age-related neuroinflammation. Free radical scavenging activity of *B. monnieri* has been investigated by many researchers (Meena et al. 2012; Jain et al. 2016). Anabasari et al. (2005) revealed that aqueous extract of bacoside A inhibited lipid peroxidation, boosted the functions of adenosine triphosphatases (ATPases) and supported ionic balance in the brain of rats exposed to cigarette smoke. Russo et al (2003) revealed antioxidant potential of methanolic extract of whole plant *in vitro* through DPPH (2,2-diphenyl-1-picrylhydrazyle) assay. Vijayan and Helen (2007) have investigated hepatoprotective role of *B. monnieri* via normalizing the values of hepatic glutathione, alkaline phosphatase and glutathione-S-transferase.

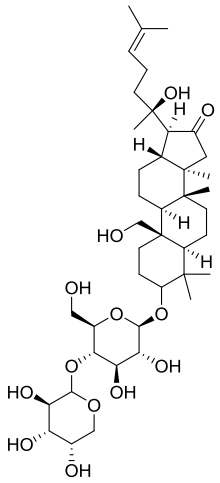
iii. Phytochemical constituents

Pharmacological value of *B. monnieri* is due to the presence of saponins that are potent nervine activator, such as bacoside A, bacoside B, bacoside C, jujubognin and

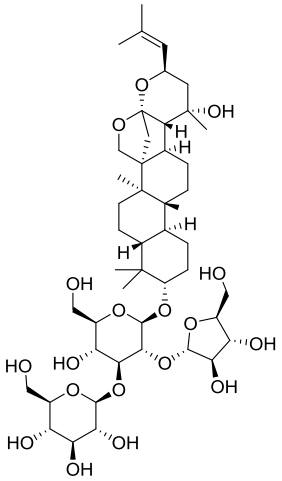
pseudojujubognin (Rastogi et al., 1994). *B. monnieri* also contains alkaloids such as nicotine, brahmine, herpestine including several other chemicals like b-sitosterol and stigmastanol (Basu et al., 1967). Saponins bacopasides I – XII have also been isolated by researchers (Garai et al 1996; Chakravarty et al. 2001; Chakravarty et al. 2003). Bacoside A is a mixture of bacoside A_3, bacopacide II, bacopasaponin C and jujubogenin (Deepak et al. 2005). Agrawal et al. (2006) confirmed the presence of bacoside A3 and bacoside II in the extract of *B. monnieri* through HPTLC. Pawar and Jadhav (2015) identified and quantified bacoside A from methanolic extract of *B. monnieri.* Whole plant is widely used in *Ayurvedic* formulations and various commercial formulations such as 'Brahmirasayanam', 'Brahmighritam', 'Mentat' and 'Memory plus' are popular drugs available in the market. Structures of major bacosides are shown in Fig 2.1.



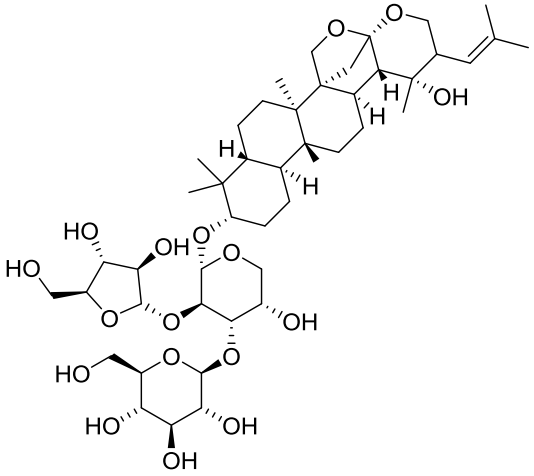
Bacoside A



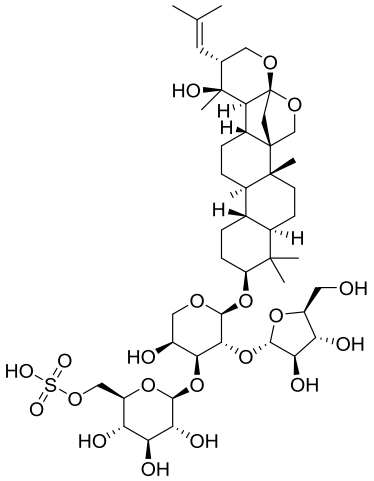
Bacoside B



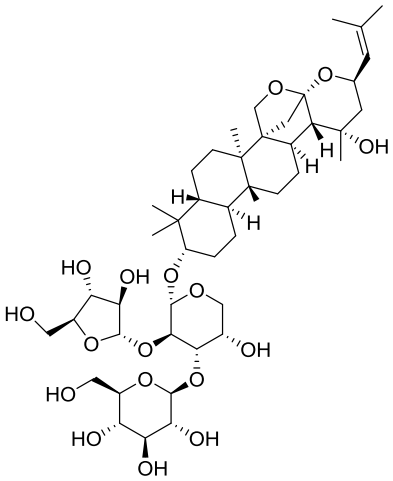
Bacoside A_3



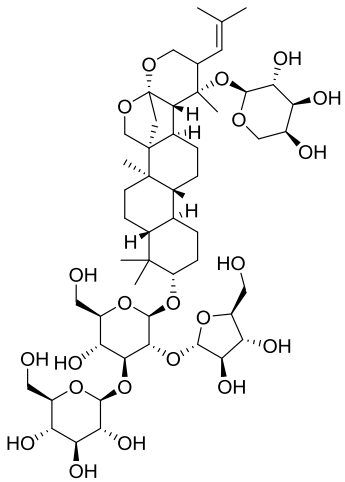
Bacosaponin C



Bacopaside I



Bacopaside X



Bacopaside XII

Fig. 2.1: Major bacosides from *B. monnieri*

2.2.2. *Chlorophytum borivilianum*

Common name of *C. borivilianum* is Safed Musli and it belongs to family Liliaceae. The herb *C. borivilianum* is also acknowledged as '*Vajikaran Rasayana',* which acts as rejuvenator and aphrodisiac. *C. borivilianum* is an immune booster possess aphrodisiac, antimicrobial, antiinflammatory, antitumor (Thakur et al. 2009), hepatoprotective activity (Sunitha et al. 2001), hypocholesteromic (Battaab et al. 2006), antidiabetic (Md. Mujeeb et al. 2009), thyroid inhibitory, antiperoxidative, hypoglycemic (Panda et al. 2009) and antioxidant, anticancer, antiartheritis (Bathoju and Giri 2017). Dried roots are marketed as 'Safed Musli Powder', 'Safed Musli' or 'Shwet Musli'.

i. *In vitro* conservation

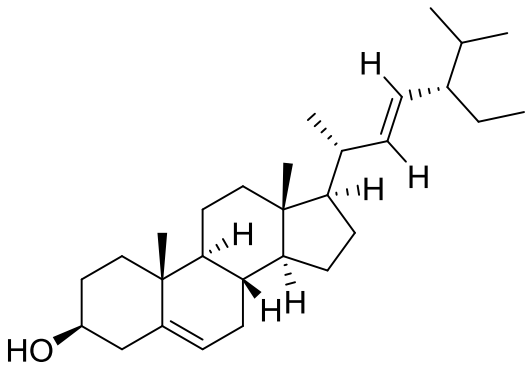
High pharmaceutical value of the tubers has created the need to conserve this medicinal plant. *In vitro* propagation has proved an appropriate technique for conservation (Jauhari et al., 2014). Purohit et al. (1994) reported *in vitro* multiplication on MS media supplemented with 22.2 μM BA using young shoot bases as explants. Subculturing was practiced at every 3 weeks interval. Roots were obtained after transferring the shoots onto rooting media with 3/4-strength and 9.8 μM IBA. Maruthi et al. (2007) evaluated various concentrations of BA alone and BA in combination with NAA on MS media using shoot bud as explants. MS fortified with 5.11 mg/L BA showed maximum number of shoots (17.82). Rizvi et al. (2010) reported somatic embryogenesis as an important aspect for *in vitro* propagation of this plant. This *in vitro* aspect is achieved by germination of seed of this herb on MS media in combination with 57.74 μM gibberellic acid. The hypocotyl was used as explants for the induction of callus. Better callus induction was observed on MS medium supplemented with 1.16 μM kinetin and 1.13-2.26 μM 2,4-dichlorophenoxyacetic acid.

ii. Pharmacological significance

Giribabu et al. (2014) reported that aqueous root extract of *C. borivilianum* regulated blood glucose level and protect the pancreas from oxidative-stress in diabetes. Root extract of *C. borivilianum* was administrated to both normal as well as hypercholestraemic rats through the diet, which showed reduction in lipid profile of plasma as well as hepatic system of albino rats (Visavadiya and Narasimhacharya, 2007). Larvicidial activity of crude extract of *C. borivilianum* tubers was exhibited by Deore (2009). Chakraborthy and Aeri (2009) showed antimicrobial potential of methanolic root extract of the plant against *Candida albicans* and *Aspergillus niger*. *C. borivilianum* root extract inhibit intracellular ROS formation, reducing lipid peroxidation level maintaining cellular antioxidant enzymes activity (Sharma and Kumar 2011). Aqueous extract of the tuberous roots showed antioxidant potential and cholesterol lowering potential (Kenjale et al. 2007).

iii. Phytochemical constituents

High repute of tubers of *C. borivilianum* in traditional Indian medicine is largely due to the presence of phytosteroidal compound, Stigmasterol (Bathoju and Giri 2012). Structure of stigmasterol is shown in Fig. 2.2.



Stigmasterol

Fig. 2.2: Stigmasterol from *C. borivilianum*

More than 25 alkaloids, calcium and protein components are also present in tubers of *C. borivilianum* (Manjunatha 2008). *C. borivilianum* also contains triterpenoids, gallo-tennins, vitamins, potassium, calcium, magnesium, zinc, copper, phosphorus, resins and large amount of simple sugars such as sucrose, glucose, fructose, galactose, mannose and xylose (Kokate et al. 2004; Thakur and Dixit 2005; Singh et al. 2012). *C. borivilianum* have immense pharmacological use for preparation of formulations to cure various ailments.

2.3. Biochemical importance of therapeutically active phytocompounds

Pharmacological activity of medicinal plants is attributed to their therapeutically active phytocompounds. Analysis of major phytocompounds responsible for remedial effects in different accessions of *B. monnieri* and *C. borivilianum* is essential to find elite accession. The elite accession can further be exploited for increased production of medicinally important phytocompounds (bacosides and stigmasterol) with the use of elicitors. Various pharmacological activities are affected by the presence and amount of these phytocompounds such as antioxidant potential of *B. monnieri* depends on the total bacoside content and total phenol content.

Many researchers reported reactive oxygen species scavenging potential of *B. monnieri*. Increased free radical scavenging activity was noticed by *B. monnieri* in *in vivo* model (Bhattacharya et al. 2000). Alamgir et al. (2014) evaluated antioxidant potential of leaf extract of *B. monnieri* through 2,2-diphenyl-1-picrylhydrazyle (DPPH) method and reported that antioxidant activity of the plant is attributed to secondary metabolites produced by the plant.

Total phenol content also significantly affects the antioxidant potential of the plant (Alam et al. 2012). Djeridane et al. (2006) assessed total phenol content in Algerian plants using Folin-Ciocalteu's reagent and antioxidant potential using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{+}$). A positive correlation was noticed between total phenol content and antioxidant activity with R = 0.7931.

Presence of phenolic compounds in the extract of *B. monnieri* has been reported by many researchers. Volluri et al. (2011) confirmed the presence of total phenol content and assessed *in vitro* antioxidant potential using DPPH method in methanolic extract of whole plant of *B. monnieri*. Visavadiya et al. (2010) reported antioxidant activity of aqueous root extract of *C. borivilianum* using FRAP, DPPH and ABTS radical scavenging potential. In the study conducted by Ashraf et al. (2012) antioxidant activities through DPPH, ferrous ion chelating (FIC), and β-carotene bleaching (BCB) from crude extract and total saponin fraction of *C. borivilianum* tubers were performed. Crude extract of the roots showed antioxidant activity (2578 ± 111 mg/100 g ascorbic acid) with bleaching effect. Cytotoxicity for MCF-7, PC3, and HCT-116 cancer cell lines was also noticed.

These pharmacologically active secondary metabolites are distinctive reserve for pharmaceutical industry. Direct extraction of secondary metabolites from plants does not meet the demand of present times. Plant cell culture has proved a promising substitute to obtain these phytocompounds. Low yield of plant secondary metabolites is the major biotechnological hindrance in plant cell culture technique.

Since secondary metabolites play important role in plant protection from the attack of insects and pathogens or to sustain biotic and abiotic stresses, some techniques have been developed to increase the secondary metabolite production. These comprises use of elicitors and abiotic stresses (Yukimune et al. 1996; Zhao et al. 2000, 2001a,b,c; Zhang et al 2004). Increased accumulation of target compounds has been achieved with treatment of elicitors. Elicitors for the plants may be referred as the substances, which when present in small amount trigger the biosynthesis of secondary metabolites or accumulation of phytoalexin.

Elicitors signal starts a signal transduction network, which activates synthesis of transcription factors. The transcription factors control the expression of genes involved in secondary metabolite production and the enzymes produced in the process are responsible for production of target secondary metabolites (Zhao et al. 2005). It is anticipated that efforts to increase the yield of secondary metabolite can be utilized by pharmaceutical companies. However, it is important to understand about biosynthetic genes, their regulation and involvement in metabolic engineering of secondary metabolite production. Hence, more knowledge about signal transduction will significantly boost the production of metabolic flux of secondary metabolites.

Several elicitors have been studied by the researches to increase the yield of secondary metabolites. Many researchers have used elicitors like jasmonic acid, salicylic acid and some other chemicals to increase the therapeutically important secondary metabolites. Adventitious roots of Ginseng were treated with methyl jasmonate (MJ) up to 150 µM and then cultured for 40 days. Up to 100 µM MJ inhibited the growth of the root but

showed increased ginsenoside accumulation (Kim et al. 2004). An experimental study was conducted to analyze the effect of SA on the metabolism of *Achillea millefolium* through biometric parameters such as growth and biochemical. Elicitation with 0.5 mM showed enhanced biomass of roots and chlorophyll content. The application of SA at 0.50 and 1.00 mM elicitated the production of essential oils and total phenols, with a consequent improvement of the antioxidant activity (Gorni and Pacheco 2016).

2.4. Principal component analysis

Data of therapeutically important secondary metabolites or different variables (phyto-pharmaceuticals) contributing medicinal value of the plants can be assessed to identify major contributors of variability in different accessions through Principal Component Analysis (PCA).

Simple linear correlation analysis indicates the measure of correlation and strength of the relationship between variables using Pearson's correlation. PCA using correlation matrix applied to evaluate the relative contribution of different variables like moisture content, total bacosides or stigmasterol, total phenol content and antioxidant property. Simple linear correlation and cluster analysis describe the correlation and strength of the relationship among different evaluated variables.

2.5. Genetic diversity analysis

Genetic diversity among different accessions can be assessed by using molecular marker technology as genetically diverse accessions can be used for crop improvement through breeding programs. Genetic diversity is the total number of genetic features

present in the genome of the species. 30-90 % genome of all the species is comprised of repetitive DNA. These regions of DNA are highly polymorphic in nature and susceptible to induce mutations. Genetic mutations contribute in evolution or polymorphism. These mutational forces and DNA regions together form the basis of many marker systems, which can be utilized for plant genome analysis. Molecular markers cannot determine specific gene activity. They can be employed for the analysis of diversity among different accessions.

Functional process of molecular markers depends on highlighting differences (polymorphisms) within the genome sequence between different accessions. Molecular markers are not affected by environmental factors and generate reliable results. Plant breeders can develop the elite accessions with the utilization of genetically diverse accessions. Elite accessions of superior quality and high yield if utilized by the farmers and pharmaceutical companies can certainly enhance the quality and quantity of the drug. Therefore, analysis of genetic diversity is an eminent approach for characterizing and producing elite accessions to get the high-grade herbal drugs.

Presently production of genetically similar large numbers of plants is in practice with the reported *in vitro* propagation techniques. Still, there is a gap between demand and supply. Therefore, study of different accessions of pharmacologically important medicinal plants for the production of therapeutically important phytocompounds through plant tissue culture techniques is the call for present times.

2.6. *In silico* methods of drug discovery

Drug discovery and drug development is an enormous, prolonged and an interdisciplinary endeavor. Drug discovery is largely characterized as sequential process that begins with target and lead discovery, subsequently lead optimization and pre-clinical *in vitro* and *in vivo* research to explore the efficiency of compound for clinical development. Traditionally, a pharmaceutical industry covers a long journey to launch a drug from discovery to market that is around 12-14 years with the cost of up to 1.2 - 1.4 billion US dollars. Now a days, the procedure of drug discovery has been changed radically with the development of genomics, proteomics and bioinformatics.

Many biologically active phytocompounds could serve as scaffolds for drug design. These compounds are commonly used as traditional medicine and thus their safety are comparatively better judged than chemical entities, which are novel for human use (Patwardhan et al., 2004).

In silico approaches helps in searching drug targets through bioinformatics tools. They are also utilized to assess active/binding sites in target structures, examining drug likeliness, docking of drug molecule with target etc. *In silico* approaches permeates structure-based drug designing with the use of computers and computational methods.

Bioinformatics techniques render novel drug candidate in short span of time at a lower cost. In the recent past, several targets of bacosides and stigmasterol have been reported like acetylcholine esterase (AChE) for bacoside and tumor necrosis factor α converting

enzyme (TACE). AChE, an enzyme present in muscles and nervous system, is important in humans and other vertebrates (Massoulie et al 1993). It hydrolyses acetylcholine, which is a molecule responsible for proper functioning of memory and cognitive functions. Upon hydrolysis in synapses, acetylcholine produces choline and acetyl group. Thereby, levels of acetylcholine in synapses reduced (Voet and Voet, 1995), which leads to memory loss. Inhibition of the enzyme can be a probable cure for AD, which is associated with the deficiency of acetylcholine.

Different studies associate TACE with colon cancer (Ramirez et al., 2007). It is responsible for producing the soluble form of TNF-α from its membrane-bound precursor i.e. pro TNF-α. Since the enzyme plays an important role in converting TNF-α in soluble form, targeting the enzyme could be a potential therapeutic strategy for colon cancer. A study conducted by Grover et al. (2011) on chaperone Hsp90 takes part in cancer disease. Cdc37 is a kinase-targeting co-chaperone of Hsp90 and is responsible for interaction between Hsp90 and protein kinase. Duel inhibition of Hsp90/Cdc37 was explored by a phytocompound withaferin A and 17-DMAG (17-dimethylamino ethylamino-17-demethoxygeldanamycin) through molecular docking and molecular dynamic simulation studies. Ramasamy et al. (2015) performed docking of bacoside A and bacoside A_3 with tryptophan hydroxylase and suggested that the complex might enhance the biosynthesis of 5-HT, which is responsible for elevating the memory function.

However, the remedial effects of bacosides and stigmasterol are well documented, in depth specific action mechanism on their corresponding disease related targets are not

known. In present scenario, demand of raw material for pharmacopeias is fulfilled from natural resources and therefore extensive pressure building on wild sources and plants are categorized under endangered and critically endangered. Thus, the utilization of *in vitro* plant cultures of elite accessions is advocated for commercial production, exploitation of drug preparation as well as for future research.

CHAPTER 3

IN VITRO PROPAGATION OF MEDICINAL PLANTS

3.1. Introduction

Ethnobotanical plants are vital contributors to healthy human life for thousands of years. *B. monnieri* and *C. borivilianum* are used as alternate therapies worldwide. These plants are categorized as important medicinal plants that require immediate attention by National Medicinal Plants Board and Technology Information Forecasting and Assessment Council, 2017. The major constraints leading to continuous depletion of their natural population are high pharmacological efficiency, inadequate availability of seed in the genebank, time-consuming process of traditional breeding and short seed viability (Tiwari et al. 2001; Maiti and Geetha, 2005). Hence, there is an urgent need for proactive understanding for the replenishment, conservation and sustainable usage of these medicinal plants using biotechnological tools through plant tissue culture techniques. *In vitro* propagation strategies have many advantages over traditional approaches like rapid multiplication, disease free and on-demand availability of plant material. In addition, *in vitro* plant cultures help in conserving and maintaining elite germplasm and have the potential for supplying high-quality planting material.

Optimum concentrations of plant growth hormones and selection of basal medium plays an important role in proliferation and multiplication of *in vitro* grown plants. Basal media differ from each other primarily in the presence and quantity of various salts and organic supplements. Different plants need different types and amount of

salts and organic supplements for *in vitro* propagation. Several basal media like MS (Murashige and Skoog, 1962), Nitsch (Nitsch and Nitsch 1956), B5 (Gamborg et al., 1968), Schenk and Hildebrandt (SH, Schenk and Hilderbarndt, 1972) and White (White, 1943) have been used for *in vitro* mass multiplication of various plants. All these media contains different type and amount of salts and organic supplements. Thus, the optimization of basal medium is a vital step to achieve rapid and high rate of *in vitro* propagated plants.

The present study is an attempt to explore the optimum medium, effect of different concentrations of auxins and cytokinins and to identify the elite accession based on growth parameters such as number of shoots, shoot length and number of propagules among 13 accessions of *B. monnieri*.

3.2. *In vitro* propagation of *Bacopa monnieri* accessions

Under this, 3 experiments were conducted:

i. *In vitro* propagation of 13 accessions of *B. monnieri* with varying concentration of auxins and cytokinins to find out the optimum combination and elite accession based on growth parameters

ii. *In vitro* regeneration of plant through callus from selected elite accession

iii. *In vitro* multiplication of selected elite accession on different media to find out the optimum medium based on growth parameters

In the present study, 13 accessions of *B. monnieri* were used, which were collected from different states (Table 3.1).

Table 3.1: List of *B. monnieri* accessions used for the study.

Sl. No.	Accession No.	State of Collection
1	IC 439118	Jharkhand
2	IC 426442	Punjab
3	IC 426447	Madhya Pradesh
4	IC 468878	Kerala
5	IC 373640	Kerala
6	IC 344312	Karnataka
7	IC 531621	Jharkhand
8	IC 375976	Jammu & Kashmir
9	IC 353203	New Delhi
10	IC 554588	Madhya Pradesh
11	IC 554586	Madhya Pradesh
12	IC 554587	Madhya Pradesh
13	IC 554585	Amarkantak

3.2.1. Material and Method

i. Plant material

Thirteen accessions of *B. monnieri* were procured from ICAR-National Bureau of Plant Genetic Research (ICAR-NBPGR) and maintained in Tissue Culture Laboratory at Delhi Technological University, Delhi, India.

ii. *In vitro* propagation with varying concentration of auxins and cytokinins

In vitro maintained shoot cultures were the source of nodal explants (single node with 1 cm of length), which were inoculated on semisolid MS medium with varying concentration of auxins {Benzyl Aminopurine (BA), Kinetin (Kn)} and cytokinins

{Indole Butyric Acid (IBA)} to find out the best combination based on maximum number of propagules. 16 combinations were tested including basal medium (control). The cultures were kept at 25 ± 2°C under 16-h photoperiod. Shoot cultures of the accessions were established *in vitro* and shoot number, shoot length and number of propagules were recorded after 8 weeks of culture to identify the elite accession. Each treatment includes 10 cultures in triplicate.

iii. *In vitro* regeneration through callus from selected accession

Leaf, internode, and nodal segment of selected accession obtained from section 3.2.1 ii were cultured on MS media fortified with different combinations and concentrations of BA and 2,4- dichlorophenoxy acetic acid (2,4-D). Cultures were maintained at 25 ± 2°C under 16-h photoperiod. Callus diameter and morphological features were recorded after 4 weeks of culture. Each treatment includes 10 culture.,s in triplicate.

iv. *In vitro* multiplication of selected accession on different media

The selected accession (obtained from experiment 3.2.1.ii) was inoculated on five different media: MS, Nitsch, B5, SH, and White for rapid propagation. All the media were supplemented with 0.4 BA and growth was observed after 8 weeks of culture. Single node explants were transferred to culture tubes (Borocil; 25 × 150 mm) having 15 ml of culture medium. Medium was autoclaved at 121°C for 15 minutes and pH 5.8 was adjusted before autoclaving. Cultures were kept for 16-h photoperiod at 25 ± 2°C. Growth on different media was compared with MS + 0.4 BA as MS medium is well established for *in vitro* propagation of *B. monnieri*. The optimum medium based on maximum number of propagules was identified for *in vitro* propagation and conservation purposes. Each treatment includes 10 cultures in triplicate.

3.2.2. Result and Discussion

i. *In vitro* propagation with varying concentration of auxins and cytokinins

All the combinations were observed for the number of shoots, shoot length and number of propagules. Shoots appeared within 7 days of culture in all the tested combinations. The emergence of 2 - 4 shoots was observed in all the treatments after 4 weeks. The difference in shoot proliferation in relation to various treatments became noticeable by 8 weeks (Table 3.2 and Fig. 3.1). Analysis of variance exhibited significant effect of treatments ($p < 0.01$) with respect to shoot multiplication. MS basal medium showed growth of 3.2 shoots from the nodal segment. Addition of BA revealed a promotory effect on the number of shoots (20 - 30 shoots/explants) at a concentration of 0.2 - 1.0 mg/L BA. More than 1.0 mg/L BA was associated with reduction in axillary shoot proliferation. High amount of BA (> 2 mg/L) resulted in browning of shoots together with basal callus (Sharma et al., 2007). Sharma and coworkers (2016) achieved highest shoot proliferation on MS fortified with 0.2 mg/L with single node explants. Supplementation of media with Kn showed enhancement in number of shoots (20 - 22 shoots/explants). No significant difference in a number of shoots was recorded with further increase in Kn. Incorporation of IBA along with BA and Kn produced multiple shoots with appearance of callus at the cut ends and decrease in shoot length. Highest shoot growth was recorded with 1.0 mg/L whereas maximum number of shoots was observed with 0.4 mg/L BA (Table 3.2). Fig 3.1 shows highest shoot multiplication with 0.4 mg/L BA as compared to MS (basal), MS + 0.2 mg/L BA and MS + 1.0 mg/L BA . *In vitro* regeneration of single node explants to multiple shoots on 0.4 mg/L BA after 8 weeks of culture is shown in Fig 3.2. Multiple shoot proliferation was recorded with an average of 30.4 shoots/explants of 7.6 cm shoot length and 26.8 number of roots/explants after 8 weeks of culture when 0.4 mg/L BA was used. Callus formation was not observed in this medium.

Table 3.2: Effect of various growth hormones on shoot multiplication and number of roots after 8 weeks of culture of *B. monnieri*

Treatments (mg/L)	Number of shoots/ explants	Shoot length (cm)	Root number
MS basal	3.25 ± 0.7^{l}	3.55 ± 0.8^{de}	7.40 ± 0.5^{i}
MS + 0.2BA	22.25 ± 0.5^{b}	8.97 ± 0.6^{ab}	23.2 ± 0.7^{b}
MS + 0.4BA	30.45 ± 0.6^{a}	7.96 ± 0.4^{bc}	26.85 ± 0.6^{a}
MS + 1.0BA	20.25 ± 0.5^{c}	9.56 ± 0.4^{a}	20.12 ± 0.6^{c}
MS + 0.2BA + 0.2IBA	11.2 ± 0.8^{g}	6.96 ± 0.4^{c}	12.10 ± 0.7^{f}
MS + 0.2BA + 0.5IBA	12.84 ± 0.5^{ef}	$3.21\pm0.4d^{ef}$	12.49 ± 0.6^{f}
MS + 0.4BA + 0.2IBA	11.80 ± 0.6^{fg}	3.75 ± 0.4^{de}	10.96 ± 0.5^{g}
MS + 0.4BA + 0.5IBA	16.20 ± 0.7^{d}	2.87 ± 0.4^{ef}	19.23 ± 0.6^{c}
MS + 1.0BA + 0.2IBA	13.70 ± 0.6^{e}	4.34 ± 0.4^{def}	15.62 ± 0.6^{d}
MS + 1.0BA + 0.5IBA	20.25 ± 0.6^{c}	3.79 ± 0.6^{de}	14.36 ± 0.5^{e}
MS + 0.5Kn	20.20 ± 0.5^{c}	6.86 ± 0.6^{c}	9.45 ± 0.7^{h}
MS + 1.0Kn	22.7 ± 0.8^{b}	6.87 ± 0.6^{c}	16.25 ± 0.7^{d}
MS + 0.5Kn + 0.2IBA	7.55 ± 0.5^{h}	3.89 ± 0.4^{de}	3.97 ± 0.7^{jk}
MS + 0.5Kn + 0.5IBA	3.74 ± 0.6^{jk}	1.97 ± 0.4^{fg}	3.53 ± 0.6^{kl}
MS + 1.0Kn + 0.2IBA	5.55 ± 0.6^{i}	2.86 ± 0.4^{ef}	2.46 ± 0.6^{l}
MS + 1.0Kn + 0.5IBA	4.75 ± 0.4^{ij}	1.01 ± 0.4^{g}	4.8 ± 0.5^{j}

Data given as Mean ± SE. different letters in superscripted are significantly different at $p<0.05$ level using DMRT.

Fig. 3.1: *In vitro* shoot multiplication of *Bacopa monnieri* on MS (basal), MS + 0.2 mg/L BA, MS + 0.4 mg/L BA and MS + 1.0 mg/L BA (L-R) after 8 weeks of culture

Vijayakumar and coworkers (2010) observed MS supplemented with 0.06 mg/L thiodizuron for maximum number of roots. Tiwari and Singh (2010), observed MS fortified with 0.44 µM BA and 1.14 µM IAA more effective for shoot elongation (7.8 cm). Another study conducted by Vijay and coworkers(2016) explored media MS in addition with 0.1 mg/L BA and 0.5 mg/L IAA for maximum number of shoots (10) and highest shoot length (6.1 cm) with nodal segments as explant. In the present study, roots initiated after 8 - 10 days of culture, primarily from lower cut end of nodal explants and occasionally from nodes. Root growth was noticed on all media tested for multiplication. However, rooting has not been the limiting factor for the multiplication of shoots. All the tested concentration of BA and BA with IBA supported root development. Kn alone facilitated a moderate number of roots (9 - 16) however addition of IBA with Kn showed less number of roots (2 - 5). Supremacy of BA over Kn in *B. monnieri* was also discussed by Rao et al (2012) and Tiwari et al. (2001). Optimum multiplication medium (0.4 mg/L BA) revealed maximum root induction (26.8 roots/culture) with the absence of callusing after 8 weeks of culture. Vijay and coworkers (2016) used 1/2 MS with 100 mg/L activated charcoal for root induction and achieved 12.4 roots/ plantlet after 20 days of culture.

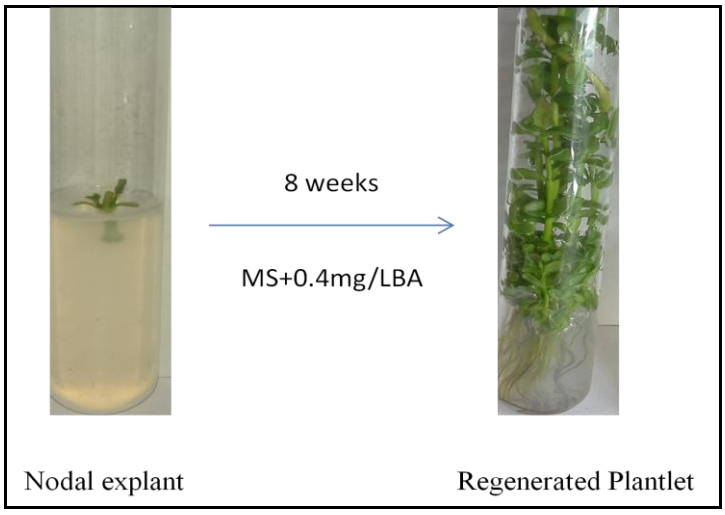


Fig. 3.2: Plantlet regeneration from nodal explants on MS + 0.4 mg/L BA after 8 weeks of culture

In vitro propagation is essential for germplasm conservation, which was achieved in *B. monnieri* by enhanced multiplication. Multiplication response on optimal medium (MS + 0.4mg/L BA) was observed on 13 accessions of *B. monnieri* (Table 3.3).

Table 3.3: Shoot multiplication in 13 accessions of *B. monnieri* on selected media (MS+0.4 BA) after 8 weeks of culture

Accession No.	Number of Shoots/ Explant	Shoot Length (cm)	Number of Propagules
IC439118	24.65±0.7^{c}	7.29±0.8^{bc}	71.5±0.6^{b}
IC426442	19.10±0.7^{d}	7.02±0.5^{bc}	54.6±0.5^{f}
IC426447	19.20±0.7^{d}	6.79±0.5^{bcd}	58.2±0.8^{e}
IC468878	19.25±0.5^{d}	7.82±0.3^{b}	56.70±.9^{e}
IC373640	24.99±0.5^{c}	6.08±0.4^{cd}	64.6±0.6^{c}
IC344312	15.90±0.6^{e}	5.59±0.6^{d}	38.7±0.4^{h}
IC531621	18.87±0.6^{d}	6.27±0.4^{cd}	41.5±0.5^{g}
IC375976	16.90±0.7^{e}	5.96±0.4^{cd}	57.0±0.7^{e}
IC353203	23.55±0.5^{c}	6.80±0.6^{bc}	70.1±0.6^{b}
IC554588	30.45±0.6^{a}	9.65±0.4^{a}	78.4±0.5^{a}
IC554586	27.20±0.7^{b}	7.19±0.4^{bc}	64.7±0.7^{c}
IC554587	29.35±0.6^{a}	7.15±0.3^{bc}	61.6±0.4^{d}
IC554585	24.20±0.7^{c}	6.55±0.4^{bcd}	70.4±0.5^{b}

Data given as Mean ± SE. different letters in superscripted are significantly different at $p<0.05$ level using DMRT.

Analysis of variance exhibited significant genotypic difference ($p<0.01$) in terms of multiplication response. Average number of shoots ranged from 15 - 30 whereas shoot length and number of propagules varied from 5.59 - 9.65 cm and 38 - 78 respectively on MS + 0.4 BA. Maximum number of shoots (30), shoot length (9.65 cm) and number of propagules (78) were recorded in IC 554588 on MS+0.4 BA. Tiwari et al (2001) observed highest number of adventitious shoot buds on medium containing 2.2 µM BA

with leaf explants. Sharma et al., (2007), obtained similar results with 22.2 shoots/explants and 75 propagules on 0.2 mg/L after 8 weeks of culture. Higher concentration of BA is associated with the production of a closely-packed mass of shoots, which were not easily separable while it was not applicable to a low amount of BA. Various researchers have also used different concentration of BA for shoot propagation in Bacopa (Tejavathi and Shailaja, 1999).

ii. *In vitro* regeneration through callus

Callus induction was observed using 3 explant, node, internode and leaf explants. Compact callus was observed in leaf explants by supplementing varying concentrations of BA and 2,4-D on MS medium. Leaf explants were inoculated on 5 different media combinations including MA basal. No callus was noticed on MS basal medium. The average time observed for callus initiation varied from 14 to 20 days. Callus was first seen in leaf explants (14 days) followed by internode and nodal segment. Dark green compact callus with maximum diameter (3.6 cm) was observed from leaf explants after 4 weeks when cultured on MS + 0.4 Kn 0.2 2,4 D (Table 3.4). After 2 subculture callus was transferred to plantlet regeneration medium (MS + 0.4 mg/L BA). Rapid proliferation (2 weeks) was observed from callus of leaf explants (Fig 3.3) compared to node and internode as callus induced from node and internode explants showed proliferation after 3 weeks and 3.4 weeks of culture respectively. Rapid growth of callus was observed on MS medium supplemented with 0.5 mg/L mg/L NAA, 0.5 mg/L 2,4-D and 0.25 thiodizuron individually (Vijayakumar et al. 2010). Mehta and coworkers (2012), noticed 3.6 and 3.2 cm callus diameter on 0.25 mg/l 2,4-D + 0.5 mg/l Kn and 0.25 mg/l 2,4-D + 0.1mg/l BAP respectively with leaf petiole explants. In the present study, leaf explants showed 100% callus formation on MS + 0.4 Kn + 0.2 2,4-D. 76% and 84% callus induction was observed with node and internode explants respectively

on the same medium. Verma et al. (2012) observed callus induction on MS supplemented with 3 mg/L BA and 1 mg/L IAA after 2 weeks of culture. Mehta (2017) revealed that MS with varying concentrations of 2,4-D showed only 50% response for callus induction. However, they found MS + 1.5 mg/L 2,4-D to be optimum for callus induction with all the three explants, internode, leaf, and node. The data of the present study revealed supremacy of leaf explants over internode and nodal explants in terms of callus feature, diameter, and proliferation.

Table 3.4: Effect of BA and 2,4-D on callus induction from leaf explants of *B. monnieri* after 4 weeks of culture

Concentration of growth hormone (mg/L)	Callus diameter (cm)	Callus proliferation	Callus feature	% of callus formation
MS	0	0	0	0
MS + 0.2 BA + 0.2 2,4-D	2.7±0.6	Good	Brownish green, friable	92
MS + 0.4 BA + 0.2 2,4-D	2.8±0.9	Moderate	Green, nodular	84
MS + 0.2 Kn + 0.2 2,4-D	2.8±0.4	Good	Green, friable	87
MS + 0.4Kn + 0.2 2,4-D	3.6±0.5	Excellent	Dark Green, compact	100

Data presented as Mean ± SE.

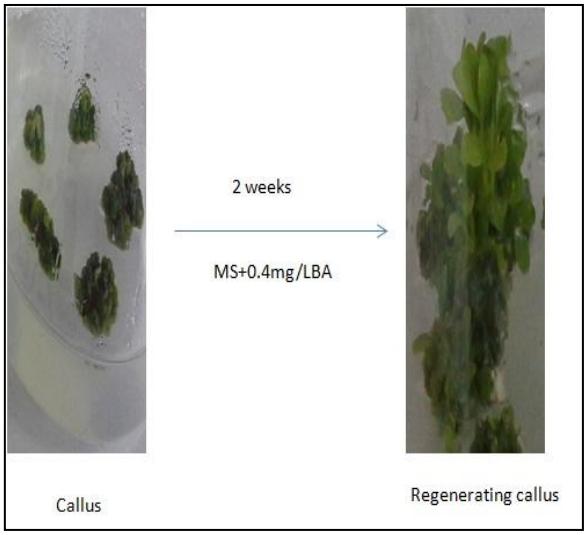


Fig 3.3: Proliferation of callus on MS + 0.4mg/L BA after 2 weeks of culture

iii. *In vitro* propagation on different media (MS, Nitsch, B5, SH, White)

Nodal explant of accession IC554588 selected from the above study was cultured on 5 different media supplemented with 0.4 BA to check the efficiency of *in vitro* propagation. Shoot emergence was noticed within 12 days of culture. Analysis of variance exhibited significant effect of treatments ($p < 0.01$) in relation to shoot multiplication. MS medium exhibited maximum number of shoots (23 shoots/explant) and shoot length (8 cm) followed by Nitsch medium with 16 shoots/explants and shoot length (6 cm). Root induction was first recorded in MS medium with maximum number of roots (19 roots/culture) along with the highest mean number of propagules (65) followed by Nitsch medium (45 propagules/culture). Table 3.5 shows effect of different media on growth parameters of *B. monnieri*. Sharma et al., 2017 observed that SH recorded higher number of shoots and shoot length as compared to B5 after 4 weeks of culture. In the present study, maximum number of propagules and maximum number of shoots were obtained on MS medium compared to other tested media and this is in accordance with earlier reports of Mohapatra and Rath (2005).

Table 3.5: Effect of different media on growth parameters of *B. monnieri* after 8 weeks of culture

Media	Number of shoots/ explants	Shoot length (cm)	Root number	Number of propagules
MS + 0.4 BA	23.35±0.5	8.15±0.5	19.1±0.7	65.1±0.85
Nitsch + 0.4 BA	16.85±0.9	6.13±0.3	15.3±0.7	45.4±0.8
B5 + 0.4 BA	13.3±0.8	5.54±0.5	11.85±0.8	36.25±0.7
S&H + 0.4 BA	9.55±0.7	4.69±0.5	9.2±0.6	21.25±0.5
White + 0.4 BA	9.15±1.1	2.17±0.4	6.55±0.5	19.8±0.6

Data given as Mean ± SE. different letters in superscripted are significantly different at $p<0.05$ level using DMRT.

3.3. *In vitro* propagation of *C. borivilianum* accessions

3.3.1. Material and Method

i. Plant material

Three accessions of *C. borivilianum* Sant and Fern. were procured from ICAR-National Bureau of Plant Genetic Research (ICAR-NBPGR) and maintained in the tissue culture laboratory at Delhi Technological University, Delhi, India. Accessions IC 266708, IC 558379 and IC 539860 belong to Rajasthan, Uttar Pradesh, and Madhya Pradesh respectively.

ii. *In vitro* shoot proliferation

Shoot base of three accessions was multiplied on ten different media with varying combinations of BA, Kn, IBA and Naphthalic Acetic Acid (NAA) for *in vitro* shoot proliferation. Cultures were kept at 25 ± 2°C under 16-h photoperiod. Observations were taken after 6 weeks of culture. Best-grown accession based on growth parameters (maximum number of shoots and highest shoot length) was selected for further study. All treatments were performed in triplicate and each treatment consisted of 10 cultures.

iii. Shoot regeneration through callus

Shoot base of selected accession obtained from section 3.3.1.ii were cultured on MS media with different combinations and concentrations of NAA, 2,4-D and IBA. Cultures were maintained at 25 ± 2°C under 16-h photoperiod. Callus diameter and morphological features were recorded after 4 weeks of culture. Each treatment includes 10 culture in triplicate.

iv. Effect of different media on shoot multiplication

The aforementioned selected accession (shoot base explant) was cultured on five different media MS, Nitsch, B5, SH and White for optimization of *in vitro* propagation. All five media were supplemented with 3.0 mg/L BA separately (Jauhari et al., 2014). Optimum medium based on maximum number of shoots and shoot length was identified after 6 weeks of the culture period. All treatments were performed in triplicate and each treatment consisted of 10 samples.

v. *In vitro* root induction

Healthy shoots of 6-week-old accessions were cultured on MS medium supplemented with 10 combinations of BA, IAA, and NAA for root induction. Cultures were incubated at 25±2 °C under total darkness for 14 days for root induction. After the appearance of roots, cultures were maintained under 16-h photoperiod for elongation of roots for 6 weeks. Optimum medium and the accession exhibiting maximum number of roots and root length were identified for elicitation study. All treatments were performed in triplicate and each treatment consisted of 10 samples.

vi. Hardening and acclimatization

Plantlets obtained from elicitor-treated and non-elicited 6-week-old root culture were washed thoroughly with running tap water to remove agar. Subsequently, plantlets were planted in the pots filled with sand, soil and decomposed cow dung in equal proportion. Small holes were made into polythene bags to cover the potted plants. These holes help in maintaining humidity and ventilation of air at early stages. After removal of polythene bags (2 weeks) pots were kept in a greenhouse for acclimatization before transferring to the field. Tubers obtained from the field transfer plants were evaluated for biochemical analysis.

3.3.2. Result and Discussion

i. *In vitro* shoot proliferation

In vitro shoot emergence in three accessions of *C. borivilianum* was observed after 10 days of culture using shoot base explant. The emergence of 2-3 shoots was observed in all treatments after 20-25 days of culture. The difference in shoot proliferation in relation to various treatments became noticeable by 6 weeks. Analysis of variance exhibited significant effect of treatments ($p<0.05$) in relation to shoot multiplication. 3.5 shoots/explants with 4.9 cm of shoot length were observed on MS basal medium. Addition of BA showed a promotary effect on the number of shoots (7.4-11.8 shoots/explants) at a concentration of 2-4 mg/L BA (Table 3.6).

Table 3.6: *In vitro* shoot multiplication in 3 accessions of *C. borivilianum* after 6 weeks of culture

Treatment (mg/L)	Shoot Number			Shoot Length		
	IC 539860	IC 266708	IC 558379	IC 539860	IC 266708	IC 558379
MS Basal	2.9±0.7^{i}	3.3±0.6^{f}	3.5±0.5^{g}	5.1±0.5^{ef}	4.1±0.7^{e}	4.9±0.6^{h}
MS + 2BA	6.1±0.6e^{f}	5.3±0.5^{de}	7.6±0.4^{d}	8.5±0.8^{b}	7.1±0.5^{a}	9.4±0.3^{b}
MS + 3BA	9.0±0.6^{a}	8.6±0.7^{a}	11.8±0.6^{a}	9.8±0.6^{a}	7.5±0.6^{a}	10.7±0.5^{a}
MS + 4BA	5.9±0.7^{f}	4.9±0.8^{e}	7.4±0.5^{d}	6.8±0.7^{c}	5.9±0.5^{c}	7.5±0.6^{d}
MS + 2Kn	3.5±0.5^{h}	2.9±0.6^{f}	5.5±0.6^{f}	4.9±0.6^{ef}	3.8±0.6^{e}	5.4±0.5^{g}
MS + 3Kn	6.6±0.4^{de}	5.8±0.6^{dc}	6.5±0.5^{e}	5.9±0.5^{d}	5.0±0.7^{d}	5.6±0.7^{g}
MS + 4Kn	7.5±0.6^{bc}	5.5±0.6^{cd}	8.9±0.7^{c}	7.0±0.5^{c}	5.6±0.6^{cd}	8.1±0.6^{c}
MS + 3BA + 0.5IBA	7.9±0.6^{b}	7.0±0.6^{b}	9.3±0.5^{bc}	6.9±0.7^{c}	5.6±0.6^{cd}	7.4±0.5^{d}
MS + 3Kn + 0.5IBA	7.0±0.7^{cd}	8.3±0.9^{a}	9.7±0.8^{b}	7.3±0.6^{c}	6.5±0.8^{b}	6.9±0.5^{e}
MS + 3BA + 0.5IBA + 0.5NAA	5.2±0.6^{g}	5.5±0.5^{cde}	8.9±0.5^{c}	4.7±0.5^{f}	3.9±0.6^{e}	5.8±0.4^{g}
MS + 3Kn + 0.5IBA + 0.5NAA	6.8±0.6^{d}	5.9±0.7^{c}	7.4±0.5^{d}	5.5±0.6^{de}	4.9±0.6^{d}	6.4±0.7^{f}

Data given as Mean ± SE. different letters in superscripted are significantly different at $p<0.05$ level using DMRT.

IC 558379 was identified as promising accession in terms of shoot growth and development. Cultures were subcultured after every 30 days interval to increase the number of shoots. These results were in conformity to the earlier published studies with different concentrations of BA (Sharma and Mohan 2006; Sunita et al. 2015). In the present study, maximum shoot multiplication from shoot base explant was noticed on media MS + 3mg/L BA after 6 weeks of culture (Fig 3.4).

Fig. 3.4: Shoot multiplication from shoot base explant on MS + 3mg/L BA after 6 weeks of culture

It was observed that higher concentration of BA (> 3.0 mg/L) was associated with browning and thus subculturing at 20 days interval would give better results. Supplementation with 2-4 mg/L Kn showed an increase in a number of shoots (5.5-8.9 shoots/explants). Further increasing the concentration of Kn does not affect the number

of shoots. MS fortified with BA and IBA showed better results compared to MS supplemented with BA, IBA, and NAA. Same observation in terms of a number of shoots and shoot length was noticed when Kn was replaced with BA. However, highest shoot length was exhibited in 3 mg/L BA followed by 2 mg/L BA and 4 mg/L Kn with IC 558379. Based on shoot growth parameters, IC 558379 was identified for maximum shoot growth on all the tested media.

ii. Shoot regeneration through callus

Shoot proliferation of *C. borivilianum* via callus induction was achieved with varying concentrations of auxins (NAA, 2,4-D, and IBA). MS basal medium served as control. No callus was observed on control and medium supplemented with NAA and IBA. The appearance of significant callus was observed after seventh week (54 days) of culture on media supplemented with 2,4-D (Table 3.7).

Table 3.7: *Chlorophytum.borivilianum*: Effect of NAA, 2,4-D, and IBA on callus induction from shoot base explants of IC 558379 (culture duration 54 days)

Treatment (mg/L)	Days of callus induction	Callus observation	% of callus formation
MS	0	0	0
MS + 1NAA	0	0	0
MA + 2.5NAA	0	0	0
MS + 5NAA	0	0	0
MS + 12,4,D	58	Yellowish friable	48.7±0.4
MS + 2.52,4,D	58	Yellowish friable	52.2±1.0
MS + 52,4,D	54	Yellowish friable	67.6±0.9
MS + 1IBA	0	0	0
MS + 2.5IBA	0	0	0
MS + 5IBA	0	0	0

However, all the tested concentrations of 2,4-D produced yellow friable callus, 5 mg/L 2,4-D remarkably enhanced the callus induction response. Nakasha and coworkers (2016) observed callus induction on MS fortified with varying concentrations of 2,4-D after 8 weeks of culture. They also noticed that NAA and IAA have no influence on callus induction. Another experiment conducted by Rizvi et al. (2010) observed callus induction on MS + 1.13 μM 2, 4-D + 1.16 μM Kn.

In the present study, subculture has been practiced at every 4 weeks of the interval. MS + 5 mg/L 2,4-D was found optimum medium for maintenance of the callus and MS + 3 mg/L BA (shooting media) was used for proliferation of callus after 3 subculture (Fig 3.5). Callus proliferation was observed after 2 weeks and a significant number of shoots were obtained after 6 weeks of transfer to shooting medium.

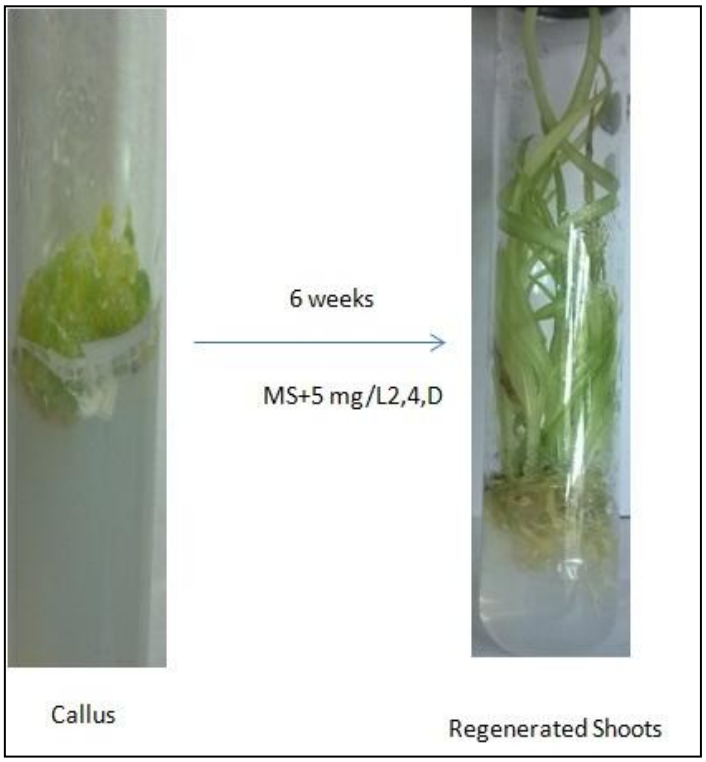


Fig. 3.5: Shoot regeneration from callus on MS + 3 mg/LBA after 6 weeks of culture

iii. Effect of different media on shoot multiplication

The efficiency of different media MS, SH, B5, White, and Nitsch supplemented with 3 mg/L BA were tested for shoot multiplication of *C. borivilianum.* A significant difference was observed regarding number of shoots and shoot length among all five tested media after 6 weeks of culture. Analysis of variance exhibited significant effect of treatments ($p < 0.01$) in relation to shoot multiplication. MS media was observed to be the best culture medium with a maximum number of shoots (12.3) and shoot length (8.1 cm) followed by SH medium. Nitsch medium was recorded with less number of shoots and shoot length. SH has shown better results in terms of number of shoots and shoot length as compared to B5, and White (Fig 3.6). Several researchers (Purohit et al., 1994; Sharma and Mohan 2006) have described the supremacy of MS medium for *in vitro* propagation of *C. borivilianum.* This may be due to the presence of high amount of macronutrients and use of EDTA as an iron chelate. The data was in agreement with Sharma and Mohan (2006) who examined Nitsch with 2 mg/L BA. Similar results have been obtained with B5, SH and White media tested for the growth of *C. borivilianum* by Prasad and coworkers (2007).

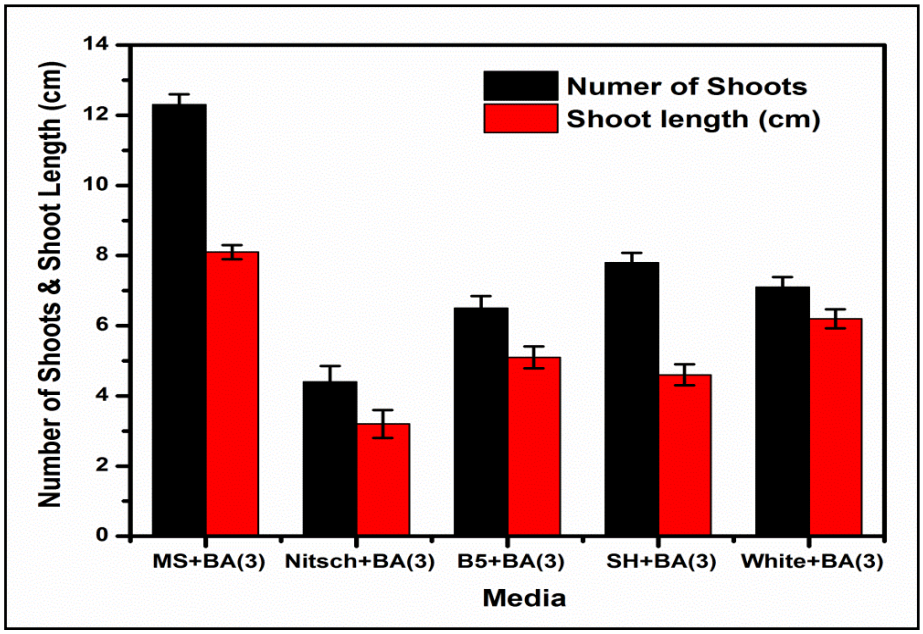


Fig. 3.6: Effect of various media on the production of a number of shoots and shoot length of *C. borivilianum* IC 558379 compared to control after 6 weeks of culture. Data represent mean±standard error of three replicates; each experiment was repeated thrice ($P < 0.05$).

iv. *In vitro* root induction

Root induction was first noticed in IC 266708 (10 days) followed by IC 558379 (14 days) and IC539860 (14 days). Fig 3.7 shows *in vitro* root induction (14 days) and significant root growth (4 weeks) in IC 558379 on media MS +3 mg/L BA + 1 mg/L IAA + 1 mg/L NAA + 6% sucrose. Optimum response of *in vitro* induced roots in terms of root length and number of roots was recorded on MS + 3 mg/L BA + 1 mg/L IAA + 1 mg/L NAA + 6% sucrose after 6 weeks of culture (Table 3.8). Analysis of variance exhibited the significant effect of treatments ($p < 0.01$) in relation to root development. Adventitious roots were observed after 10-14 days of culture in all the accessions. Media ¾ MS + 3 mg/L BAP + 1 mg/L IAA + 1 mg/L NAA showed moderate number of roots (12.8) as compared to MS + 3 mg/L BA + 1 mg/L IAA + 1 mg/L NAA + 6% sucrose with maximum roots (22.9). Slight variation in root length (7.0-7.3 cm) was noticed in all the three accessions.

Table 3.8: *Chlorophytum borivilianum*: Growth parameters of roots studied in IC 558379 (culture duration: 6 weeks)

Treatments	Number of roots	Root length	% of shooted roots
MS basal	0^{h}	0^{g}	0^{g}
MS + BAP(2) + IAA(1) + NAA(1)	$9.0\pm0.^{f}$	2.5 ± 0.6^{e}	65^{c}
MS + BAP(3) + IAA(1) + NAA(1)	6.4 ± 0.5^{g}	1.1 ± 0.8^{f}	71^{b}
MS + BAP(4) + IAA(1) + NAA(1)	11.0 ± 0.7^{e}	1.3 ± 0.5^{f}	74^{b}
3/4MS + BAP(2) + IAA(1) + NAA(1)	11.1 ± 0.7^{e}	5.2 ± 1.1^{d}	59^{d}
3/4MS + BAP(3) + IAA(1) + NAA(1)	12.3 ± 0.8^{d}	6.3 ± 0.4^{c}	43^{e}
3/4MS + BAP(4) + IAA(1) + NAA(1)	13.5 ± 0.8^{c}	6.1 ± 0.4^{c}	40^{f}
MS + BAP(2) + IAA(1) + NAA(1) + Sucrose(6%)	17.3 ± 1.0^{b}	5.1 ± 0.8^{d}	63^{c}
MS + BAP(3) + IAA(1) + NAA(1) + Sucrose(6%)	22.9 ± 1.1^{a}	7.3 ± 0.8^{a}	91^{a}
MS + BAP(4) + IAA(1) + NAA(1) + Sucrose(6%)	18.1 ± 1.3^{b}	7.0 ± 0.7^{ab}	58^{d}

Data given as Mean ± SE. different letters in superscripted are significantly different at $p<0.05$ level using DMRT.

Fig. 3.7: *In vitro* root induction of accession IC 558379 on MS + 3 mg/L BA + 1 mg/L IAA + 1 mg/L NAA + 6% sucrose after 6 weeks of culture

It was observed that IC 558379 exhibited better root development and elongation compared to IC 266708 and IC 539860 on all the tested media. The basal medium provides less amount of nutrition, which can affect the root growth. ¾ concentrations of MS were observed more effective than full strength of MS medium while MS with 6% of sucrose exhibited a maximum number of roots and root length. It is evident from the present study that increasing amount of sucrose was responsible for root induction. Auxin plays important roles in root establishment (Torrey 1976). Application of lower salt concentration in rooting medium is more appropriate compared to higher salt levels, which is often found inhibitory for root induction (George et. al., 2008). Ashraf and coworkers (2012), reported an increase in number of tubers by the incorporation of 60 mg/L of sucrose in root culture of *C. borivilianum*. Jakkulwar and Wadhai (2012) observed root proliferation on MS supplemented with 1 mg/L IAA and 3 mg/L NAA after 22 days of culture.

3.4. Conclusion

A remarkable variation was noticed in terms of shoot multiplication in all the studied accessions and significantly high multiplication rate was noticed in thirteen accessions of *B. monnieri*. Affect of different accessions on the tissue culture outcome is well defied and genotypic variation regarding tissue culture response is in the record for many years. The present study exhibited successful multiplication of *B. monnieri* with a solitary medium desirable for the proliferation and multiplication of shoots. The result of the study is important, as a simple method is noticed appropriate for 13 different accessions. The mean highest shoots and number of propagules were noticed in accession IC 554588 followed by IC 439118. Thus, results of *in vitro* propagation of 13 accessions of *B. monnieri* on 16 media combinations of auxins and cytokinins (on MS

medium) proved MS + 0.4 BA as the optimum media and IC 554588 the elite accession for further study.

A significant difference among different accessions of *C. borivilianum* was noticed in shoot multiplication and root induction and development. However, all the accessions showed significantly high rate of multiplication, accession IC 558379 was noticed with the maximum shoot and root growth. It is evident from the present study that MS medium supplemented with different combinations of auxins and cytokinins is a reliable approach for multiplication and conservation of *C. borivilianum* for the rapid shoot and root development. The accession IC 558379 due to exhibiting higher shoot growth and better root development as compared to other accessions was selected for further study.

CHAPTER 4

BIOCHEMICAL ANALYSIS OF MEDICINAL PLANTS

4.1. Introduction

Pharmacological effects of herbal plants are known from the earliest annals of human habitancy. Therapeutic properties of plants are attributed to the secondary metabolites including antioxidant properties (Chin et al. 2009). Antioxidants support human defense system to fight age-related degenerative diseases and other oxidative damage (Atoui et al. 2005). Synthetically produced antioxidants have adverse affects when consumed *in vivo*. Elgazar (2013) revealed that Butylated Hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are synthetic phenol compounds that are used as antioxidants. BHT and BHA are safe when used at recommended levels but overdose of these compounds can produce carcinogenic effects. They also observed that BHT and BHA reduced N-acetylation of carcinogens and form DNA-carcinogen adducts *in vivo*. Although the living creatures have the capacity to balance the fatalistic effect of free radicals, it is necessary to provide some supplements through diet to maintain the radical concentration to a lesser extent. Considering the above facts, consumption of herbal drugs rich in antioxidants is an alternate way to strengthen the antioxidant potential of the body. Secondary metabolites of plants offer a safe alternative for antioxidants (Walton and Brown, 1999). Secondary metabolites are the natural source of antioxidants and can cure many other disorders. Bacosides are formulated as curative agent for cognitive diseases and age-related disorders while stigmasterol has potential to cure aphrodisiac, cancer and arthritis. Growing demand of herbal drugs in national and international market justifies the analysis of secondary metabolites present in different

accessions of *B. monnieri* and *C. borivilianum*. Elite accession can be used by various pharmaceutical companies to produce superior quality of herbal drugs with same expense as earlier.

4.2. Biochemical analysis of *B. monnieri*

Whole plant of *B. monnieri* is widely used in *Ayurvedic* formulations since ancient times to enhance memory or cognitive functions (Kumar et al. 2016). The renowned pharmacological effects are attributed to biologically active secondary metabolites such as brahmins, stigmasterol, beta-sitosterol, triterpenoid saponins, bacosides and bacosaponins (Basu et al. 1967). Various researchers reported the antioxidant property of bacosides (Kapoor 1990; Meena et al. 2012; Alamgir et al. 2014) including free radical scavenging potential (Anbarasi et al. 2005). Bacoside A3 and bacosaponine C acts as the inhibitor of superoxide release from the polymorphonuclear cells (Pawar et al. 2001).

It is well-known that plant tissue culture based propagation methods play an important role in the production of secondary metabolites (Ahmad et al. 2013). In *B. monnieri* higher bacoside A content was reported in *in vitro* cultures compared to field grown plants (Sharma et al. 2012). The yield of secondary metabolites is strongly affected by environmental conditions (Gorelick et al. 2014). It has been noticed that the production of secondary metabolites increases in stress conditions. In literature, elicitors have been applied to increase the yield of secondary metabolites in *in vitro* plant cultures (Hussain et al. 2012; Karuppusamy, 2009). Although, several abiotic chemical and plant hormones such as jasmonic acid (JA) (Farmer et al. 2003; Sharma et al. 2015), salicylic Acid (SA) (Ali et al. 2006; Sharma et al. 2015), malt extract (ME) (Kundu et al. 2016)

and $CuSO_4$ (Sharma et al. 2015) have been used as elicitors to enhance the secondary metabolite production, their effects are species specific. JA and SA are hormonal elictors involved in the stress response and affect the production of secondary metabolites like terpenoids, flavonoids and alkaloids (Farmer et al. 2003; Shabani et al. 2009). As ME contains the malt content, effect of ME on secondary metabolite production as natural mixture can be evaluated.

High therapeutic value of the plant and less availability for future use, justify the investigation of different accessions of *B. monnieri* for higher bacosides content and other pharmaceutically important metabolites to identify elite accession. The strategy could be further used to enhance bacosides for commercial exploitation and standardization of the formulations prepared from the plant. In the present study, thirteen accessions of *B. monnieri* (Table 3.1 Chapter 3) were evaluated for total bacoside production, total phenol content and antioxidant potential to find the elite accession. The study also investigates affects of total bacosides and total phenol content on antioxidant potential. Accession IC 554588, selected among thirteen accessions of *B. monnieri* (Chapter 3), was elicited with JA, SA or ME for the assessment of their effect on total bacosides, total phenol content and antioxidant potential compared to non-elicited plants. Apart from this, methanolic extract of the selected accession (IC 554588) was investigated for the presence of pharmaceutically important metabolite bacoside through Fourier Transform Infrared Spectroscopy (FTIR). Data were analyzed to identify major relative contributors of variability among all the studied parameters in different accessions through Principle Component Analysis and cluster analysis.

4.2.1. Material and Method

i. *In vitro* shoot induction and elicitation

For elicitation study, stock solutions were prepared in 90% ethanol for jasmonic acid (JA) and in sterile Mili-Q water (Mili-Q, USA) for salicylic acid (SA) and malt extract (ME) (Sigma-Aldrich USA). Filter sterilized 1.0 mg/l of JA, SA or ME were added to the autoclaved medium. Stem segments 1.0-2.0 cm (2-3 nodes) of selected accession from *in vitro* propagated plants were transferred to Erlenmeyer flasks (100 ml) containing MS + 0.4 mg/l BA (20 ml) with or without elicitor (control). The cultures were maintained at 25 ± 2°C under 16-h photoperiod. After 4 weeks, the elicitor-treated and non-elicited cultures were evaluated for growth parameters (number of shoots/explant and shoot length), moisture content and biochemical parameters for the presence and quantity of major secondary metabolites (total bacosides, total phenol content and antioxidant potential). All treatments were performed in triplicate and each treatment consisted of 10 samples.

ii. Biochemical analyses

For estimation of total bacosides, total phenol content and antioxidant potential, elicited and non-elicited *in vitro* cultured *B. monnieri* accessions were studied. Cultures from the following treatments were used for sample preparation: (i) non-elicited 4-week-old cultures of 13 accessions grown on MS + 0.4 BA and (ii) 4-week-old cultures of selected accession grown on MS + 0.4 BA supplemented with elicitors and control (without elicitor).

Sample preparation: Approximately 5 g of green shoots were harvested, shade-dried and crushed to fine powder. The powdered sample was extracted with HPLC grade

methanol (Merck Chemicals) using Soxhlet apparatus (5 cycles) at room temperature. The extracted solvent was dried on rotary evaporator and volume was made up to 2 ml. The sample was filtered through 0.22-micron filter and used for estimation of total bacosides, total phenol content and antioxidant potential (DPPH and CUPRAC). Methanolic extract of accessions IC 554588 prepared from above-mentioned method was used for FTIR to investigate the presence of bacoside.

FTIR (Fourier Transform Infrared Spectroscopy): FTIR spectra were recorded for the bacoside sample using Perkin Elmer Model Spectrum RXI-Mid IR spectrometer operating in the range of 650–4000 cm^{-1} with a resolution of 4 cm^{-1}.

Quantification of total bacosides: Total bacosides were quantified through HPLC using the method described by Mishra et al. (2013). HPLC experiments were performed on Agilent HPLC system equipped with 1260 Infinity Binary Pump (G1312B). ZORBAX ODS C18 column (5 µm 4.6 x 250 mm), 1260 infinity diode array detector (G4212B). The mobile phase consisted of Sodium acetate buffer and Acetonitrile 0.1 M (65:35 v/v), pH 3.2 was adjusted with acetic acid. The flow rate was 0.5 ml/min for isocratic elution of bacosides with 30 min run time. Detection was done at 205 nm and sample injection volume was kept at 2.5 µL. 1 mg of the bacoside mixture (Sigma-Aldrich) comprising bacoside A, bacoside A3, bacoside II, bacopaside X and bacosaponin C was dissolved in 2 ml methanol and used as a standard solution to quantify total bacosides.

Linearity and precision: We injected different concentrations of bacoside standard and standard curve has been drawn. Linear standard curve with $R^2 > 0.9$ was obtained. We

observe good precision based on three replication. Standard deviation is within the reasonable level.

Limit of detection and limit of quantification: Limit of detection and limit of quantification were calculated 0.0306 µg/5 ml and 0.0918 µg/5 ml respectively.

Determination of total phenol content (TPC): Determination of TPC in the extract was done with Folin-Ciocalteu (FC) reagent using Gallic acid as a standard solution. TPC was measured according to the method described by Singleton et al. (1999) with slight modifications. 100 µL of the extract was added to 900 µL of distilled water, 200 µL of 1 N FC reagent and 2.0 mL of sodium carbonate (7% w/v). The contents were mixed and allowed to stand for 30 min at room temperature (25 ± 1°C) in dark. The absorbance was measured at 750 nm using UV-Vis spectrophotometer (LAMBDA 25 UV/Vis Systems, PerkinElmer, Inc. MA 02451, USA). Total phenol content was expressed as µg/g GAE (Gallic acid equivalent).

Determination of antioxidant potential: Antioxidant potential of samples was assessed using DPPH (2,2-diphenylpicrylhydrazyl) and CUPRAC (Cupric ion reducing antioxidant capacity) assay.

DPPH assay was done according to the method of Brand-Williams et al (1995) with slight modification. DPPH assay was performed by pipetting out 800 µL (100mM) of Tris HCl buffer (pH 7.4) to which 200 µL of standard solution (Gallic Acid) of varying concentration was added. Different volumes of sample extract were dried under nitrogen and redissolved in 200 µL of methanol, which was then added to 0.8 ml of DPPH reagent. The reaction mixture was vortexed and left for 30 min in dark condition.

Absorbance was recorded at 517 nm using UV-Vis spectrophotometer (LAMBDA 25 UV/Vis Systems, PerkinElmer, Inc. MA 02451, USA) with methanol as the blank.

$$50\%\ \text{Inhibition} = \frac{\text{A(control)} - \text{A(sample)}}{\text{A(control)}} \times 100$$

where A (control) is absorbance of blank control (containing all reagents except the extract) and A(sample) is absorbance of the test sample.

CUPRAC of the extract was determined according to the method of Apak et al. (2004). In reaction mixture 1 ml $CuCl_2$ solution (0.01 M), 1 ml neocuproine alcoholic solution (7.5 mM), 1 ml ammonium acetate buffer (1.0 M) of pH 7.0, 100 µL extract and 1 ml water were added (total volume, 4.1 ml) and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min.

iii. Statistical analysis

Data of *in vitro* propagation and elicitation was analyzed using ANOVA with SPSS version 21.0 statistical software package. Results were expressed in terms of means and standard error, while means were compared with Duncan's multiple range test (DMRT, 9) at $P<0.05$ level. Analysis of variance was carried out using PROC GLM to determine significant differences in parameters studied among the *B. monnieri* accessions. Multilinear regression (MLR) has been used for statistical analysis to check the correlation among total bacosides, total phenols and antioxidant potential.

Simple linear correlation analysis was performed to indicate the measure of correlation and strength of the relationship between variables using Pearson's correlation. The sample similarities were calculated on the basis of pair-wise Euclidean distance and the

unweighed pair-group method with arithmetic means (UPGMA) algorithm was used for establishing cluster to search natural groupings among the accessions for different parameters. Principal Component Analysis (PCA) using correlation matrix was carried out to evaluate the relative contribution of moisture, CUPRAC, DPPH, total bacosides and total phenols to the total variability in the *B. monnieri* accessions.

4.2.2. Result and Discussion

i. *In vitro* shoot induction and elicitation

Shoot multiplication and proliferation were assessed for thirteen accessions of *B. monnieri* on MS medium supplemented with 0.4mg/L BA using nodal explants. Various researchers have reported the growth of *B. monnieri* on different media (Sharma et al. 2007; Jauhari et al. 2016). In the current experiment, maximum shoot length was observed in IC554588 (2.8 cm) followed by IC375976 (2.2 cm) whereas a maximum number of shoots/explant were recorded in IC344314 (8.7 shoots/explants) followed by IC554588 (8.3 shoots/explants) (Table 4.1). Overall, maximum shoot growth was observed in IC554588, which was selected for further elicitation experiments. No alteration in the morphology of the plant and no callus formation were observed in the elicitor-supplemented medium. A slight reduction in a number of shoots/explants (Fig 4.1) and shoot length (Fig 4.2) was observed in elicitor-treated plants. This may be due to increase in the level of the enzyme β-amyrin synthase, responsible for increasing structural components, up to 2-3 weeks of elicitation with methyl jasmonate and reduction after 4 weeks (Mangas et al. 2009). Similar elicitation effect has been reported in earlier studies for bacoside A and triterpenoid saponins (Namdeo, 2007; Sharma et al. 2012). In the present study, explants grown on media supplemented with ME often-displayed necrosis of the meristematic area. Thus, the

growth of ME-treated plants was inhibited and less number of shoots/explants and shoot length was noticed compared to SA and JA. Plants elicited with SA, exhibited higher number of shoots/explant compared to JA and ME. Shoot length of SA-treated plants was comparable with control while a slight decrease was observed with JA and ME. In a similar study, maximum number of shoot regeneration has been reported in *Centella asiatica* elicited with ME as compared to JA and SA (Kundu et al. 2015). Thus, the response to elicitors may be species specific. As far as moisture content is concerned, it was more or less constant across all the accessions and only non-significant differences were observed in non-elicited 13 accessions of *B. monnieri* (Table 4.1).

Table 4.1: Total bacoside amount, total phenol content and antioxidant potential (DPPH and CUPRAC) studied in 13 accessions of *B. monnieri* after 4 weeks of culture

Accession No.	Moisture (%)	CUPRAC (µg/g)	DPPH (µg/g)	Bacosides (µg/g DW)	Phenol (µg/g)	No of shoots/ Explant	Shoot length (cm)
IC 439118	86.0±0.5^{e}	60.7±0.4^{j}	0.978±0.6^{g}	324.1±0.6^{h}	17.7±0.6^{b}	7.0±0.8^{cde}	2.1±0.6^{ab}
IC 426442	89.0±0.4^{b}	67.8±0.6^{i}	0.856±0.4^{g}	250.9±0.5^{k}	9.81±0.7^{e}	8.1±0.7^{abc}	2.1±0.6^{ab}
IC 426447	87.5±0.5^{d}	69.9±0.2^{h}	0.681±0.4^{g}	434.0±0.7^{c}	7.58±0.4^{f}	5.6±0.8^{fg}	1.2±0.7^{bc}
IC 468878	88.4±0.7^{cb}	35.1±0.4^{l}	2.32±0.6^{f}	433.4±0.4^{c}	1.49±0.5^{i}	4.5±0.6^{g}	1.1±0.5^{bc}
IC 373640	88.4±0.4^{cb}	51.7±0.9^{k}	3.82±0.5^{e}	407.2±0.5^{f}	5.57±0.5^{g}	6.4±0.6^{def}	1.3±0.6^{bc}
IC 344312	90.1±0.6^{a}	193.0±0.6^{b}	6.49±0.6^{b}	455.1±0.6^{b}	18.3±0.6^{b}	8.7±0.6^{a}	2.1±0.4^{ab}
IC 531621	87.6±0.3^{dc}	88.9±0.4^{f}	3.79±0.6^{e}	328.5±0.7^{g}	5.77±0.7^{g}	5.4±0.7^{fg}	2.1±0.5^{ab}
IC 375976	87.4±0.4^{d}	126.1±0.5^{d}	5.26±0.4^{c}	313.5±0.9^{j}	3.31±0.7^{h}	5.3±0.5^{fg}	2.2±0.7^{ab}
IC353203	89.1±0.5^{b}	108.2±0.4^{e}	4.37±0.9^{e}	208.9±0.1^{l}	15.3±0.4^{c}	7.2±0.6^{bcd}	1.6±0.4^{bc}
IC 554588	88.0±0.6^{dc}	173.4±0.7^{c}	7.15±0.5^{a}	515.4±0.6^{a}	11.9±0.5^{d}	8.3±0.7^{ab}	2.8±0.5^{a}
IC 554586	82.7±0.5^{g}	126.4±0.5^{d}	3.62±0.5^{e}	432.2±0.6^{d}	8.98±0.8^{e}	2.2±0.5^{h}	0.8±0.3^{c}
IC 554587	84.7±0.3^{f}	78.1±0.6^{g}	2.07±0.6^{f}	412.9±0.5^{e}	16.2±0.5^{c}	4.9±0.6^{g}	1.2±0.5^{bc}
IC 554585	85.5±0.6^{e}	231.0±0.5^{a}	4.25±0.4^{d}	318.7±0.4^{i}	22.3±0.6^{a}	5.8±0.5^{efg}	1.5±0.4^{bc}

Data given as Mean ± SE. different letters in superscripted are significantly different at $p<0.05$ level using DMRT.

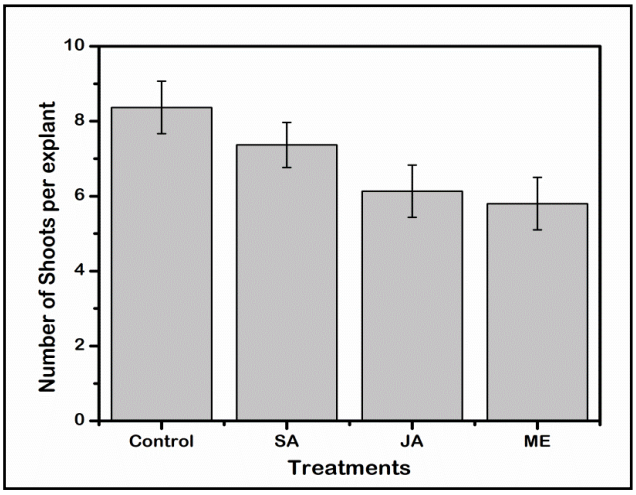


Fig. 4.1: Effect of elicitors JA, SA and ME (1.0 mg/L) on a number of shoots/explant of *B. monnieri* (IC 554588) after 4 weeks of culture. Data represent mean±standard error of three replicates; each experiment was repeated thrice ($P < 0.05$)

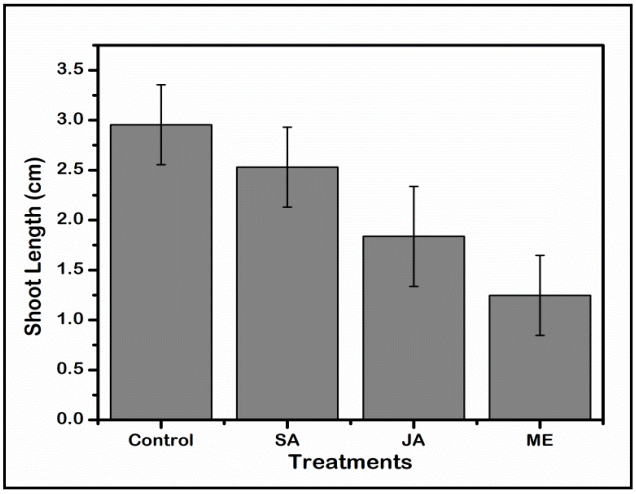


Fig. 4.2: Effect of elicitors JA, SA and ME (1.0 mg/L) on shoot length (cm) of *B. monnieri* (IC 554588) after 4 weeks of culture. Data represent mean±standard error of three replicates; each experiment was repeated thrice ($P < 0.05$)

ii. Biochemical Analysis

FTIR: IR spectra acquired for bacosides showed characteristic molecule bands and peaks: 3379-3087 cm^{-1} (O-H), 1639 cm^{-1} (C=O) and 1571 cm^{-1} (C=C) (Thakkar et al. 2017). Prominent bands were observed in FTIR spectra (Fig. 4.3) The strong broad band appearing at 3323 cm^{-1} can be associated with the stretching vibrations of O–H. A weak band at 1667 cm^{-1} could be assigned to C=O band frequency (a bacoside

characteristic band) of bacoside overlapped with its C=C absorption. Another absorption of C=C in the cis plane was observed at 694 cm^{-1} in bacoside.

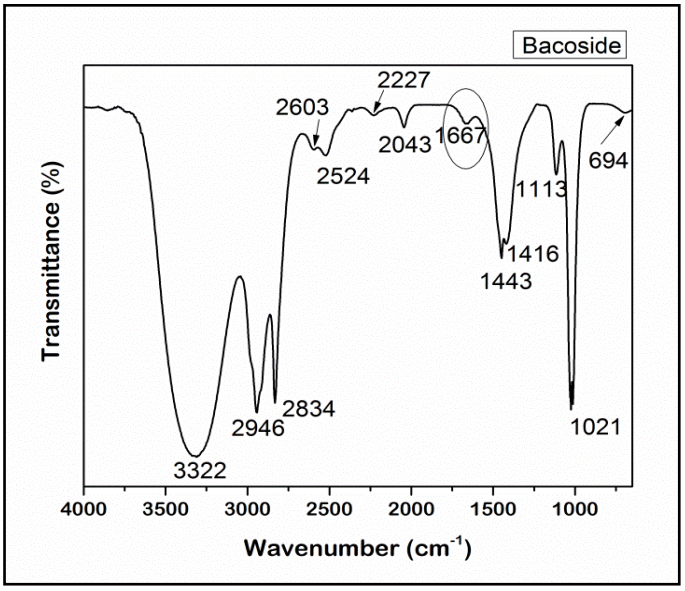


Fig. 4.3: FTIR spectra of methanolic extract of *B. monnieri* (IC 554588)

Quantification of total bacosides: Bacoside content of *in vitro* propagated shoots of 13 non-elicited accessions of *B. monnieri* using HPLC technique is depicted in Table 4.1. Wide variation in bacoside amount (208.93-515.47 µg/g dry weight (DW) basis) was exhibited in 13 accessions based on Duncan's Multiple Range Test (DMRT). Although accession IC554588 produced maximum amount of total bacosides, no correlation of growth parameters on the yield of bacosides was noticed. Accessions belonging to Madhya Pradesh (IC 554588) and Karnataka (IC 344312) produced highest amount of bacosides followed by the accessions of Kerala (IC 648878), Madhya Pradesh (IC 426447, IC 554586) and Jharkhand (IC 531621, IC 439118). Bacoside A3 is the major active constituent of saponin fraction, which has memory enhancing effect (Pal et al. 1998). Mundkinajeddu et al. (2005) assessed samples of *B. monnieri* for the presence of bacoside A3, bacopaside II, jujubognin isomer of bacopasaponin and bacopasaponin. Mishra et al. 2013 reported the presence of bacoside A_3 in *B. monnieri*.

In the present study, IC554588 produced maximum amount of total bacosides (515.4 µg/g DW), which was remarkably influenced by elicitors. Standard of bacoside is a mixture of five types of bacosides (bacoside A, bacoside A3, bacoside II, bacopaside X and bacosaponin C) shown at Retention Time 1.7, 2.3, 20.3, 22.8, 27.3 min in chromatogram (Fig 4.4a). After analyzing the peaks of 13 accessions, few peaks were below LOQ but above LOD. Five peaks of accession IC 426447 have been observed but only four can be quantified. Likewise, in accessions IC 375976 and IC 353203, two out of three peaks and three out of five peaks were quantified respectively.

Applied elicitors (JA, SA and ME) exhibited different effects on bacoside production. The study exhibited that bacoside profile has been changed after the elicitor treatment. Standard of bacoside shown five peaks as given in Fig. 4.4a. Two types of bacosides are present in control at Retention Time 1.7, 2.3 min (Fig 4.4b). After the elicitation of JA, increased amount of bacoside was noticed at Retention Time 1.7, 2.3, 22.8, 27.3 min (Fig 4.4c). Despite a slight reduction in growth parameters, increased bacoside content was recorded in all the elicited samples compared to control (Fig 4.5a). Maximum amount of total bacosides was obtained in the plant elicited with JA (3.9 fold) followed by ME (3.2 fold) and SA (2.7 fold) after four weeks of the elicitation. Sharma et al. (2014) reported 3.08 and 1.32 fold increase in bacoside content after elicitation with JA (1.0 mg/L) and SA (50 mM) respectively in *B. monnieri* after 9 days of elicitation. 2-3 fold enhancement in bacoside A content was achieved with MeJ (50 µM) and SA (50 µM) respectively after three weeks of elicitation (Largia et al. 2015). Mi-Hyun and coworkers (2004) reported that treatment with methyl jasmonate overexpresses the gene *PgSS1*, which is responsible for upregulation of triterpene saponins. This may be the possible reason for the enhanced production of bacoside with the treatment of JA.

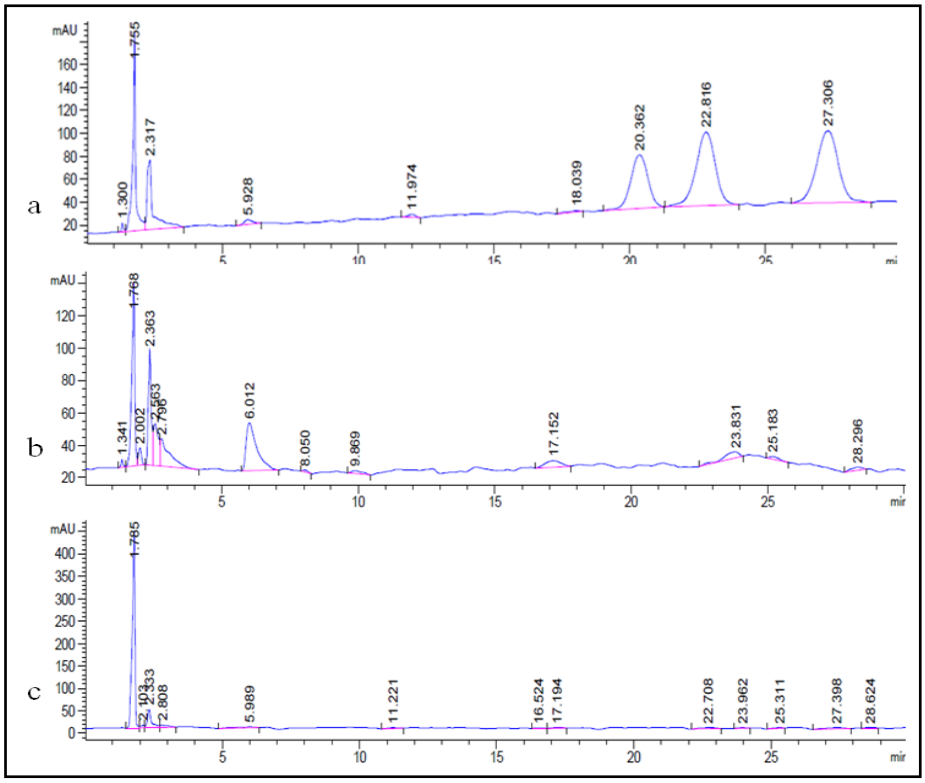


Fig. 4.4: Chromatogram of bacosides obtained through HPLC (a) Standard, (b) control, (c) elicitor treated (JA)

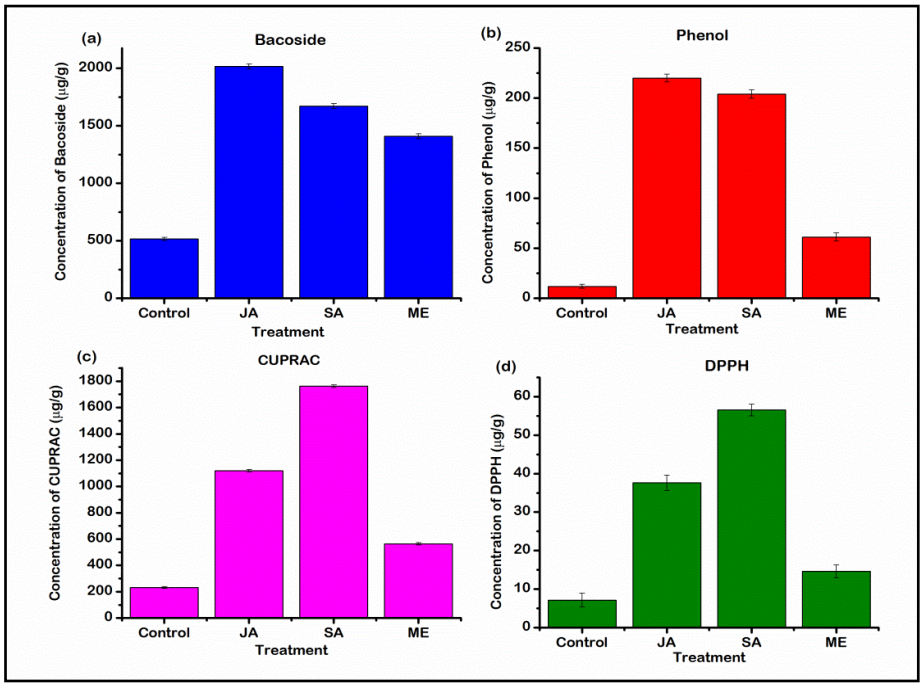


Fig. 4.5: Effect of elicitors JA, SA and ME (1.0 mg/L) on CUPRAC, DPPH, total bacoside content and total phenol content of *B. monnieri* (IC 554588) after 4 weeks of culture. Data represent mean±standard error of three replicates; each experiment was repeated thrice ($P < 0.05$)

Determination of total phenol content (TPC): 13 *Bacopa* accessions were investigated for Total Phenol Content. Phenolic compounds play an important role in free radical scavenging potential of plants and are responsible for antioxidant property (Gottlieb and Borin, 2000). The present study exhibited 1.5-22.4 µg/g GAE TPC in thirteen accessions (Table 2). Maximum phenol content was observed in the accession belonging to Amarkantak (IC 554585) and Karnataka (IC 344312). Jharkhand (IC 439118) Madhya Pradesh (IC 554587), New Delhi (IC 353203) and accessions from other states showed comparatively lower amount of phenol content. Elicitors were applied on the selected (IC 554588) having 11.9 µg/g GAE phenol content, which was further increased to 219.9, 61.2 and 203.2 µg/g GAE in plants elicited with JA, ME and SA respectively (Fig 4.5b). Results of present investigation confirmed earlier reports of phenol concentration in ethanolic and aqueous extract of *B. monnieri*, which was 3.18 µg/ml and 3.71 µg/ml GAE respectively (Mukherjee et al. 2011). The present study clearly indicates that elicitation increases accumulation of total phenol content in *in vitro* cultures of *B. monnieri*.

Antioxidant potential: In the present study, presence of significant amount of total bacosides and total phenol content in the samples of *B. monnieri* suggested that bacosides and phenols might be the contributor to antioxidant activity, which can be enhanced with the use of elicitors. Though, antioxidant compounds reduce free radicals by single electron and hydrogen atom transfer mechanisms, evaluation of antioxidant potential of the plants using different methods (DPPH, ABTS, FRAP) showed variation in antioxidant activity (Thaipong et al. 2006). Different methods vary in terms of principle and environmental conditions. It is difficult to precisely measure the antioxidant activity of a system having various components using single test. Therefore, different mechanisms are needed to confirm antioxidant potentiality of the plants. In the

present study antioxidant potential was studied using DPPH and CUPRAC method. Maximum antioxidant activity was 7.15 µg/g GAE (IC 554588) for DPPH and 231 µg/g GAE (IC 554588) for CUPRAC, among all the thirteen accessions of *B. monnieri*. Antioxidant potential (CUPRAC and DPPH) of all the samples of *B. monnieri* is given in Table 4.1. Considering both the methods (CUPRAC and DPPH), accession of Madhya Pradesh (IC 554588), Karnataka (IC 344312) and Amarkantak (IC 554585) exhibited maximum antioxidant potential compared to other states.

Few researchers have evaluated effect of bacosides on antioxidant potential. Tripathi et al. (1996) compared the alcoholic extract of *B. monnieri* with Tris, EDTA and vitamin E and found that 100 µg of antioxidant potential is equivalent to 247 µg of EDTA and 58 µg of vitamin E. Russo et al. (2003) reported antioxidant potential using DPPH method and reduced hydrogen peroxide-induced cytotoxicity of methanolic extract of whole plant. Administration of 10 mg/kg aqueous bacoside A remarkably enhanced levels of glutathione, vitamin C, vitamin A and vitamin E in brain of rat model exposed to cigarette smoke. Free radicals produced during the normal metabolic reaction of aerobic cells are extremely reactive and at unsteady state that have tendency to react with food lipids, nucleic acid, sugars and sterols (Lee et al. 2004), which lead to various physiological modifications. Bacoside A has been found to increase superoxide dismutase, catalase, glutathione peroxide and glutathioin reductase (Anabasari et al. 2006). Jain and coworkers, (2016) reported free radical scavenging activity using CUPRAC assay equivalent to 0.226 TE mM at 1000 µg/ml.

Many researchers reported that enhanced phenol content leads to increased antioxidant capacity (Cai et al. 2004; Rahman et al. 2007; Ahmad et al. 2013). Saxena et al. (2016)

reported that phenol content (using Folin-Ciocalteu reagent) present in methanolic and ethnolic extract of the plants *Eclipta* and *Plumbago* is responsible for antioxidant potential. In the present study, increase or decrease in total bacosides, phenol content and antioxidant potential was not influenced by growth parameters (Table 4.1). However, all the tested elicitors, when used separately, stimulated yield of total bacosides, total phenols and antioxidant potential, with a significant difference. Nevertheless, total bacosides and total phenol content were much higher with JA-elicited plants while elicitation with SA showed highest increase in antioxidant potential. Antioxidant activity for CUPRAC assay showed highest 231 µg/g GAE (IC 554588), which was enhanced to 1119.8, 563 and 1762 µg/g GAE by the application of elicitors JA, ME and SA respectively (Fig 4.5c). DPPH assay increased from 7.15 µg/g GAE to 37.6, 14.6 and 56.5 µg/g GAE with the elicitation of JA, ME and SA respectively (Fig 4.5d). Chemical elicitation treatment significantly enhanced the free radical scavenging potential of the extract. The present study clearly indicates that elicitation increases accumulation of total bacosides, total phenol content and antioxidant potential of *in vitro* cultures of *B. monnieri*.

Effect of total bacosides and total phenols on antioxidant potential: According to statistical analysis (MLR), antioxidant potential is highly correlated to total bacoside and total phenols as R-sq(adj) is 65.8% and 90.1% respectively. It is also evident that phenol is more correlated to antioxidant potential than bacoside. As per regression equation, the coefficient of phenol is higher than the coefficient of bacoside, which employs that antioxidant potential is more sensitive to phenol in comparison to bacoside. The sign of coefficients indicate that both the factors are directly correlated to

antioxidant potential. The R-adj value for the regression equation is 89%, which shows very strong correlation of both factors with antioxidant potential.

iii. Correlation, variation, principal component and cluster analysis

Statistical analysis revealed negative correlation of moisture content with bacoside content, antioxidant potential and total phenol content (Table 4.2). Antioxidant CUPRAC method exhibited strong correlation with DPPH, bacoside and phenol content. Among all the components phenol is the major contributor to variation. In addition, phenol is the major contributor to antioxidant activity measured by CUPRAC and DPPH method. Phenols and bacosides are highly correlated with strong positive correlation. Elicited samples exhibited an increase in bacoside content as well as phenol component.

Table 4.2: Linear correlation between moisture content, antioxidant potential using CUPRAC and DPPH method, bacosides and phenol content of 13 *B. monnieri* accessions.

		CUPRAC	**DPPH**	**Bacosides**	**Phenol**
Moisture	Pearson Correlation	-.815	-.820	-.574	-.832
	Sig. (2-tailed)	.000	.000	.000	.000
CUPRAC	Pearson Correlation		.994	.811	.951
	Sig. (2-tailed)		.000	.000	.000
DPPH	Pearson Correlation			.798	.954
	Sig. (2-tailed)			.000	.000
Bacosides	Pearson Correlation				.871
	Sig. (2-tailed)				.000

Principal components analysis (PCA) has been carried out to find total variation in all the samples. In the present study, five parameters have been taken into account for the same. PCA revealed that Moisture, CUPRAC, and DPPH together governed 83% of total variability among all five components (Table 4.3). Therefore, these three components are

considered as Principal Component (PC). PC1 represents moisture while PC2 and PC3 represent CUPRAC and DPPH respectively in the component matrix (Table 4.4). In PC1 CUPRAC (0.644) and DPPH (0.689) have high positive loading and are considered as a major contributor to variability. In PC2, CUPRAC is major contributor followed by phenols and bacosides. The maximum contribution of bacoside was noticed in PC3. However, the overall picture exhibited that antioxidant potential as assessed by CUPRAC and DPPH method are major traits responsible for variability amongst all the tested accessions. Antioxidant potential was also noticed as a major contributor in cluster grouping, and their influence is distinctly reflected in the primary grouping of 13 *B. monnieri* accessions.

Table 4.3: *Bacopa monnieri*: Eigenvalues for all the five components in 13 accessions.

Component	**Initial Eigen Values**		
	Total	**% of Variance**	**Cumulative %**
Moisture	2.816	40.232	40.232
CUPRAC	1.725	24.643	64.875
DPPH	1.261	18.016	82.891
Bacosides	0.628	8.971	91.862
Phenols	0.432	6.172	98.034

Extraction Method: Principal Component Analysis

Table 4.4: *Bacopa monnieri*: Component matrix of five components in 13 accessions.

	Component		
	PC1	**PC2**	**PC3**
Moisture	.584	-.679	.229
CUPRAC	.644	.692	-.095
DPPH	.689	.419	.431
Bacosides	-.015	.431	.725
Phenol	.448	.453	-.683

Extraction Method: Principal Component Analysis. (Table denotes PC1-Principal Component 1, PC2- Principal Component 2, PC3- Principal Component 3)

The dendrogram generated, based on judged parameters revealed 5 distinct clusters containing 5 accessions in Cluster I, 2 accessions in each Cluster II and Cluster III, 3 in Cluster IV and 1 accession in Cluster V at the linkage distance of 10 (Fig .4.6). In general, accessions present in Cluster I, Cluster IV and Cluster V have moderate amount of bacoside content. Accessions of Cluster II (IC 344312 and IC 554588) exhibited highest amount of bacoside while accessions of Cluster III had lowest amount of total bacosides. Accessions of Cluster II also have high phenol content and antioxidant potential. IC554585 of Cluster V showed high antioxidant potential (by CUPRAC method) and phenol content with the moderate amount of bacoside. Hence, amongst the 13 studied accessions, IC 554588 and IC 344312 IC 554585 are best suited for exploitation for pharmaceutical purposes. Further, based on principal component analysis and cluster grouping it is indicated that major contribution to antioxidant activity is of total phenols, although contribution of bacosides cannot be ruled out.

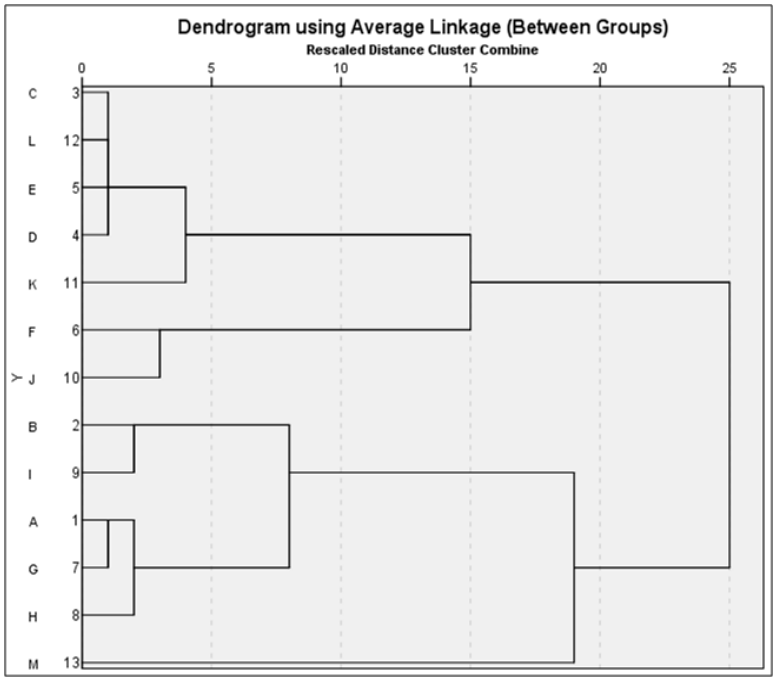


Fig. 4.6: *Bacopa monnieri*: Clustering of 13 accessions based on components moisture content, antioxidant potential using CUPRAC and DPPH method, bacosides and phenol content, number of shoots/explants and shoot length. (Refer table 3.1 of Chapter 3 for the corresponding accessions)

4.3. Biochemical analysis of *C. borivilianum*

Tubers (roots) of *C. borivilianum* are widely utilized for diverse therapeutic applications. Various pharmacological activities of the plant have already been discussed in Chapter 1. Antioxidant potential is an important therapeutic property, which is still a topic of interest for research community. Pharmacological properties of *C. borivilianum* are attributed to stigmasterol, a phytosterol. Stigmasterol is an active constituent of the herb and is a major secondary metabolite present in tubers (Bathoju and Giri 2012). Along with other medicinal properties, it is also known to possess antioxidant potential. Limited work has been done regarding quantification of stigmasterol from *C. borivilianum* (Bathoju and Giri 2012; Panigrahi and Patel 2016). It is well known that environmental conditions strongly affect the yield of secondary metabolites (Gorelick et al., 2014). According to literature, elicitation is an effective strategy for the stimulation of secondary metabolite production (Hussain et al., 2012). Although enhanced production of secondary metabolites with the application of JA, SA and ME has been studied widely (Kim et al. 2004; Sakunphueak and Panichayupakaranant 2010; Kundu et al., 2016), the effects are species specific. Therefore, the evaluation of JA, SA and ME on the production of stigmasterol, phenol content and thereby the pharmacological effects including the antioxidant potential of the species is required. Biochemical analysis including quantification of stigmasterol from different accessions is prudent for standardization of the preparations developed from the plant for therapeutic use. Diverse curative properties of the plant and less availability for future use, justify the exploration of different accessions of *C. borivilianum* for higher stigmasterol production for commercial exploitation. No study has been published so far on biochemical analysis of root extract using elicitors and principal component

analysis from the studied accessions of *C. borivilianum* to identify the promising germplasm. The aim of the present study is to enhance pharmaceutically important compound stigmasterol production, total phenol content and antioxidant potential of the methenolic extract of tubers with the use of elicitors. The selected accession among three accessions (Chapter 3) was elicited with three elicitors (JA, SA and ME). Methanolic extract of hardened elicited tubers was examined for the enhanced stigmasterol production, total phenol content and antioxidant potential as compared to non-elicited plants. Data were analyzed to identify major relative contributors of variability among all the studied parameters in different accessions through Principle Component Analysis.

4.3.1. Material and Method

Plant material is taken as given in section 3.3.1i.

i. *In vitro* elicitation for shoot and root growth

Elicitation study was performed on selected accession (IC 558379) with JA, SA and ME. A stock solution of JA was prepared in 90% ethanol and sterile Mili-Q water (Mili-Q, USA) was used to prepare SA and ME (Sigma-Aldrich, USA). Filter sterilized 1.0 mg/L of JA, SA or 500 mg/L of ME were added to the autoclaved medium.

Shoot base from 6 weeks culture of selected accession, were transferred to the optimum shooting medium with or without elicitors (control). The cultures were incubated at 25 ± 2°C under 16 h photoperiod. After 6 weeks of culture, the effect of elicitor-treated and non-elicited samples were evaluated for growth parameters (number of shoots/explant and shoot length). For root induction, 6-week-old healthy shoots of selected accession were transferred to optimum rooting media and incubated at 25 ±2°C under total

darkness for 14 days. Subsequently, cultures were transferred to optimum rooting medium with or without elicitors (control). The concentration of JA and SA were kept 1 mg/L while 500 mg/L of ME was added to the medium. Cultures were incubated at 25 ± 2°C under 16 h photoperiod for development of roots. After 6 weeks, elicitor-treated and non-elicited adventitious roots were acclimatized and hardened for estimation of biochemical parameters (stigmasterol production, total phenol content and antioxidant potential). All the treatments were performed in triplicates and each treatment consisted of 10 samples.

ii. Biochemical analysis

Hardening and acclimatization was done as given in Chapter 3 section 3.3.1v. For biochemical analysis, stigmasterol estimation, total phenol content and antioxidant potential, of elicited and non-elicited roots, following treatments were used for the sample preparations: (i) non-elicited roots (hardened) of 3 accessions grown on MS + 1 mg/L IAA + 1 mg/L NAA + 6% sucrose and (ii) hardened roots of selected accession, grown on MS +1 mg/L IAA + 1 mg/L NAA + 6% sucrose supplemented with elicitor and control (without elicitor).

Sample preparation: Root extract was prepared following the method of Visavadiya et al. (2010) with slight modifications. Acclimatized and hardened roots (10 g each) collected from the greenhouse were washed thoroughly with tap water and peeled slightly. After measuring the fresh weight, roots were dried at 37°C for 2-3 days and weighed. Subsequently, roots were sliced into small pieces and ground to powder. The powder was extracted with 200 ml methanol using Soxhlet extractor for 8 hours. The extracted solvent was dried on rotary evaporator and volume was made up to 2 ml and

centrifuged at 5000 rpm for 20 min. The supernatant was then filtered through 0.22-micron filter and used for the estimation of stigmasterol, total phenol content and antioxidant potential (DPPH and CUPRAC). Methanolic extract of accessions IC 558379 prepared from above-mentioned method was used for FTIR to investigate the presence of stigmasterol.

FTIR: FTIR spectra of methanolic root extract were recorded for the presence of stigmasterol using Perkin Elmer Model Spectrum RXI-Mid IR. Spectrometer operating in the range of 650-4000 cm^{-1} with a resolution of 4 cm^{-1}.

Quantification of stigmasterol: Stigmasterol was quantified using HPLC with the method used by Sivanandhan et al (2012). HPLC experiments were performed on Agilent HPLC system equipped with 1260 Infinity Binary Pump (G1312B). ZORBAX ODS C18 column (5 µm 4.6x250 mm), 1260 infinity diode array detector (G4212B) and isocratic elution with methanol/water (65:35) at a flow rate of 0.5 ml/min with 30 min run time. Detection was done at 205nm and sample injection volume was kept at 2.5 µL. 1 mg stigmasterol was dissolved in 2 ml of methanol and used as a standard solution to quantify stigmasterol.

Linearity and precision: We injected different concentrations of bacoside standard and standard curve has been drawn. Linear standard curve with $R^2 > 0.9$ was obtained. We observe good precision based on three replication. Standard deviation is within the reasonable level.

Limit of detection and limit of quantification: Limit of detection and limit of quantification were calculated 1928.7 µg/2.5 ml and 5786.1 µg/2.5 ml respectively.

Determination of total phenol content (TPC): Determination of TPC in the extract was done using Folin-Ciocalteu (FC) assay as described by Singleton et al (1999) with slight modifications. Briefly, 100 µL of the extract was added to 900 µL of distilled water, 200 µL of 1 N FC reagent and 2.0 mL of sodium carbonate (7% w/v). Further, the contents were mixed and allowed to stand for 30 min at room temperature (25 ± 1°C). The absorbance was measured at 750 nm using UV-Vis spectrophotometer (LAMBDA 25 UV/Vis Systems, PerkinElmer, Inc. MA 02451, USA). Total phenol content was expressed as µg/g GAE (Gallic acid equivalent).

Determination of antioxidant potential: Antioxidant potential of samples was assessed using DPPH (2,2-diphenylpicrylhydrazyl) and CUPRAC (Cupric ion reducing antioxidant capacity) assay.

DPPH assay: DPPH assay was done according to the method of Brand-Williams et al (1995). DPPH assay was performed by pipetting out 800 µL (100mM) of Tris HCl buffer (pH 7.4) to which 200 µL of standard solution (Gallic Acid) of varying concentration was added. Different volumes of sample extract were dried under nitrogen and redissolved in 200 µL of methanol, which was then added to 0.8 ml of DPPH reagent. The reaction mixture was vortexed and left for 30 min in dark condition. Absorbance was recorded at 517 nm using UV-Vis spectrophotometer (LAMBDA 25 UV/Vis Systems, PerkinElmer, Inc. MA 02451, USA) with methanol as blank.

$$50\%\ \text{Inhibition} = \frac{\text{A(control)} - \text{A(sample)}}{\text{A(control)}} \times 100$$

where A (control) is absorbance of blank control (containing all reagents except extract) and A(sample) is absorbance of the test sample.

CUPRAC assay: Cupric ion reducing antioxidant capacity (CUPRAC) of the extract was determined according to the method of Apak et al (2004). 1 ml CuCl2 solution (0.01 M), 1 ml neocuproine alcoholic solution (7.5 mM) and 1 ml ammonium acetate buffer (1.0 M) of pH 7.0 and 0.1 ml extract were added to the reaction mixture followed by 1 ml water (total volume, 4.1 ml) and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min.

iii. Statistical analysis

Data for *in vitro* propagation and elicitation was analyzed using ANOVA with SPSS version 21.0 statistical software package. Results were expressed in terms of means and standard error, while means were compared with Duncan's multiple range test (DMRT, 9) at $P<0.05$ level. Analysis of variance was carried out using PROC GLM to determine significant differences in parameters studied among the *C. borivilianum* accessions. Correlations among data obtained were calculated using Pearson's correlation. Simple linear correlation analysis was performed to indicate the measure of correlation and strength of the relationship between variables. The sample similarities were calculated on the basis of pair-wise Euclidean distance and UPGMA algorithm was used for establishing cluster to search natural groupings among accessions for different parameters. Principal Component Analysis (PCA) using correlation matrix was carried out to evaluate the relative contribution of moisture, CUPRAC, DPPH, stigmasterol, and phenols to total variability in *C. borivilianum* accessions.

4.3.2. Result and Discussion

i. *In vitro* elicitation for shoot and root growth

For the assessment of elicitation on the accession IC 558379, number of shoots and shoot length were recorded on MS + 3 mg/L BA fortified with JA, SA or ME. The

addition of elicitors exhibited a varying effect on shoot growth. Slight decrease in shoot growth was observed with treatment of JA and ME as compared to non-elicited cultures. SA did not inhibit the number of shoots and shoot length significantly. Sharma et al. 2012 reported decreased growth of MJ treated shoots with enhanced bacoside A production compared to non-elicited shoots. Similar elicitation effects of MJ have been revealed earlier for other triterpinoid saponin compounds (Namdeo 2007). The responses of adventitious root growth in non-elicited cultures in relation to JA, SA and ME were different. Root growth was higher in the cultures treated with JA compared to ME. On the other hand, SA did not inhibit the root biomass significantly. Elicited cultures showed enhancement of roots compared to control (Fig 4.7).

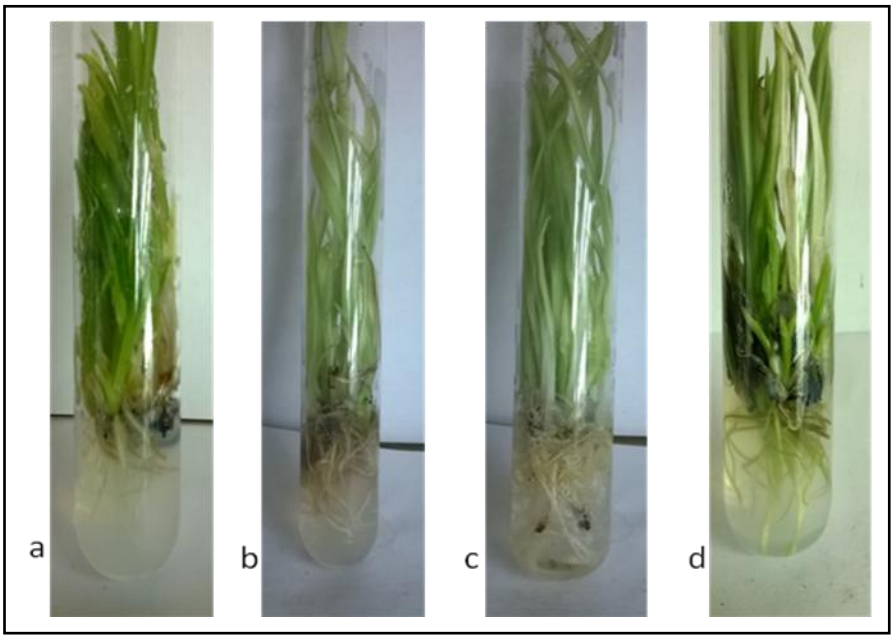


Fig. 4.7: Effect of elicitors on root growth of *C. borivilianum* accession IC 558379 (a) control, (b) JA, (c) SA and (d) ME on the media MS+3mg/l BA+1mg/l IAA+1mg/l NAA+6%sucrose after 6 weeks of culture

Slight decrease in shoot growth was noticed in elicited cultures. Analysis of variance exhibited significant effect of treatments ($p < 0.01$) in relation to shoot multiplication and root induction. Results of the present study are in agreement with Kang et al. (2004), who

reported that there is no adverse effect on root growth of *Scopolia parviflora* elicited with SA. Apart from elicitation effect on shoot and root growth, browning in cultures was noticed after 4 weeks of elicitation with JA. This may be due to the release of phenolic compounds due to the elicitor-induced-stress (Zhao et al., 2001).

ii. Biochemical analysis

FTIR: Prominent bands were observed in FTIR spectra (Fig 4.8). Strong broad band appearing at 3323 cm^{-1} can be associated with the stretching vibrations of O–H. A weak band at 1658 cm^{-1} could be assigned to C=O band frequency overlapped with its C=C absorption. Another absorption of C=C out of the plane was observed at 756 cm^{-1}. Similar spectra have been obtained by Giribabu et al. (2014) from the root extract of *C. borivilianum.*

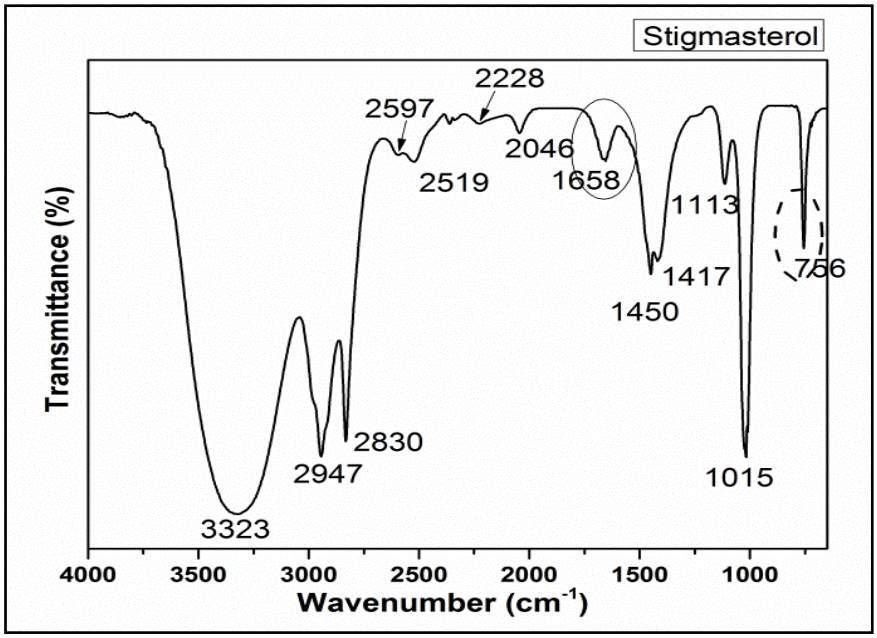


Fig. 4.8: FTIR spectra of the root extract from *C. borivilianum* IC 558379 (culture period 6 weeks).

Determination of stigmasterol: Stigmasterol content was estimated in hardened roots of *in vitro* propagated accessions of *C. borivilianum* as well as hardened elicited roots

(IC 558379) along with control. Roots of all the tested accessions showed varied amount of stigmasterol (1.18-1.58 mg/g DW) (Table 4.5). Standard of stigmasterol has shown only one peak at retention time 2.88. Analyses of the peaks of all the samples are above LOQ. The study exhibited that bacoside profile has changed after elicitor treatment. Chromatogram of stigmasterol standard (RT-2.88), control (RT-2.92) and elicitor (SA) (RT-2.92) treated is shown in Fig 4.9 (a), (b) and (c) respectively. Maximum stigmasterol content was noticed in IC 558379, which was enhanced after elicitation with JA, SA and ME to 2.4 fold, 3.2 fold and 1.9 fold respectively (Fig 4.10a). Panigrahi and Patel (2016) extracted 7.69 µg/g and 13.97 µg/g of stigmasterol from *in vitro* roots and callus respectively through HPTLC. In the present study, SA showed highest increase in stigmasterol production with no adverse effects on growth. JA and ME exhibited higher stigmasterol production with slight reduction in growth compared to non-elicited plants. The results of present study are in conformity with earlier findings and exhibit significant elicitation on stigmasterol content. Similar results are shown with the studies of Kim et al. (2004) for ginsenoside production from *Pinax ginseng* with the use of methyl jasmonate. Mi-Hyun and coworkers (2004) reported that treatment with methyl jasmonate overexpresses the gene *PgSS1*in adventitious roots. The gene is responsible for upregulation of phytosterol. This may be the possible reason for the enhanced production of stigmasterol in JA treated roots.

Table 4.5: Biochemical parameters studied in 3 accessions of *C. borivilianum* roots.

Accessions and treatments	Moisture (%)	CUPRAC (µg/g)	DPPH (µg/g)	Stigmasterol (µg/g DW)	Phenol (µg/g)
IC539860	84.3±0.3^{b}	125±0.2^{c}	12.9±0.1^{c}	1364.5±0.4^{b}	93.6±0.4^{b}
IC266708	85.7±0.0^{a}	164±0.1^{a}	13.5±0.1^{b}	1187.4±0.3^{c}	89.0±0.2^{c}
IC558379	85.3±0.5^{a}	156±0.3^{b}	16.0±0.2^{a}	1584.4±0.9^{a}	142±0.2^{a}

Data represent as Mean ± SE. different letters in superscripted are significantly different at $p<0.05$ level using DMRT.

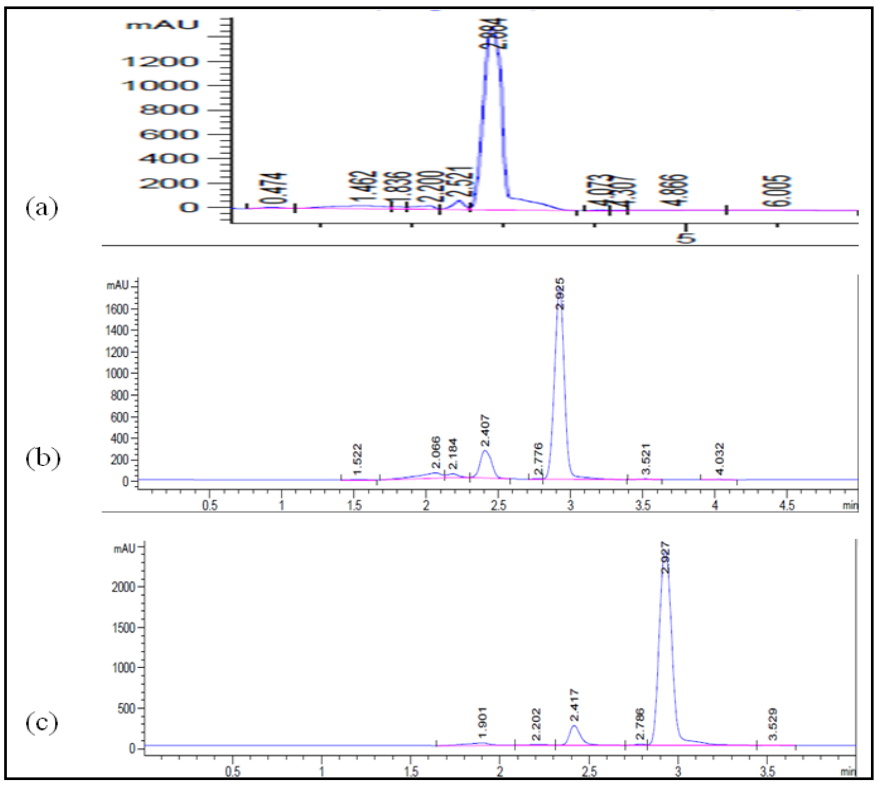


Fig. 4.9: Chromatogram of stigmasterol obtained through HPLC (a) Standard, (b) control, (c) elicitor treated (SA)

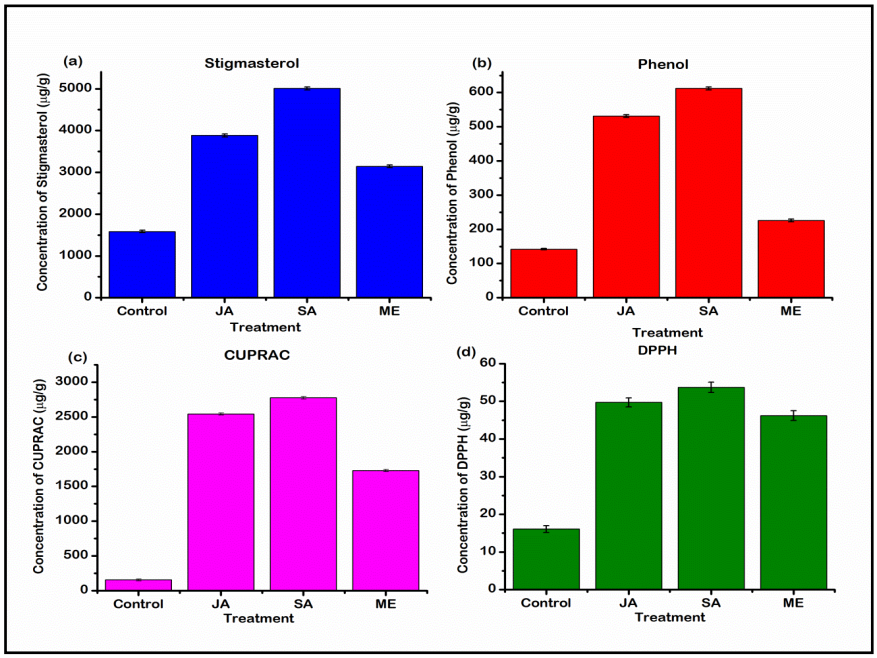


Fig. 4.10: Effect of elicitors (1.0 mg/L) on production of stigmasterol, total phenol content and antioxidant potential (CUPRAC and DPPH) of *C. borivilianum* (IC 558379) after 4 weeks of culture

Determination of total phenolic content (TPC): In the present study, total phenol content using non-elicited and elicited root cultures of *C. borivilianum* was assessed. Phenol content in different accessions of *C. borivilianum* (non-elicited) is shown in Table 4.5. All the elicited samples resulted in higher amount of total phenols compared to non-elicited samples. SA-elicited roots contained higher level of total phenols than JA and ME. Elicitor treated plants showed increased production of phenols 3.7, 4.3 and 1.5 fold with JA, SA and ME respectively compared to non-elicited roots (Fig 4.10b). Studies conducted by Chung et al. (2016) and Gorni and Pacheco (2016) are in conformity with the present results. It is evident that ME is prepared from barley, a good source of phenolic compounds (Goupy et al. 1999). This could be the reason for enhancing phenols in the samples elicited with ME. Our results suggested that elicitation with JA, SA and ME supports the enhancement of TPC in root cultures of *C. borivilianum*.

Determination of antioxidant potential: Antioxidant potential of the plants is mainly due to the presence of phenol compounds (Jang et al. 2010). Phenols play a vital role in determining antioxidant potential of various plants. Redox property of phenolic compounds is responsible for absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxidases (Weremczuk-Jezyna et al. 2013). Studies have been done to report the presence of phenols in *C. borivilianum* (Singh et al. 2006). Due to variation in principle and environmental conditions, it is difficult to precisely measure the antioxidant activity of a system having various components using single test. Antioxidant potential of different accessions of *C. borivilianum* is shown in Table 4.5.The present study evaluated the antioxidant

potential of non-elicited and elicited roots of *C. borivilianum*. Non-elicited plants showed 125-164 µg/g and 12-16 µg/g antioxidant activity with CUPRAC and DPPH method, which was increased to 1729-2775 µg/g and 46-53 µg/g respectively with the use of elicitors. In earlier studies, aqueous extract of *C. borivilianum* roots showed antioxidant activity (Visavadiya et al. 2010). Chemical elicitation treatment significantly enhanced the antioxidant potential of the extract. Fig 4.10c and 4.10d show the antioxidant activity of JA, SA and ME treated plants with CUPRAC and DPPH assay respectively. Consistent with our reports JA and SA-elicited hairy root culture and suspension cells increased the antioxidant potential in *Momordica charantia* and *Artemisia absinthium* respectively (Chung et al. 2016; Ali et al. 2015). Elicitation by SA acts as major contributor to upregulate the biosynthetic enzyme antioxidants (Dong et al. 2010). Malt plays a vital role as a natural antioxidant. Antioxidants from malt are responsible to scavenge oxygen-free radicals and prevent oxidative reactions, thus could be used to maintain the oxidative stability (Vanderhaegen et al. 2006). The present study suggests that phenolic compounds present in *C. borivilianum* are responsible for antioxidant activity. The study demonstrated the role of JA, SA and ME in the stimulated production of stigmasterol, TPC and antioxidant potential from field transferred roots of *C. borivilianum*.

iii. Correlation, variation, principal component analysis

Statistical analysis revealed positive correlation of moisture with antioxidant potential (CUPRAC and DPPH) and total phenol content. This signifies that high moisture content increases the antioxidant potential and phenol content as flashy tubers are supposed to be healthy. Healthy thick roots having high moisture content are considered

good-quality material for the pharmaceutical industry due to the presence of high amount of phytocompounds (Singh et al. 2012). CUPRAC exhibited positive correlation with DPPH and phenol content. DPPH showed strong positive correlation with stigmasterol and phenol. Stigmasterol exhibits strong positive correlation with phenol (Table 4.6). Phenol shows high positive correlation with DPPH and stigmasterol. It signifies that synthesis of stigmasterol increases total phenol content and antioxidant potential of the plant and high amount of stigmasterol can be achieved by elicitation of *C. borivilianum* with JA, SA and ME.

Table 4.6: Linear correlation between moisture content, antioxidant potential using CUPRAC and DPPH method. stigmasterol and phenol content of 3 *C. borivilianum* accessions.

		Moisture	**CUPRAC**	**DPPH**	**Stigmasterol**	**Phenol**
Moisture	Pearson Correlation	1	.906	.389	-.185	.159
	Sig. (2-tailed)		.001	.301	.633	.683
CUPRAC	Pearson Correlation		1	.510	-.128	.252
	Sig. (2-tailed)			.161	.744	.513
DPPH	Pearson Correlation			1	.783	.955
	Sig. (2-tailed)				.013	.000
Stigmasterol	Pearson Correlation				1	.928
	Sig. (2-tailed)					.000

Principal components analysis (PCA) has been carried out to find total variation in all the samples. In the present study, five parameters have been taken into account to reveal the component responsible for variation in different accessions. PCA showed that first two components (moisture and CUPRAC) together governed 97% of total variability (Table 4.7). Therefore, moisture and CUPRAC have been considered as Principal Component (PC) PC1 and PC2 respectively in the component matrix. In PC 1

antioxidant potential (CUPRAC) and phenol have high positive loading more than 0.9 and are a major contributor to variability followed by stigmasterol. In PC 2 moisture content and antioxidant potential (DPPH) are the major contributors. However, the overall picture exhibited that antioxidant potential is major traits followed by phenol and stigmasterol are responsible for variability in accession collections from different states (Table 4.8).

Table 4.7: Eigenvalues for all the five components in 3 accessions of *C. borivilianum*

Component	**Initial Eigenvalues**		
	Total	**% of Variance**	**% of Cumulative Variance**
Moisture	2.967	59.331	59.331
CUPRAC	1.924	38.487	97.819
DPPH	0.104	2.075	99.894
Stigmasterol	0.005	0.105	99.998
Phenol	0.000	0.002	100.000

Extraction Method: Principal Component Analysis

Table 4.8: Component matrix of five components in 3 accessions of *C. borivilianum*

	Component	
	PC1	**PC2**
Moisture	0.448	0.862
CUPRAC	0.536	0.823
DPPH	0.994	-0.044
Stigmasterol	0.767	-0.636
Phenol	0.950	-0.311

Extraction Method: Principal Component Analysis. (Table denotes PC1-Principal Component 1, PC2- Principal Component 2)

4.4. Conclusion

The study revealed wide variability among thirteen accessions of *B. monnieri*, collected from different states of India, with respect to total bacoside content, total phenol content and antioxidant potential. In addition, highly significant and strong correlation was found between total bacosides, total phenols and antioxidant activity, which were the major contributors in cluster grouping. IC 554588, IC 344312 and IC 554585 have been identified elite accessions with higher bacoside conent and antioxidant potential as compared to others. Use of elicitors enhanced total bacosides and total phenol content, which are responsible for higher antioxidant potential. Statistical analysis proved strong correlation of phenols and bacosides to antioxidant potential.

A significant difference among different accessions of *C. borivilianum* was observed with regard to shoot multiplication and root induction, but reasonably high multiplication rate was obtained in all the accessions. The present study clearly reveals successful multiplication and conservation of *C. borivilianum*. JA, SA and ME-elicited roots produced a higher amount of stigmasterol, total phenol content, and antioxidant potential when compared to non-elicited root cultures. The enhanced level of phenolic compounds contributes to antioxidant potential of Safed Musli. This result suggested that the application of elicitors permits the optimized production of stigmasterol, phenolic compounds and higher antioxidant potential in root cultures of *C. borivilianum*. Hence, IC 558379 of *C. borivilianum* can be used as a lead for further research to investigate antioxidant compound from the plant resulting in the development of the new pharmaceutical product.

CHAPTER 5

PHYTOREMEDIATION POTENTIAL

5.1. Introduction

Industrial development, especially in developing countries deals with new challenges to the biosphere. Among the available resources, water is a precious commodity that requires special attention. Majority of water near industrial areas is highly polluted thereby not suitable for human consumption and agriculture (Hettick et al. 2016). The world is heading towards water crisis (Rosegrant and Cai 2001) due to the burgeoning population which is exhausting the available fresh water resources. Improvement of living conditions often comes at a cost of environment. Heavy metal contaminated water from industries is added to rivers making it unfit for human consumption. Hazardous heavy metals found in industrial wastewater are Cr, Cd, Cu, Ni, Pb, As and Zn. A density of more than 5 g per cubic centimeter is generally considered as heavy metal determining factor. Ingestion of heavy metals beyond the permitted concentration, can adversely affect the human health (Chang et al. 1996; Wang and Shi 2001; Beyersmann and Hartwig 2008). One of the toxic heavy metal usually present in the contaminated water is chromium (Cr) which usually exists in two forms - trivalent [Cr (III)] or hexavalent [Cr (VI)]. Hexavalent Cr occurs in the form of chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) which has more toxicity than other valency states. Trivalent Chromium is non-toxic but absorbed less readily than hexavalent Chromium (Sharma and Forster 1995). High exposure to Cr (VI) can lead to cancer in the digestive tract and lungs, gastric pain, nausea, vomiting, severe diarrhea, fatigue, irritability, hemorrhage, and damage to nervous system (Rosegrant and Cai 2001; Singh et al. 2011). Cadmium

(Cd) is also a heavy metal found in contaminated water. It is commonly used in industrial practices as an anticorrosive agent, a stabilizer in polyvinyl chloride products, as a color pigment and in the fabrication of Ni-Cd batteries (Godt et al. 2006). Continued consumption of food or water, containing even low levels of Cd may result in renal failure (CSEM 2008).

Conventional methods for removing heavy metal ions from industrial wastewater demands high investments and operational costs (Rich 1987) and therefore, not suitable for small scale industries. On the contrary, *in vitro* technique like adsorption through activated carbon (AC) is an effective and economical substitute to remediate wastewater from toxic heavy metals. Used contaminated biomaterial can be disposed off or processed for further use without harming the environment depending on the contaminant characteristics (Kim and Qi 1995; Wilbur et al. 2012). Environment friendly technique of utilizing *in vitro* culture plants for the uptake of heavy metals has an advantage in metabolic studies like the ease of adding inhibitors and inducers in the culture medium, which can be helpful in identifying the enzymes- involved in xenobiotic metabolism (Doran 2009). However, not much has been explored so far about the metabolic pathways associated in the transformation of xenobiotic compounds and capacity of certain plants to bear, detoxify and gather a significant amount of heavy metals (Couselo 2012), moreover dissimilarity in xenobiotic metabolism between *in vitro* culture and soil-grown plants was noticed (Doran 2009). In a greenhouse pot experiment, a biomaterial biochar decreased the bioavailability of Cd, Cr and Zn with the promotion in the growth of *Machilus pauhai* plant (Guo et al. 2017). As the *in vitro* cultures are maintained in microbial free environment, they can be exploited to distinguish the responses and metabolic capabilities of tissue-culture

plants from those encountered with microorganisms in their natural environment (Chaudhry et al. 2005; Lebeau et al. 2008). *In vitro* technique namely hairy root culture was found to be particularly noteworthy in understanding the enzymatic process involved in phytoremediation of phenols and thereby exploring a cost effective system to remediate the polluted environments (Paola et al. 2006). Waoo et al. (2013) standardized an *in vitro* technique to propagate large number of *Lantana camera* for the removal of heavy metals from the environment. The small amount of biomaterial generated by the plant species that have inherent competence to accumulate or metabolize various pollutants could be enhanced by *in vitro* culture material without sacrificing the natural habitat. In addition, soil-grown plants have a limited lifespan whereas *in vitro* plant cultures can be supplied on demand without disturbing the natural habitat. Therefore, use of plant tissue culture has increased in phytoremediation research as model plant system to discover the biochemical response of plant cells to environmental pollutants.

The plant species *Bacopa monnieri* (brahmi), family Scrophulariaceae, is a weak wetland plant known as memory enhancer in *Ayurvedic* systems of medicine. Various studies on uptake of single or mixed metal removal by *B. monnieri* through *in vitro* techniques have been successfully done by various scientists till date (Sinha et al. 1996; Sinha 1999; Shukla et al. 2007). Acknowledging above facts, the present study is focused on removal of heavy metals like Cr (VI) and Cd by *in vitro* plant cultures and soil-grown plants of *B. monnieri* from synthetic solution as well as industrial wastewater with respect to time. Multilinear regression (MLR) has been used for statistical analysis.

5.2. Phytoremediation potential of *B. monnieri*

5.2.1. Material and Method

Accession IC 554588, selected from previous *in vitro* propagation study was evaluated for phytoremediation potential for Cr (VI) and Cd. Isolated green parts of the plants were air-dried and crushed using mortar and pestle. AC was prepared by the method used by Singanan and Singana (2007). To the powdered samples, concentrated sulfuric acid was added in the ratio of 1:1.8. Samples were then heated in a hot-air oven at 160˚C for 6 hours, to release black, coarse AC. After cooling, the AC was washed with double distilled water by repeated centrifugation at 5000 rpm (Medline Scientific). Once pH 7.0-7.5 is attained, the samples were dried in the hot air oven at 105˚C for 2-3 hours until moisture was completely removed. The dried samples were sieved through 90µm sieve to obtain only fine AC particles. The prepared biomaterial i.e. AC samples were weighed, characterized and stored at room temperature. Table 5.1 shows the characteristics of AC derived from *in vitro* propagated and agro net grown plant of *B. monnieri*. Morphologically AC prepared from *in vitro* plant cultures and soil-grown plants are same. AC prepared from *in vitro* plant cultures and soil-grown plants is shown in Fig 5.1.

Table 5.1: Characteristics of AC prepared *in vitro* culture and soil-grown plants of *B. monnieri*.

Sl. No.	Parameters	Value	
		In vitro	**Soil-Grown**
1	Moisture (%)	0.18	0.15
2	Ash (%)	0.37	0.41
3	pH	7.2	7.4
4	Particle Size	≤90µm	≤90µm
5	Iodine Number	720mg/g	690mg/g

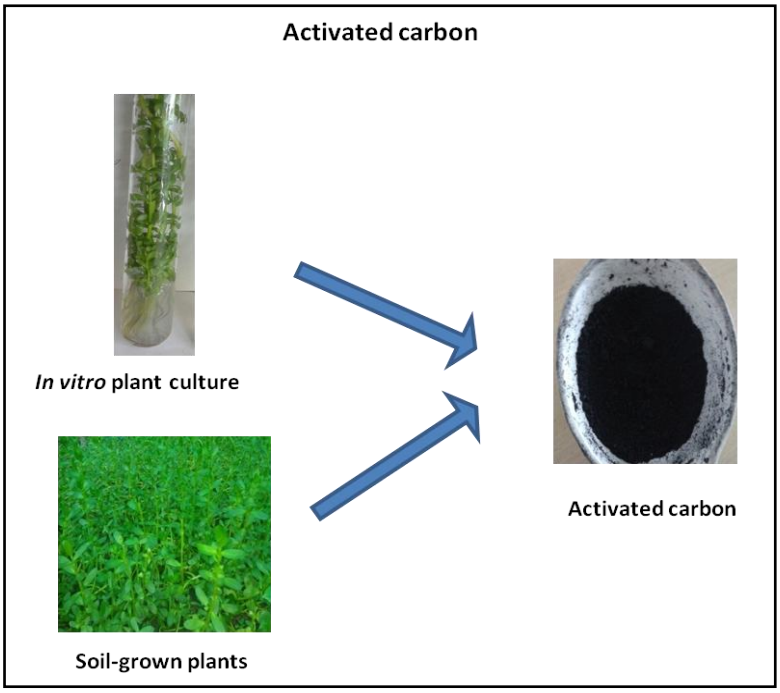


Fig. 5.1: AC prepared from *in vitro* plant cultures and soil-grown plants

Synthetic wastewater containing Cr and Cd was prepared by using analytical grade $K_2Cr_2O_7$ and $CdCl_2$ (Sigma-Aldrich), at the concentrations of 100-500-ppm and of 1000-5000-ppm respectively. Wastewater containing effluents from metal industries in Bawana Industrial Area, Delhi, India, was collected from the Western Yamuna Canal, which flows through that area. Wastewater was diluted with double distilled water to a dilution factor of 10 for the study. Samples of Cr and Cd were observed for light absorption using spectrophotometer (UV/Vis PerkinElmer LEMBDA 950) at absorption maxima 350 nm and 283 nm respectively. Characteristics of industrial wastewater used for the experiment is shown in Table 5.2. Batch mode adsorption experiment was carried out by taking 60 ml of each test solution with 1mg/ml AC in 150 ml conical flask. The conical flasks were then agitated at 100 rpm using a mechanical shaker (REMI Orbital Shaker). Periodic observation was taken from 30 min to 180 minutes until there was little or negligible decrease in concentration of Cr and Cd. Percent removal of Cr and Cd on the adsorbents was calculated as:

$$\text{Removal } \% = 100 \times \frac{(C_0 - C_f)}{C_0}$$

where C_o is the initial concentration and C_f is the final concentration in ppm.

Table 5.2: Characteristics of industrial wastewater

Sl. No.	Parameters	Value
1	pH	7.9
2	Electrical Conductivity	368 µS/cm
3	Total Dissolved Solids	328-ppm
4	Cr concentration	225-ppm
5	Cd concentration	1950-ppm

Before starting the experiment, the instrument was calibrated with optimum wavelength. Presence of any target metal in the blank solution was checked for the accuracy of results. Limit of detection and limit of quantification for Cr was 20 ppm and 60 ppm whereas for Cd it was 1000 ppm and 3000 ppm respectively. Detection of Cr and Cd was performed by the method used by Parks et al. (2004) and Gavris et al. (2013) respectively.

5.2.2. Results and Discussion

Two standard curves corresponding to the concentration for Cr and Cd in the synthetic water sample are shown in the Fig 5.2 (a) and (b) respectively. *In vitro* plant cultures adsorbed significantly higher amount of metal ion as compared to soil-grown plants. Soil grown plants showed 55% - 100% removal whereas *in vitro* plant cultures removed 86% - 100% of Cr. Cd removal from *in vitro* plant cultures and soil grown plants was observed 54% and 55% respectively. Table 5.3 and 5.4 shows the effect of *in vitro*

culture and soil-grown plants on synthetic Cr and Cd removal respectively with respect to time. Increase in the concentrations of metal ions adversely affects adsorption. Maximum amount of Cr and Cd removal was noticed up to 120 minutes. While, rate of removal was significantly higher up to 90 minutes, which was reduced afterwards. Equilibrium was attained at 180 minutes for all the concentrations studied. Similar results were obtained in an experiment conducted with AC from *Tridax procumbens* to treat tannery water containing high concentrations of Cr and obtained 95% - 97% of Cr removal after 180 minutes of treatment (Singanan et al. 2007). Another close observation was obtained from AC prepared from bamboo waste (*Oxytenanthera abyssinica*) for adsorption of hexavalent Cr (Dula et al. 2014). AC from low cost eucalyptus seeds exhibited toxic metal ion (Zinc) adsorption potential from the contaminated water (Senthil et al. 2016). Khoramnejadian and Saeb (2017) reported metal (Cr, Cd, Cu and Ni) quenching ability of *Amaranthus retroflexus* from contaminated soil after 120 days of cultivation. According to statistical analysis (MLR) of synthetic Cr, percentage removal of Cr is highly correlated with time and concentration as R-Sq(adj) is 87.2% and 93.5% for *in vitro* culture and soil-grown plants respectively. Significant differences in the removal of Cr using *in vitro* culture and soil-grown plants could be justified by the value of F being greater than $F_{critical}$ (ANOVA). Further AC of *in vitro* culture plant material removes higher amount of Cr as compared to soil-grown plants. For synthetic Cd, percentage removal is highly correlated with time and concentration as R-Sq(adj) is 92.9% and 92.2% for *in vitro* culture and soil-grown plants respectively. Since $F < F_{critical}$, it can be concluded that the removal of synthetic Cd using *in vitro* and soil-grown plants is almost equal.

Table 5.3: Effect of *in vitro* culture and soil-grown plants on the removal of synthetic Cr with respect to time

Time (min)	**Concentration of potassium dichromate**									
	100-ppm		**200-ppm**		**300-ppm**		**400-ppm**		**500-ppm**	
	In vitro	**Agro net**	***In vitro***	**Agro net**	***In vitro***	**Agro net**	***In vitro***	**Agro net**	***In vitro***	**Agro net**
0	100	100	200	200	300	300	400	400	500	500
30	70	90	80	150	130	265	200	340	250	430
60	30	65	60	95	85	230	145	285	210	370
90	10	30	20	65	50	170	100	210	130	300
120	0	0	0	40	18	163	64	200	85	250
150	0	0	0	24	0	85	40	195	70	225
180	0	0	0	16	0	59	40	195	70	225

Table 5.4: Effect of AC from *in vitro* and agro net grown plants on synthetic Cd concentration with respect to time

Time (min)	**Concentration of cadmium chloride**									
	1000-ppm		**2000-ppm**		**3000-ppm**		**4000-ppm**		**5000-ppm**	
	In vitro	**Agro net**	***In vitro***	**Agro net**	***In vitro***	**Agro net**	***In vitro***	**Agro net**	***In vitro***	**Agro net**
0	1000	1000	2000	2000	3000	3000	4000	4000	5000	5000
30	300	400	1400	1200	2300	2200	3750	2900	4500	4100
60	200	100	1100	1050	1850	1650	2950	2400	3900	3800
90	50	50	600	500	1250	1400	2500	2000	3300	3150
120	0	0	400	200	930	1750	2060	1660	2800	2650
150	0	0	280	100	620	1120	1790	1460	2400	2300
180	0	0	280	100	590	1120	1770	1460	2300	2250

Table 5.5 shows the effect of *in vitro* culture and soil-grown plants on industrial wastewater. After attaining equilibrium at 180 minutes, 64% - 93% removal of metal

ion was observed. However, *in vitro* culture rapidly removes toxic metals as compared to soil-grown plants. According to statistical analysis, $F < F_{critical}$, it can be concluded that removal of Cr and Cd from industrial wastewater using *in vitro* and soil-grown plants is almost equal.

Table 5.5: Effect of *in vitro* culture and soil-grown plants on Cr and Cd concentration in industrial wastewater

Time (min)	**Activated carbon source**			
	***In vitro* grown plants against Cr**	**Soil-grown plants against Cr**	***In vitro* grown plants against Cd**	**Soil-grown plants against Cd**
0	225	225	1950	1950
30	180	195	1300	1450
60	130	160	950	1200
90	110	120	600	800
120	89	96	325	515
150	76	81	156	360
180	74	81	136	351

Comparison of rate of removal of Cr and Cd with *in vitro* culture and soil-grown plants in industrial wastewater is shown in Fig 5.2 (c) and (d) respectively. Rate of reduction in metal ion concentration was higher with *in vitro* plant cultures as compared to soil-grown plants.

Many researchers have proved that *in vitro* cultures are reliable source for toxicity and tolerant studies (Mumma and Davidonis 1983; Azevedo et al. 2005). Singh et al. (2017) reported the potential of *in vitro* cultured *Vetiveria zizanoids* for Arsenic removal. *In vitro* cultures are also appropriate for applying inhibitors and chemical effectors as several

metabolic inhibitors and agents helped in revealing this mechanism (Doran 2009) and meets the Maximum Contaminant Level (MCL) standard 0.005 mg/L (ATSDR 1999) and 0.1 mg/L (EPA 2008) for Cd and Cr respectively. In addition, *in vitro* culture plants own the exact genomic information of their soil-grown parent plant and could be used in –omic research aimed at uptake of heavy metals like genomic, transcriptomic, proteomic and metabolic (Doran 2009). Application of this methodology need preliminary treatment, like suspended solid removal from polluted water but it can be applicable for industrial wastewater treatment.

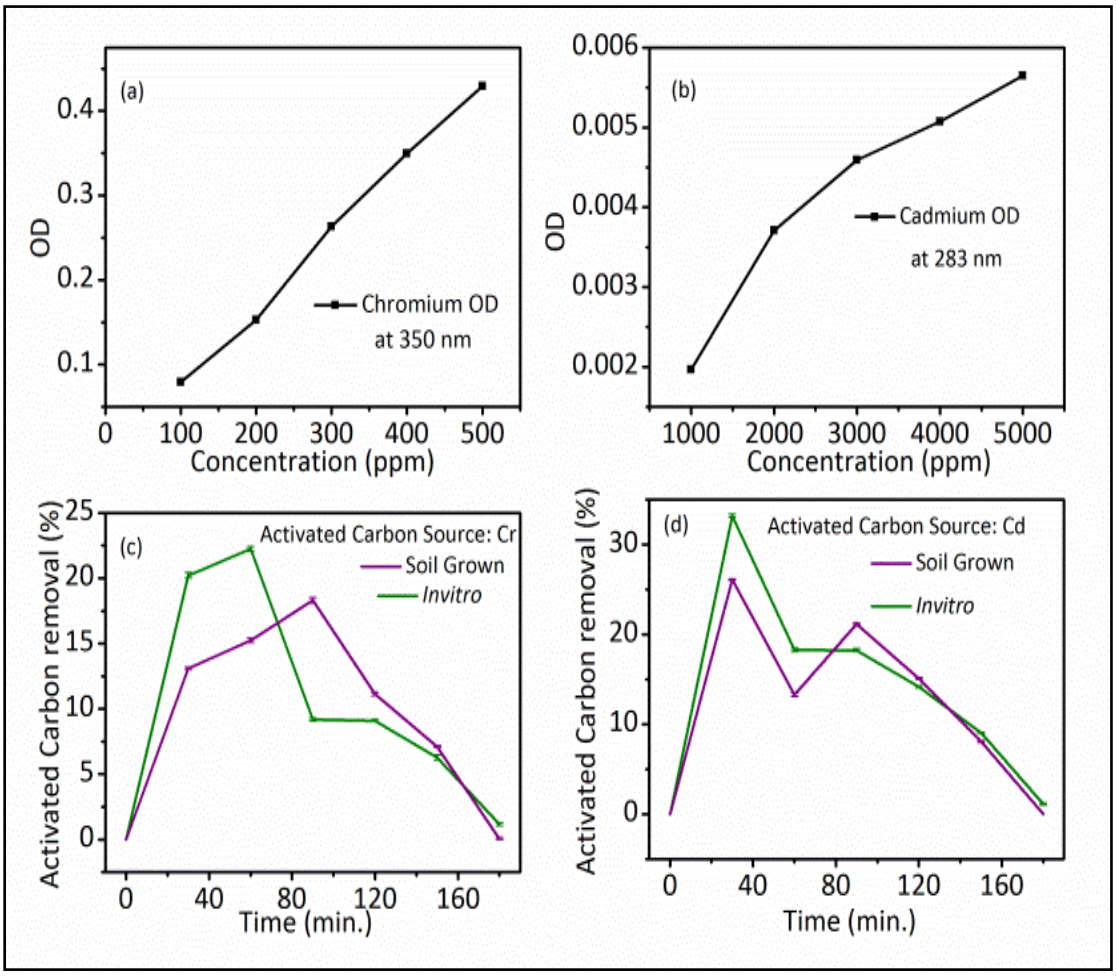


Fig. 5.2: Standard curve (a) Cr and (b) Cd. Comparison of rate of removal (c) Cr (d) Cd with i*n vitro* culture and soil-grown plants in industrial wastewater

5.3. Phytoremediation potential of *C. borivilianum*

5.3.1. Material and Method

It has been followed as given in section 5.2.1.

5.3.2. Results and Discussion

Experiments were done to check the phytoremediation potential of *C. borivilianum* to remove Cr and Cd from polluted water. The results showed there was no reduction of Cr and Cd using AC of *C. borivilianum* indicating no phytoremediation under the experimental set up used. Further studies may be required to confirm the phytoremediation using AC by standardizing various parameters like amount of AC, pH of AC and concentration of polluted water. More in depth studies are required to check the phtoremediation potential of *C. borivilianum*.

5.4. Conclusion

Present study concludes that *in vitro* culture of *B. monnieri* is more competent over soil-grown plants for reclamation of polluted water containing Cr and Cd and for large-scale remediation process for the betterment of humankind, agriculture and environment. It has been proven statistically also that both reclaimed Cr and Cd from synthetic solutions as well as from industrial wastewater. However, rate of reduction was significantly higher with *in vitro* culture as compared to soil-grown plants. *In vitro* cultures offer microbial free environment with no translocation barrier to determine intrinsic capacity of plant cells for remediation of contaminants. Technical applicability like cost-effectiveness, easily manageable biomaterial, rapid and eco-friendly set up are the key factors that play an important role in the selection of *in vitro* cultures for

detoxification of pollutants from the wastewater. IC 554588 has successfully removed the toxic heavy metals Cr and Cd from polluted water.

This study has been published in "Bulletin of Environmental Contaimination and Toxicology", (Springer) entitled **"Uptake of Heavy metals from industrial wastewater using *in vitro* plant cultures".**

CHAPTER 6

GENETIC DIVERSITY ANALYSIS

6.1. Introduction

Medicinal plants grown in different agro-climatic zones vary in the amount of secondary metabolite, as it is influenced by environmental conditions. Genetic diversity among accessions reveals genetic association among various accessions. Information on genetic diversity and relationship among accessions is necessary for germplasm collection, conservation and breeding programs. Variable accessions can be utilized by plant breeders to develop new varieties with agronomically favourable characters. In case of medicinal plants elite accessions with enhanced therapeutic value can be developed by using such breeding material. Molecular markers can be utilized for the authentication of medicinal plants and examining accessions using molecular markers improves the understanding of breeding material. Development of genomics has resulted in other novel marker system such as start codon targeted polymorphism (SCoT; Collard and Mackill 2009) and CAAT box-derived polymorphism (CBDP; Singh et al. 2014) that can be utilized to screen breeding material as selection on the basis of markers is environment independent.These markers have distinctively functional domains corresponding to conserved DNA sequences within genes. Amirmoradi et al. (2012); Gorji et al. (2011); Luo et al. (2011); Xiong et al. (2011) have studied genetic diversity in crops like potato, mango, cicer and peanut using gene targeted markers. Gene targeted markers are superior over arbitrary primer-based markers as they are obtained from genomic region of the plants so primers can bind to any of the two strands and are more reproducible (Singh et al. 2014).

To the best of our knowledge, no study has been done so far to assess genetic diversity of *B monnieri* using SCoT and CBDP markers. The present study is the first attempt to assess genetic diversity among 19 accessions of *B. monnieri* procured from NBPGR by molecular markers SCoT and CBDP.

6.2. Genetic diversity analysis of *B. monnieri*

6.2.1. Material and Method

i. DNA extraction

Two gene-based molecular markers SCoT and CBDP were used for genetic diversity assessment of 19 accessions of *B. monnieri* (procured from NBPGR, Delhi, India) belonging to different agro-climatic zones shown in Table 6.1 (Sehgal et al. 1990).

Table 6.1: All the studied *B. monnieri* accessions with their germplasm identity number, the corresponding state of the collection and agro-climatic zone (Refer Fig. 6.1for agro-climatic zones of India)

Sl. No.	Germplasm Identity No./ Accession No.	State of collection	Agro-climatic zone
1	IC 249250	Orissa	11
2	IC 256496	Orissa	11
3	IC 284992	Orissa	11
4	IC 353203	New Delhi	5
5	IC 531621	Jharkhand	7
6	IC 439118	Jharkhand	7
7	IC 392842	Cuttack	7
8	IC 554585	Amarkantak	8
9	IC 468878	Kerala	12
10	IC 373640	Kerala	12
11	IC 375976	Jammu and Kashmir	1
12	IC 426447	Madhya Pradesh	8
13	IC 554588	Madhya Pradesh	8
14	IC 554587	Madhya Pradesh	8
15	IC 554586	Madhya Pradesh	8
16	IC 426442	Punjab	6
17	IC 353204	Uttar Pradesh	5
18	IC 342108	Karnataka	10
19	IC 344312	Karnataka	10

Lyophilized leaf sample was ground to fine powder with liquid nitrogen. 50 mg of each powdered sample was used for DNA extraction using micro-extraction method as described by Prabhu et al. (1998). The DNA was diluted to a final concentration of 10ng/µl for SCoT and CBDP marker analysis.

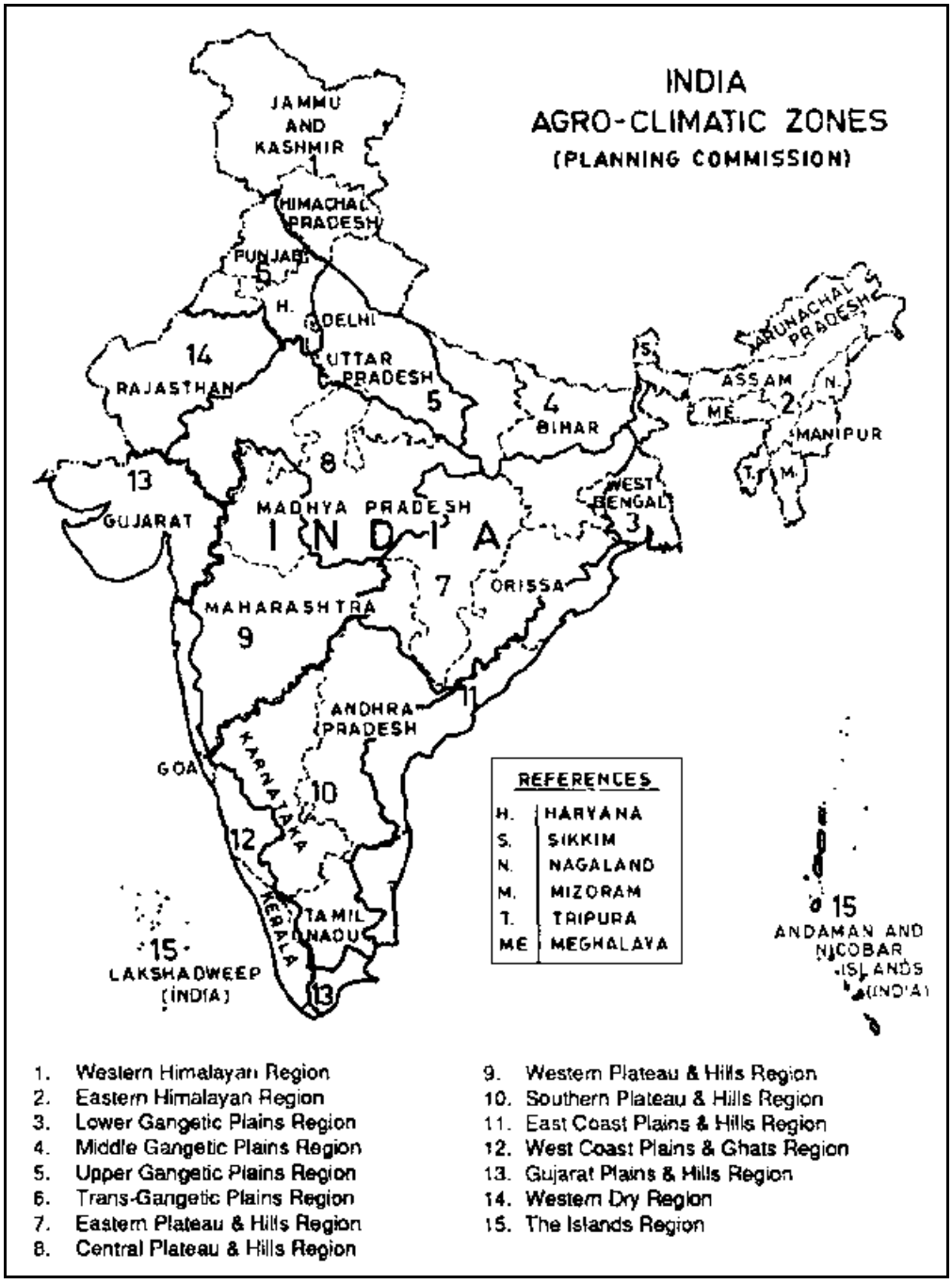
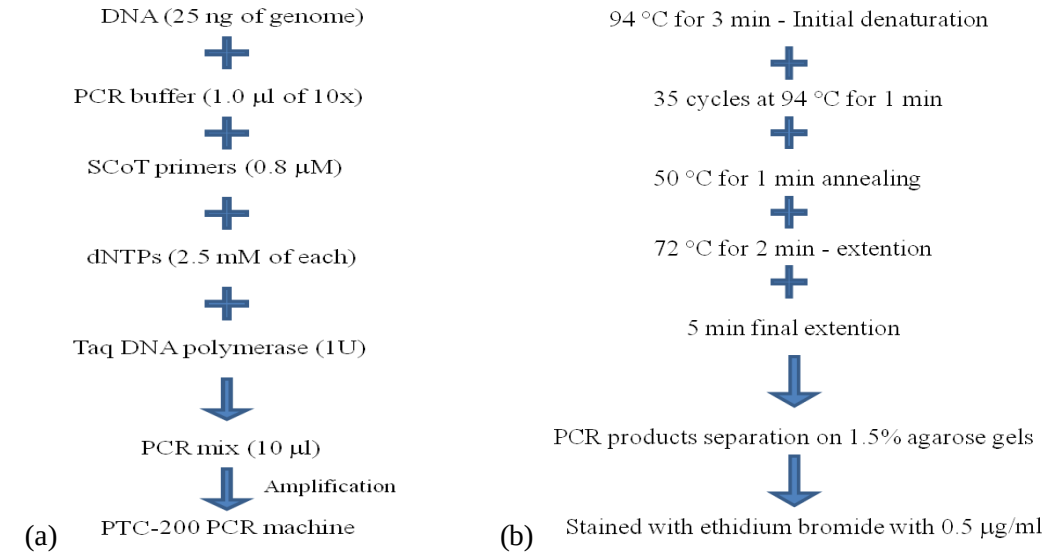


Fig. 6.1: Agro-climatic zones of India as identified by planning commission (Sehgal et al. 1990)

ii. PCR Amplification with SCoT and CBDP markers

Here is the flow charts showing amplification of SCoT and CBDP markers. PCR reaction was set as per the method of Tiwari et al. (2016) with some modifications.

SCoT markers



CBDP markers

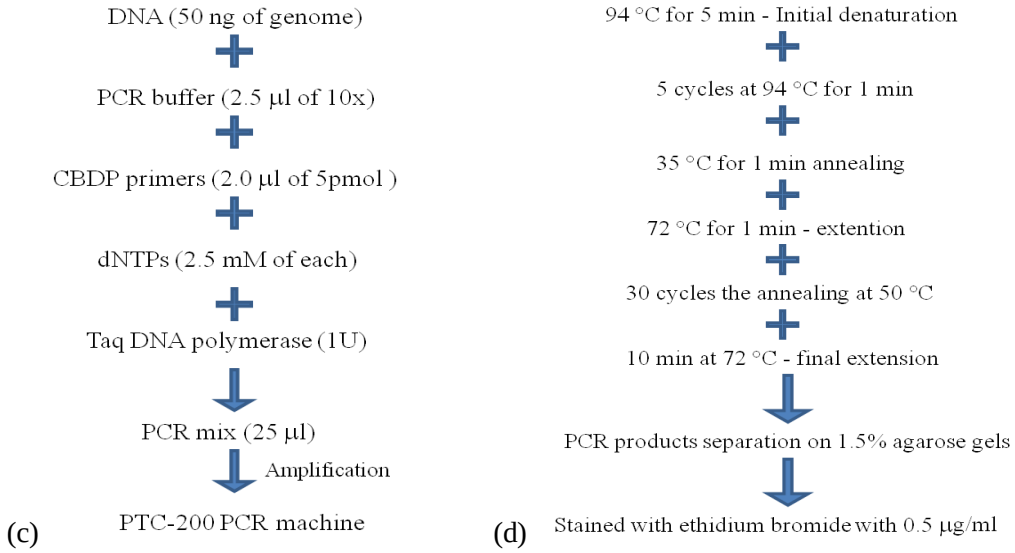


Fig. 6.2: Flow chart of SCoT and CBDP markers amplification (a) PCR mixture for SCoT, (b) PCR amplification steps for SCoT, (c) PCR mixture for CBDP, (d) PCR amplification steps for CBDP

All the primers and chemicals have been purchased from Sigma Chemicals, USA, Taq DNA polymerase from Thermoscientific, USA, PCR (PTC-200) from MJ Research, USA and 1Kb DNA ladder from Fermentas, USA. Experiment was performed with 10 SCoT and 10 CBDP markers.

iii. Statistical analysis

Scoring of band profile was done as 1 for presence and 0 for absence of bands. JSC was measured by using pair-wise comparision of genotype. Software used for genetic analysis are NTSYSpc (Numerical taxonomy and multivariate analysis system) 2.02e software, based on unweighed Pair Group Method with Arithmetic Means (UPGMA). SIMQUAL (Similarity for qualitative data program in NTSYS) module of JSC was used to estimate genetic association.

6.2.2. Result and Discussion

Analysis of genetic diversity showed remarkable variation among the brahmi accessions. All the studied parameters for SCoT and CBDP markers are given in Table 6.2 and Table 6.3 respectively. Result of PCR amplification of brahmi with SCoT (primer 10) and CBDP (primer 9) is shown in Fig 6.2. One hundred and nineteen loci were scored without any monomorphic bands. Analysis of genetic variation is a crucial factor in germplasm characterization and gene-targeted markers are the most reliable alternatives for such studies as they are not influenced by the environment. Allele number ranged from 7-14 for SCoT and 9-13 for CBDP marker system with major allele frequency ranging from 0.15-0.36 and 0.15-0.31 for SCoT and CBDP markers respectively. Gene diversity was from 0.80 (SCoT 10) to 0.91 (SCoT 3, SCOT 9) and 0.83 (CAAT 10) to 0.89 (CAAT 7, CAAT 8). Average gene diversity of 0.64 has been reported in Chickpea by Hajibarat et al. (2015) using gene-targeted markers.

Table 6.2: SCoT primer sequence with major allele frequency, allele number, gene diversity and PIC value.

No. of Primers	SCoT Primers	Sequence of Primers	Major Allele Frequency	Allele No	Gene Diversity	PIC
1	SCoT 1	CAACAATGGCTACCACCA	0.1579	12.0000	0.8920	0.8823
2	SCoT 2	CAACAATGGCTACCACCC	0.2105	7.0000	0.8255	0.8017
3	SCoT 3	CAACAATGGCTACCACCG	0.1579	14.0000	0.9141	0.9078
4	SCoT 4	CAACAATGGCTACCACCT	0.1579	12.0000	0.8975	0.8887
5	SCoT 5	CAACAATGGCTACCACGT	0.2105	12.0000	0.8864	0.8764
6	SCoT 6	CAACAATGGCTACCAGCA	0.2632	14.0000	0.8864	0.8785
7	SCoT 7	CAACAATGGCTACCAGCC	0.2105	9.0000	0.8643	0.8494
8	SCoT 8	ACGACATGGCGACCAACG	0.2632	11.0000	0.8643	0.8516
9	SCoT 9	CCATGGCTACCACCGGCC	0.1579	14.0000	0.9141	0.9078
10	SCoT 10	CCATGGCTACCACCGGCG	0.3684	10.0000	0.8089	0.7916
	Mean	NA	0.2158	11.5000	0.8753	0.8636

Table 6.3: CBDP primer sequence with major allele frequency, allele number, gene diversity and PIC value.

No. of Primers	CBDP Primers	Sequence of Primers	Major Allele Frequency	Allele No	GeneDiversity	PIC
1	CAAT 1	TGAGCACGATCCAATAGC	0.2632	11.0000	0.8643	0.8516
2	CAAT 2	TGAGCACGATCCAATAAT	0.1579	11.0000	0.8920	0.8821
3	CAAT 3	TGAGCACGATCCAATGAT	0.2632	12.0000	0.8753	0.8650
4	CAAT 4	TGAGCACGATCCAATGTT	0.2105	12.0000	0.8864	0.8764
5	CAAT 5	TGAGCACGATCCAATATA	0.2105	11.0000	0.8809	0.8697
6	CAAT 6	TGAGCACGATCCAATTGA	0.1579	11.0000	0.8864	0.8757
7	CAAT 7	CTGAGCACGATCCAATAG	0.2105	13.0000	0.8975	0.8894
8	CAAT 8	CTGAGCACGATCCAATAC	0.2105	13.0000	0.8975	0.8894
9	CAAT 9	CTGAGCACGATCCAATCA	0.2105	13.0000	0.8920	0.8831
10	CAAT 10	CTGAGCACGATCCAATGT	0.3158	9.0000	0.8310	0.8134
	Mean	NA	0.2211	11.6000	0.8803	0.8696

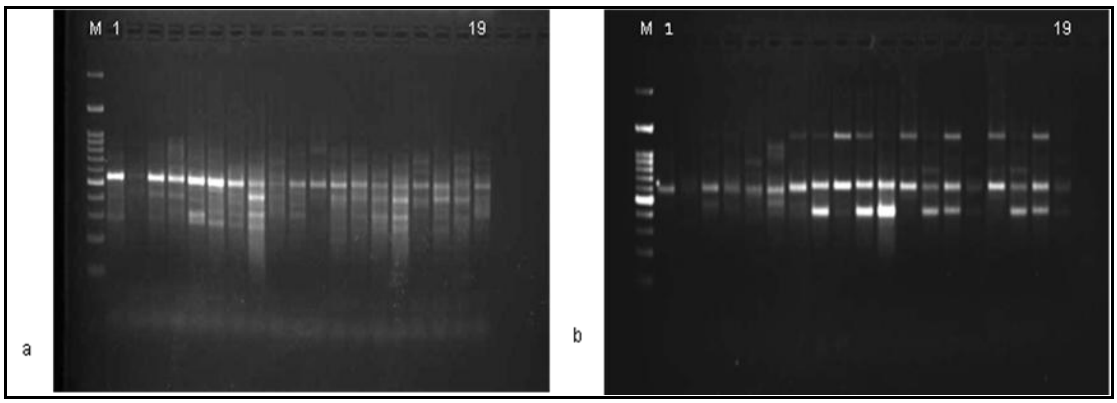


Fig. 6.2: PCR amplified product of 19 accessions of *B. monnieri* (a) SCoT (primer 10) and (b) CBDP (primer 9) with 1 Kb ladder (M)

In the combined study of SCoT and CBDP markers, *B. monnieri* accessions could be distinguished from each other at the level of 1 to 119 polymorphic alleles among individuals. 0.22 to 0.67 was the calculated similarity index for all the primers. Highly similar accessions were IC 554586 and IC 554587 with similarity index of 0.67. However, accessions IC 468878 and IC 256496 were far apart with the similarity index value of 0.22 (Table 6.4). The study revealed that highly similar accessions are present in same geographical region while the least similar accessions are found in different geographical regions.

NTSYS-pc statistical package was used for cluster analysis. Dendrogram generated by UPGMA cluster analysis based on Jaccard's similarity estimates for both the markers generated two clusters (Fig 6.3). Cluster I contained the accession IC 375976 while cluster II showed eighteen accessions. Cluster II was divided into two sub-clusters; sub-cluster I, consisted three and sub-cluster II contained remaining fifteen accessions. Accessions 12, 13, 14 and 15 belonging to same agro-climatic zone (8) were grouped into same cluster likewise accessions 9 and 10 come under same agro-climatic region (12) and grouped into same cluster (Table 6.1). This is the first study to assess genetic diversity with SCOT and CBDP markers for brahmi.

Table 6.4: Similarity matrix for Jaccard's coefficient for 19 *B. monnieri* accessions based on 119 loci obtained with SCoT, CBDP marker.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	1																		
2	0.4815	1																	
3	0.4769	0.4035	1																
4	0.4769	0.4286	0.5738	1															
5	0.4096	0.2949	0.481	0.5395	1														
6	0.3556	0.2471	0.4023	0.4023	0.6067	1													
7	0.3467	0.25	0.3289	0.3467	0.4524	0.4598	1												
8	0.4146	0.2987	0.381	0.3976	0.4731	0.4343	0.4235	1											
9	0.3099	0.2222	0.3881	0.3286	0.3735	0.3371	0.4203	0.4304	1										
10	0.3438	0.2963	0.3438	0.3871	0.4079	0.3023	0.4444	0.4324	0.4561	1									
11	0.303	0.2727	0.3651	0.3651	0.3544	0.2727	0.2817	0.2771	0.2969	0.3333	1								
12	0.4267	0.3188	0.3896	0.4459	0.4884	0.3571	0.4737	0.4941	0.3684	0.4265	0.3472	1							
13	0.4026	0.3333	0.4026	0.3846	0.5	0.4105	0.5915	0.4713	0.3816	0.4	0.3067	0.6081	1						
14	0.4444	0.3467	0.3765	0.3765	0.4227	0.3491	0.4186	0.6506	0.3571	0.3375	0.2738	0.5422	0.5926	1					
15	0.4024	0.32	0.3529	0.369	0.4783	0.41	0.4118	0.6463	0.3827	0.3636	0.3291	0.4824	0.4767	0.679	1				
16	0.338	0.339	0.3571	0.3571	0.381	0.2737	0.3158	0.369	0.3143	0.2319	0.3934	0.4324	0.4459	0.3976	0.3735	1			
17	0.359	0.2676	0.3766	0.359	0.4767	0.4194	0.4416	0.5181	0.3733	0.3521	0.28	0.481	0.5128	0.5679	0.4881	0.5441	1		
18	0.3857	0.35	0.3288	0.3662	0.439	0.3085	0.4366	0.4625	0.3623	0.4032	0.2985	0.5	0.5797	0.5325	0.4321	0.5738	0.4861	1	
19	0.3175	0.3137	0.2576	0.3387	0.3165	0.3133	0.3538	0.3377	0.3115	0.3774	0.3519	0.3429	0.338	0.3867	0.3421	0.3898	0.3676	0.5849	1

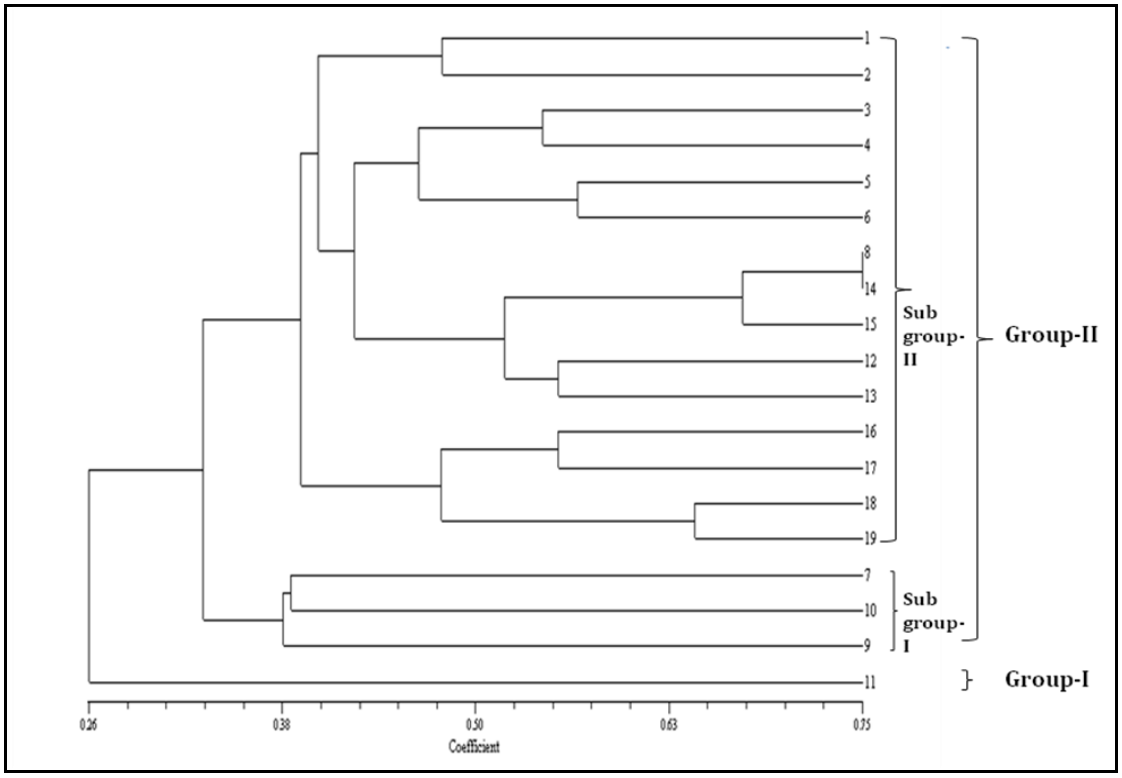


Fig. 6.3: Dendrogram generated for 19 *B. monnieri* accessions using UPGMA cluster analysis based on Jaccard's similarity estimates for SCoT, CBDP markers data

PIC of SCoT and CBDP markers is one of the parameter to check degree of genetic diversity. SCoT and CBDP markers are more capable of exhibiting information concerning DNA polymorphism. Discriminatory power of the primers can be evaluated by PIC. PIC varied from 0.79 (SCoT 10) to 0.90 (SCoT 3 and SCoT 9) for SCoT with an average of 0.86. For CBDP, it varied from 0.81 (CAAT 10) to 0.88 (CAAT 7 and CAAT 9) with an average of 0.86. Primers of high PIC value like SCoT 3, SCoT 9 and SCoT 10 along with CAAT 7, CAAT 9 and CAAT 10 contain more potential to explore polymorphism compared to other primers. Similarly, Tiwari and coworkers (2016) studied genetic diversity of *Andrographis peniculata* and noticed PIC ranged from 0.32 - 0.45 and 0.25 - 0.42 for RAPD and ISSR markers respectively whereas for SCoT it was 0.09 - 0.48 and for CBDP primer it was 0.30 - 0.46. The above study showed lesser PIC value for RAPD and ISSR primers. Data discussed here showed lesser PIC value of RAPD and ISSR primers than SCoT and CBDP markers. Molecular markers are superior to phenotypic markers as the data generated by them can be analyzed objectively that aids in establishing relationship among accessions. 2-dimensional and 3-dimensional plots are given in Fig 6.4 and Fig. 6.5 respectively. Both the plots that exhibit genetic variability among all the accessions validate the cluster groupng and similarity index.

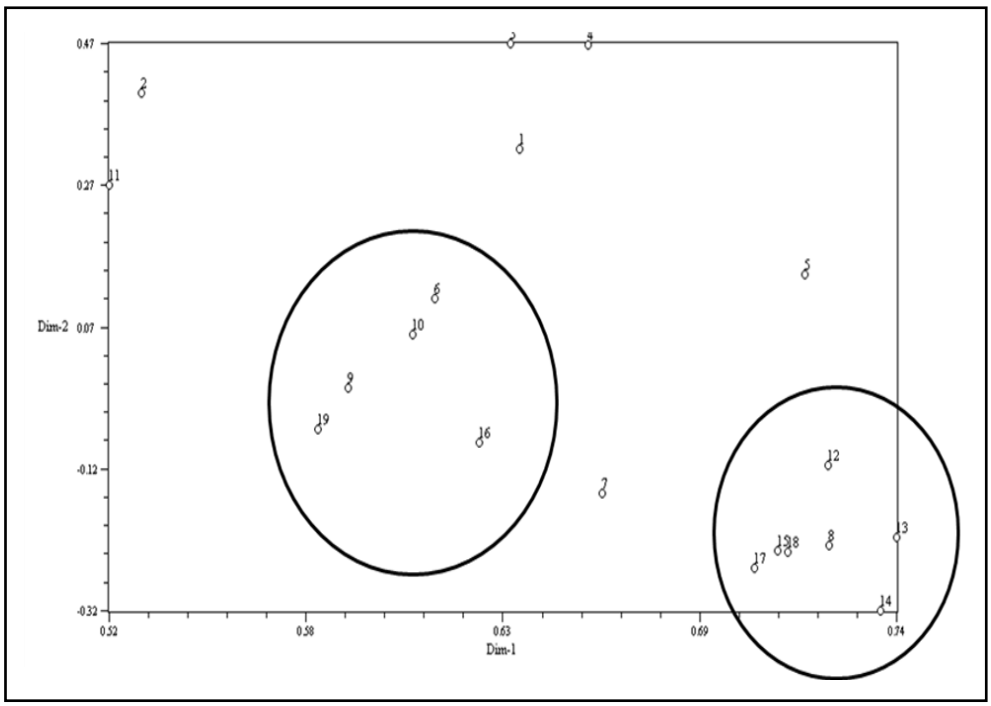


Fig. 6.4: 2-Dimensional plot generated for 19 *B. monnieri* accessions using UPGMA cluster analysis based on Jaccard's similarity estimates for SCoT, CBDP markers data

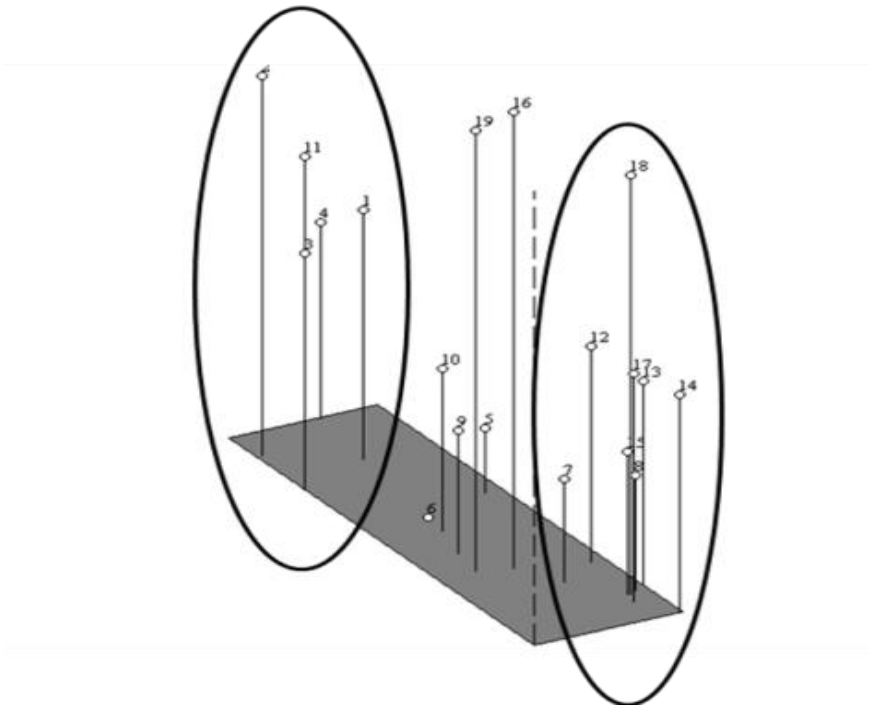


Fig. 6.5: 3-Dimensional plot generated for 19 *B. monnieri* accessions using UPGMA cluster analysis based on Jaccard's similarity estimates for SCoT, CBDP markers data

The availability of large numbers of fragments defining independent genetic loci with highly reproducible polymorphism detection enables efficient evaluation of genetic diversity. Accessions IC 249250, IC 256496, IC 284992 showed minimum similarity with IC 375976 (Jammu and Kashmir) in cluster analysis. Similarity matrix exhibited that IC 256496 (Orissa) and IC 373640 (Kerala) were not genetically related (Table 6.4). It is anticipated that above-mentioned accessions can be conserved for further studies.

6.3. Genetic diversity analysis of *C. borivilianum*

We have studied existing three accessions of *C. borivilianum* with two molecular markers SCoT and CBDP using 10 primers each.

6.3.1. Material and Method

i. Plant material

Three accessions have been used for the study as given in table 6.5.

Table 6.5: List of *C. borivilianum* accessions used for the study

Sl. No.	Accessions No.	State of Collection
1	IC 266708	Rajasthan
2	IC 558379	Uttar Pradesh
3	IC 539860	Madhya Pradesh

ii. DNA extraction

Lyophilized root sample was ground to fine powder with liquid nitrogen. 50 mg of each powdered sample was used for DNA extraction from the hardened roots has been done using micro-extraction method as described by Prabhu et al. (1998). The DNA was diluted to the final concentration of 10ng/µl for SCoT and CBDP marker analysis.

iii. PCR amplification with SCoT and CBDP markers

PCR amplification has been done as given in section 6.2.1ii.

6.3.2. Result and Discussion

For genetic diversity analysis, large number of accessions is required. Preliminary study were undertaken to test SCoT and CBDP markers for differences if any between three accessions. All the three accessions were grouped into two different groups. Accession IC 266708 and IC 558379 are more similar compared to IC 539860. Since preliminary study distinguishes between three accessions. So, these markers can be used for diversity analysis after collecting more accessions.

6.4. Conclusion

On the basis of marker study the accessions collected from different state showed wide variation. These markers can be used for assessing genetic diversity among different accessions. Genetically diverse accessions are the important source of useful genes, which can be used in breeding programs to develop improved varieties.

CHAPTER 7

ELUCIDATION OF PHARMACOLOGICAL MODE OF ACTIONS OF IMPORTANT SECONDARY METABOLITES OF MEDICINAL PLANTS

7.1. Introduction

Computational approach is a breakthrough in plant-based drug designing process that utilizes pharmacologically active metabolites as lead molecule for specific targets. Computational approach for the development of herbal drugs is fetching attention of research community to discover safe drugs for some dreadful or incurable diseases. Traditional method of producing a drug from scratch to market takes a long time of approximately 14 years with high expenditure. With a myriad of developments in 'omic' technology (transcriptomic and metabolomic), bioinformatics play a crucial role in drug discovery with the use of large databases and software. Use of bioinformatics in the process of drug designing can accelerate many steps and reduce the overall time and cost.

Understanding the role of protein or enzymes involved in metabolic processes is essential for searching the putative target of particular disease. With the help of several bioinformatics tools, complex of target and ligand (lead molecule) can be analyzed for its stability in bodily conditions. Stability of complex in the system is a crucial step for drug development process to check the drug from adsorption till excretion. Bioinformatics is a rapid and cost effective approach for the elucidation of pharmacological mode of action in the development of effective herbal drugs.

7.2. *In silico* insight into potential Anti-Alzheimer's disease activity of *Bacopa monnieri*

B. monnieri is renowned for cognitive-enhancing and neuroprotective effects. The pharmacological importance of this medicinal herb is due to the presence of saponins, potent nervine activator, such as bacoside A, B and C, jujubognin and pseudojujubognin (Rastogi et al., 1994). *B. monnieri* is studied in age-related neurodegenerative diseases (Singh et al., 2008; Stough et al., 2008) such as Alzheimer's disease (AD), Parkinson's disease and schizophrenia. AD, frequent in elderly people, is characterized by terrible memory loss, unusual personality and behavior changes along with reduction on cognitive functions. Acetylcholine plays important role in memory and cognitive function (Matthews 1996). Increase in concentration of acetylcholine in the brain is the most successful strategy for the treatment of AD (Heinrich and Teoh, 2004). Depletion in acetylcholine amount is associated with reduced cognitive activities (Francis et al 1999). Most of the FDA approved drugs for AD are AChE inhibitors. AChE is responsible for hydrolysis of acetylcholine and produces choline and acetyl group. Thus, decreases acetylcholine levels in synapses. Presently available drugs for AD donepzil (Kelly 1997) and rivastigmine (Gottwald 1999) reportedly produce harmful effects like nausea, diarrhea and liver toxicity (Schulz 2003; Melzer 1998). This demands for better AChE inhibitor from herbal source to reduce these adverse effects (Mukherjee et al. 2007). Bacosides A and B play important role in neurotransmission. Bacosides rejuvenate synapses and improve damaged neurons, which helps to improve cognitive functions (Rastogi and Kulshreshtha 1998). Researchers revealed that *B. monnieri* supports augmentation of acetylecholine (Bhattacharya et al., 2000) in the brain. Inhibition of AChE and activation of cholin

acetyltransferas through *B. monnieri* was studied by Aguiar et al (2013). Ramasamy et al. (2015) performed *in vitro* experiments along with *in silico* studies on *B. monnieri* to examine the AChE inhibition activity of bacosides and their derivatives. All the tested phytocompounds inhibited AChE effectively. Kumar et al. (2016) reported remarkable improvement in cognitive functions of medical scholars, with administration of *B. monnieri* at a dose of 150 mg for six weeks.

Literature survey revealed that interaction of bacosides and AChE is not well studied. So, exploring the inhibition of AChE through bacoside computationally to check the stability of the complex is justified. Based on the above facts, inhibition of acetylcholinesterase *in vitro* with methanolic extract of selected accession (IC 554588) and *in silico* for the development of a probable candidate for the management of neurodegenerative disease AD has been studied. Molecular dynamics simulations have been performed to better understand the interaction and stability of bacoside and Acetyl cholinesterase.

7.2.1. Material and Method

i. Sample preparation

Sample preparation is same as given in section 4.2.1.ii of Chapter 4.

ii. *In vitro* acetylecholinesterase assay

Crude methanolic plant extract was examined for AChE activity. The assay for measuring Acetylcholinesterase inhibitory activity was improvised from the method described by Ellman et al (1961) and Ingkaninan et al (2000). Acetylcholine acts as a substrate, which hydrolyses by the enzyme AChE. The reaction ends with the production of yellow color of yellow 5-thio-2-nitrobenzoate anion. Briefly, five

dilutions of 200, 400, 600, 800 and 1000 µg/ml concentration were prepared from the sample. 125 µl of 3 mM dithiobis nitrobenzoic acid (DTNB), 25 µl of 15 mM acetylthiocholine iodide (ATCI), 50 µl buffer (Tris-HCl, pH-8) and 25 µl of each diluted sample was taken and added to the wells of microplate, followed by 25 µl of 0.28 U/ml AChE enzyme. Absorbance was measured at 405 nm against blank for every 30 sec for 5 times by a CERES UV 900C micoplate reader (Bio-Tek Instrument, USA). Aqueous methanol is taken as blank and controlled sample was prepared without plant extract. Inhibition percentage was measured by comparing rates of sample with controlled sample and blank. Experiment was done in duplicates. Mean absorbance per minute (*A*) was calculated for every dilution of different samples. Each plate had one blank well and one control well. Percentage of AChE inhibition was given by concentration of sample and was calculated by using the formula:

$$\text{Percent Inhibition} = \frac{A_{control} - A_{extract}}{A_{control}} \times 100$$

iii. *In silico* analysis and molecular dynamics simulations

In order to understand inhibition capabilities of AChE through major metabolite of Brahmi i.e. bacoside, we performed molecular docking and molecular dynamics simulations. Protein structure of AChE was obtained from RCSB (www.rwsxdcsb.org) (PDB ID 1ODC). Two-Dimensional (2-D) structure of bacoside was obtained from PubChem, an open chemistry database (PubChem CID: 53398644) in SDF format (https://pubchem.ncbi.nlm.nih.gov/compound/53398644). The 2-D structure was converted to three-Dimensional (3-D) structure by using Online SMILES Translator (https://cactus.nci.nih.gov/translate/) and energy was minimized.

The 3-D structure of AChE was docked with bacoside A molecule. Molecular docking simulation was performed using the PatchDock algorithm. PatchDock docking algorithm is based on shape complementarily or geometry (Schneidman-Duhovny et al. 2005). A wide interface is ensured to include local features of the docked molecules that have complementary characteristics. The input parameters were the PDB coordinate files for the receptor (AChE), and ligand (Bacoside) molecules with default parameters. A scoring function that considers both geometric fit and atomic desolvation energy were used to evaluate each candidate transformation. Root Mean Square Deviation (RMSD) clustering was applied to the candidate solutions to discard the redundant solutions. Five best solutions were selected for further refinement and rescoring analysis by FireDock (Fast Interaction Refinement in molecular DOCKing) algorithm. FireDock (http://bioinfo3d.cs.tau.ac.il/FireDock/) algorithm is based on the optimization of side-chain conformations and rigid-body orientation followed by a high-throughput refinement (Andrusier et al. 2007). The best structure of docking complex (AChE-Bacoside) was reexamined. The physical inspection of the complex using SPDBV and UCSF CHIMERA is also supporting the complex with the highest score. The complex was subjected to molecular dynamics studies.

Dynamics of proteins can be understood by using Molecular dynamics (MD) simulations on nanosecond to microsecond timescales (Gajula et al. 2003; Gajula et al2016). Gromacs simulations suite v5.1 was used for MD simulations calculations (Spoel et al. 2005). The protein complex was placed in an orthorhombic simulation box and the box size was adjusted to maintain a minimum distance of 10 Å to the cell boundary. The box was filled with water model of Simple Point Charge (SPC). GROMOS96 force field (43a1) was

used for both the minimization procedure and the MD simulations steps (Gunsteren et al. 1996). LINCS algorithm was used to calculate Protein bond lengths (Hess et al. 1997). For both long-range coulombic interactions as well as Lennard-Jones interactions, a cut-off radius of 1.4 nm was applied. The system was energy minimized and equilibrated for 1ns each with both NPT and NVT coupling respectively. Temperature was gradually increased to 300K. Production run was carried for 30 ns with a 2 fs time step, and full periodic boundary conditions were applied throughout.

iv. Statistical analysis

Results were expressed in terms of means and standard error, while means were compared with Duncan's multiple range test (DMRT, 9) at $P<0.05$ level. PatchDock algorithm was used for molecular docking simulations. Gromacs simulations suite v5.1 was used for MD simulations calculations.

7.2.2. Result and Discussion

i. *In vitro* acetylcholinesterase assay

The extract of the selected accession IC 554588 was tested for *in vitro* AChE inhibition. According to reports, AChE is responsible for excessive degradation of acetylcholine in the synaptic cleft (Davies and Maloney, 1976; Sims et al., 1983). AChE Inhibition activity of extract ranged from 9.2% to 62.6% for concentration from 200 to 1000 µg/ml (Fig. 7.1). Inhibitory activity of methenolic extract of *B. monnieri* increased with increasing concentration of extract. Maximum activity of plant extract was noticed at 1000 µg/ml, which is 62.6%. The inhibitory activity of extract of *B. monnieri* was in conformity of earlier study (Das et al., 2002).

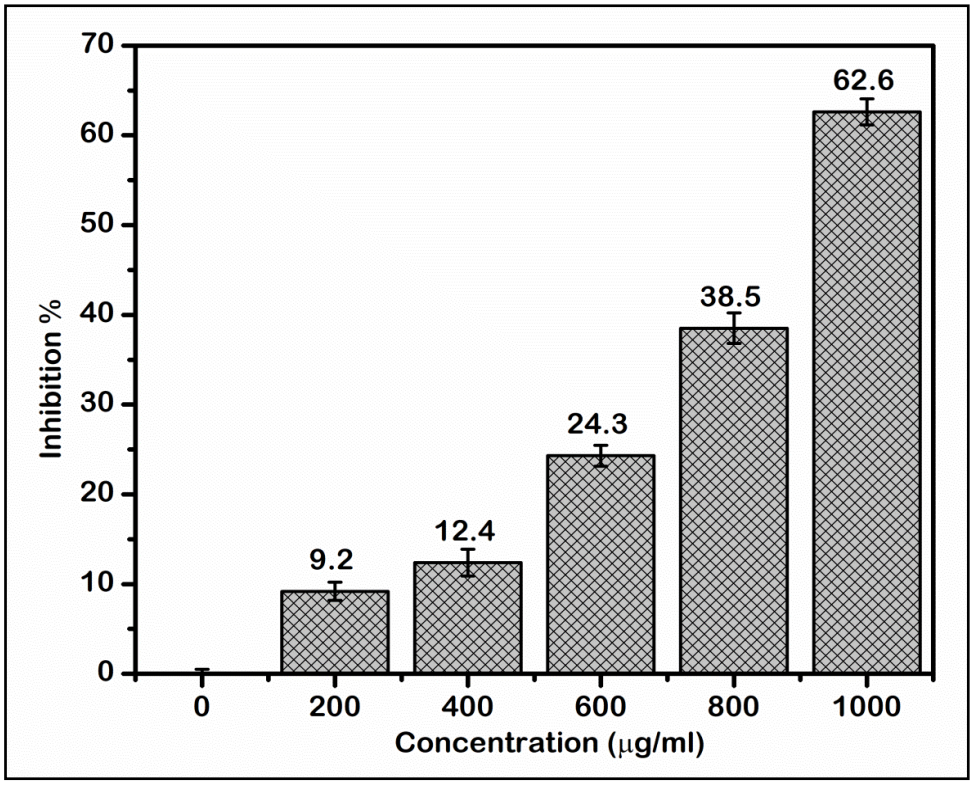


Fig. 7.1: Percentage inhibition of Acetylcholoinesterase (AChE) through methanolic extract of *in vitro* grown *B. monnieri* (IC 554588)

ii. *In silico* analysis and molecular dynamics simulation

Molecular dynamics simulation has been carried out on AChE in complex with bacoside A to evaluate the interactions in motion. Simulation provides the information of protein flexibility and movement, which is not the feature of molecular docking. Fig 7.2 depicts the interactions as a Ligplot of the docked complex of bacoside A with AChE. Fig 7.3 shows the AChE (wired ribbon) with bound Bacoside in thick grey color rendered in CPK model. H-bond interactions are present in bacoside A/AChE complex. Fig 7.4 shows H-bonding pattern of Bacoside with AChE during 30 ns simulation period. It is evident from the graph that the ligand is constantly forming hydrogen bonds with the protein complex. Computed Root Mean Square Deviation (RSMD) plot

proposes the stability of the docked complex (Fig 7.5). In the present study, the results generated by molecular docking and molecular dynamics simulations programs revealed that the AChE complex with bound Bacoside is very stable throughout the simulations period.

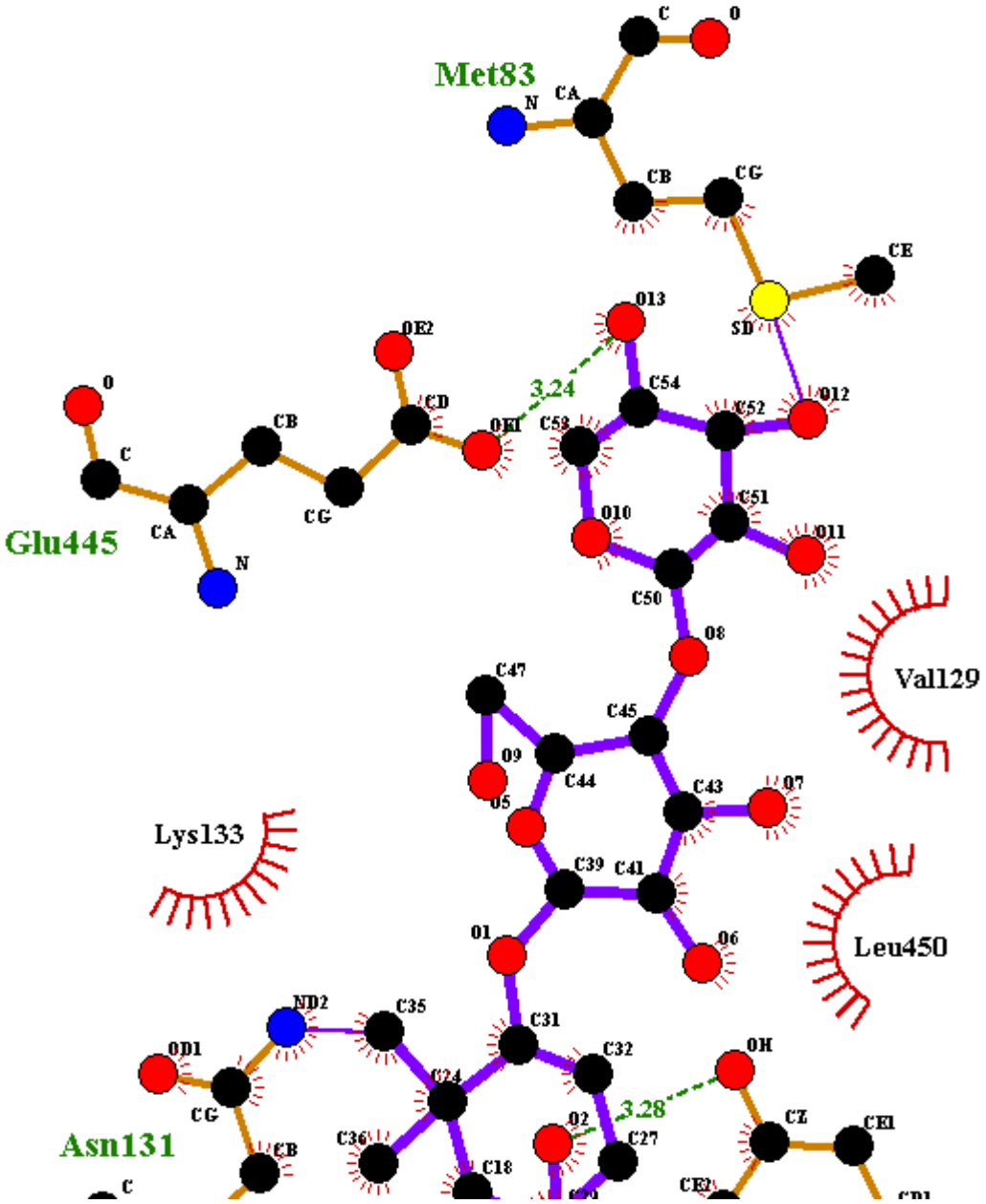


Fig. 7.2: Ligplot showing hydrogen and hydrophobic bonds between bacoside A and AChE

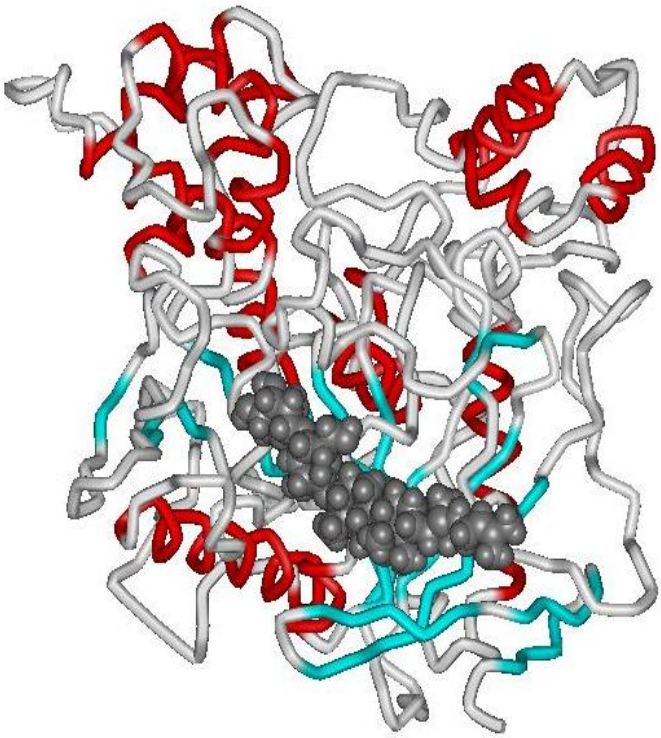


Fig. 7.3: AChE (wired ribbon) with bound Bacoside rendered in CPK model (thick grey coloured)

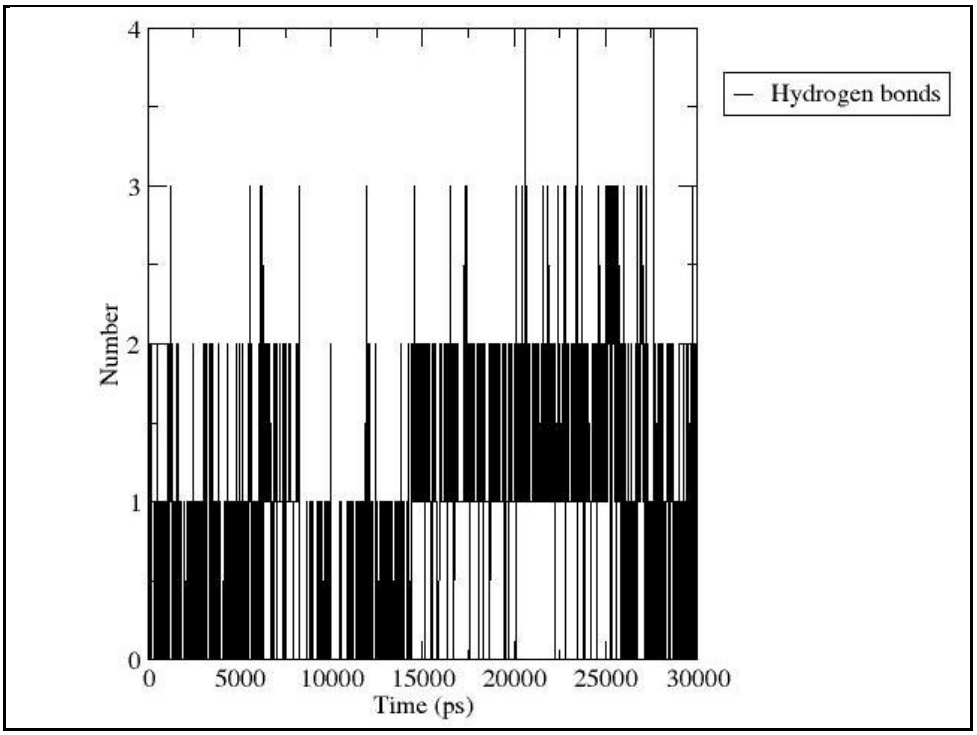


Fig. 7.4: H-bonding pattern of Bacoside with AChE

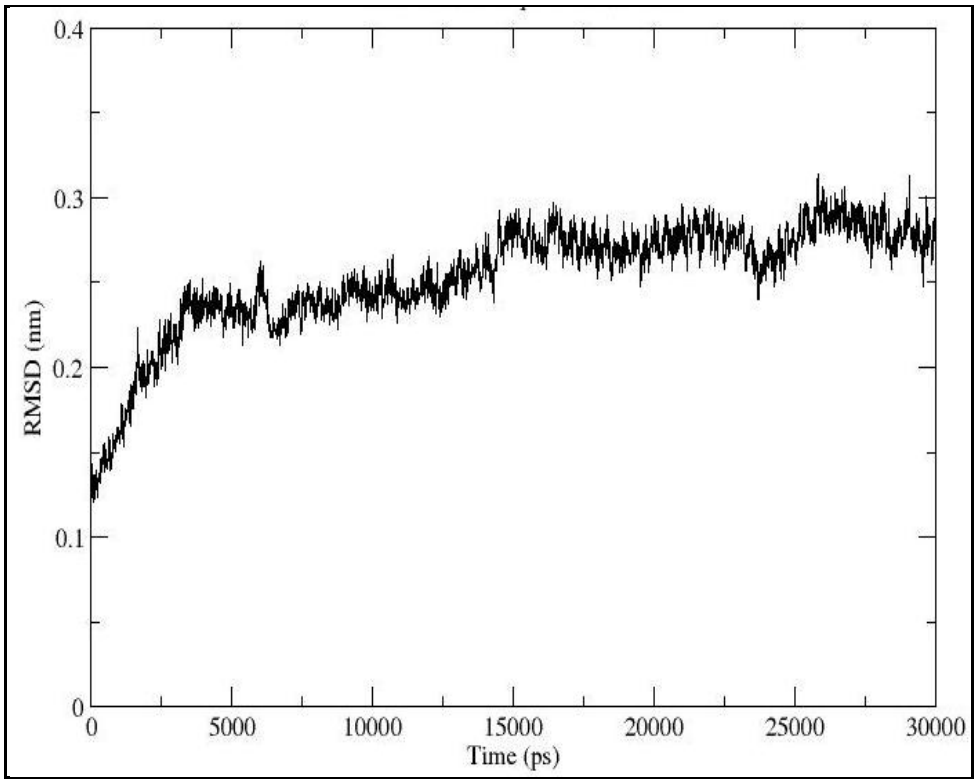


Fig. 7.5: RMSD plot

Grover et al. (2012) reported that the AChE contains a hydrophobic site, which has a number of subsites consisting Ser203, His447, Glu334, Gly121, Gly122, Ala204, Trp236, Phe295, Phe297, Phe338, Trp86, Tyr133, Glu202, Gly448, Ile451, Asp74, Tyr124, Ser125, Trp286, Tyr337 and Tyr341 residues. The active site of AChE is covered by the omega group, a disulfide-linked loop containing Cys69-Cys96, burried at base of enzyme with 20 Å deep close to in the middle of the enzyme (Wiesner et al. 2007). The visual inspection of our AChE complex shows that the bacoside is binding to the AChE protein around active site that is buried inside the protein at the end of deep narrow gorge beginning with Met83 and ending with Glu445 (Fig 7.2) capable to block the physical interactions and may inhibit the enzymatic activity of the protein. Alisaraie and Fels (2006) indicated a short channel at the bottom of the gorge, a

backdoor site that consist amino acid Trp84 at the entry and ends at the enzyme surface in a cavity close to amino acid Glu445 supports this data.

7.3. *In Silico* insight into potential anti-cancer activity of *Chlorophytum borivilianum*

Colorectal cancer is one of the third commonly diagnosed cancers. This is one of the major cause responsible for death in both men and women in developed countries. According to Siegel (2018), there are 97,220 estimated new cases of colon cancer and 50,630 estimated deaths in both the sexes. Colon is the part of the digestive system or gastrointestinal system. Most colorectal cancers originate from adenomatous polyp. Polyps are the growth that begins in the inner lining of the colon and moves toward the center. Most of the polyps are noncancerous. Only few types of polyps can become cancer known as adenomas. Colon cancer grows slowly which takes 10-15 years (Kelloff et al. 2004). Risk factors for this disease include physical inactivity, obesity, and high consumption of meat, smoking, and heavy alcohol intake. A family background of colon cancer or adenomatous polyps and personal history of inflammatory bowel disease are the other risk factors associated with colon cancer. Inflammation and tumorogenesis is a grievous combination, well supported by various researchers in last decade.

Chronic inflammatory bowel disease ulcerative colitis is an important risk factor for the development of colon cancer (Popivanova et al. 2008). Treatment of colon cancer with the oral drugs is possible only when diagnosed at the early stage of cancer development. Some FDA approved drugs for colon cancer are Avastin, Bevacizumab, Camptosar,

Cetuximab, 5FU (Fluorouracil Injection). Surgery, radiotherapy, and chemotherapy are advised at the latter stage of colon cancer, which are associated with reoccurrence of the disease and side effects like fatigue, constipation or diarrhea, a temporary or permanent colostomy, bladder irritation etc. Few advanced imaging techniques enabled the progression of treatment approach in diagnosis of hepatic metastatic disease and rectal cancer with the limitations like technical, economical and logistic challenges (Cutsem et al. 2016). Besides the advancement of therapeutic strategies; treatment of colorectal cancer need to be more specific and effective.

The most important phytosterols present in plants are sitosterol, campesterol, and stigmasterol. *C. borivilianum* has stigmasterol as major secondary metabolite with high medicinal value. Medicinal properties and various biological activities viz. aphrodisiac, antioxidant, anticancer and immune booster are attributed by its major metabolite stigmasterol. Experimental investigation revealed that few types of cancers like colon, breast, and prostate can be prevented with the use of phytosterols (Awad and Fink 2000). Ali et al. (2015) examined *in vivo* chemopreventive effects of stigmasterol on skin cancer. They noticed decreased levels of lipd peroxides and less DNA damage with the administration of stigmasterol. Stigmasterol also enhanced glutathione, superoxide dismutase, and catalase, which showed the chemopreventive potential of stigmasterol. 5.34 µg/mL stigmasterol was required to inhibit the activity of TNF-α (Chao and Lin 2010). TNF-α is a major immune-modulatory and proinflammatory cytokine that is synthesized as a membrane-anchored precursor. It supports previous studies indicating the antiinflammatory properties of stigmasterol, with low concentrations of tumor

necrosis factor-alpha (TNF-α). Monocytes and macrophages are responsible for releasing TNF-α, involved in the host immune response (Ramirez et al. 2007). Pro-inflammatory mediators such as TNF- α, IL-6, and nitric oxide are crucial for the uninterrupted functioning of immune system especially in the presence of any infection. On the contrary, this can be the reason for the development of tissue and organ injury when overproduced (Szollosi et al. 2016). Increased concentration of TNF-α is responsible for the pathogenic process of infectious as well as autoimmune diseases (Magna et al 2005). TNF- α is the mediator of tumor-associated inflammation and tumorigenesis (El-Bahrawy et al. 2016). It was observed as an important mediator of initiation and advancement of colitis-associated colon carcinogenesis (Popivanova et al. 2008). Colon inflammation was found to be responsible for the promotion colon TNF-α which is linked to increased chances of colon pathogenesis (El-Bahrawy et al. 2016). Various studies associate tumor necrosis factor alpha converting enzyme (TACE) with colon cancer (Ramirez et al. 2007). TACE is the member of A disintegrin and a metalloproteinase-containing enzyme (ADME). It is responsible for producing the soluble form of TNF-α from its membrane-bound precursor i.e. pro TNF-α. An experimental study conducted on teleost proved TNFα-converting activity of TACE (Lu et al. 2015).

Various other studies proved the involvement of TACE in the suppression of colon cancer (Ramirez et al. 2007). It is well known that high concentration of TNF-α is found in the cases of colon cancer. Inhibition of TNF-α production can suppress the signals that are responsible for releasing mature TNF-α. Although a number of

proteases have been shown to process pro-TNF-alpha, the most efficient are TNF-α converting enzyme (TACE). Since the enzyme plays an important role in converting TNF-α in soluble form, targeting the enzyme could be a potential therapeutic strategy counteracting the increase in TNF-α concentration, which has been seen in many cases of colon cancer. The present study explores a novel therapeutic perspective of stigmasterol for colon cancer with the suppression of over expressed TNF-α by stigmasterol through inhibition of TACE. Molecular docking studies have been used to identify the binding modes. A Molecular Dynamics (MD) simulation was carried out to analyze the stability of docked complex inside the bodily conditions by using Gromacs simulation suite.

Literature survey revealed that interaction of stigmasterol and TACE is not well studied. Molecular dynamics simulations has been performed to better understand the interaction and stability of stigmasterol and TNF-α converting enzyme.

7.3.1. Material and Method

i. Protein and ligand preparation

Crystal structure of TACE [PDB: 1BKC] was retrieved from PDB. Structure of ligand molecule stigmasterol (CID: 5280794) was obtained from NCBI – PubChem Compound Database.

ii. Prediction of active site

Understanding the reaction of catalytic residues is necessary in order to understand the accurate function of an enzyme. Although active site information of TACE was

partially reported (Maskos et al. 1998) earlier, current study presents more details on co-crystallized structure with its inhibitor and further validation has been done by *in silico* analysis. A most probable active site with amino acid residue information was obtained from Q-site Finder web server.

iii. Molecular docking

Molecular docking was performed with AutoDock4 (Morris et al. 1998). AutoDock4 is a one of the widely-used docking tool having an efficiency of predicting rapid and exact bound confirmations of ligand and their potential targets. AutoDock docking models are often consistent with X-ray crystal structures (Dym et al. 2002; Rao and Olson 1999).

iv. MD simulation in water

A Molecular Dynamics (MD) simulation was carried out for the docked complex to analyze its stability inside the bodily conditions by using Gromacs simulation suite (Van Der Spoel et al. 2005). AutoDock uses a grid-based method for quick evaluation of binding energy of test confirmations. AutoDock starts with preprocessing of ligand by removal of coordinates from the PDB file and water molecules. AutoDock allows the target to be embedded in the grid while the investigating atom is consecutively directed to each grid point. Interaction energy of investigating atom and target molecule is calculated and is secured in the grid, which is then referred during docking simulation. Lamarckian genetic algorithm is used for conformational searching (Morris et al. 1998). AutoDock4 uses a semi empirical free energy force field for the prediction of binding energies of ligand to macromolecular targets. Q-site Finder anticipates the information active site residues by using energy criteria and computes van der Waals interactions of methyl

probe with the target molecule. The clusters of favorable energies are graded according to the total interaction energies and the cluster of maximum energy filled the first rank (Laurie and Jackson 2005]. Polar hydrogen was added to the protein structure to provide accurate ionization. The ligand was attributed to gasteiger charges and rigid roots. 13 rotatable bonds were adjusted. Key residues were embedded in energy-container grid of 60 Å × 60 Å × 60 Å (x, y, z). The best confirmation was obtained through default Lamarckian genetic algorithm as a search protocol. The output from AutoDock4 was analyzed with Viewerlite 5.0. Ligplot generated using PDBsum determined the number and length of H- bonds between ligand and receptor. The application of MD simulations to study the protein dynamics is well described in many previous studies (Abe et al. 2011; Kumar et al. 2016). In the present study, MD simulations were performed for 30 ns and stability of protein as well as ligand as a complex was studied.

7.3.2. Result and Discussion

i. Identification of active catalytic site in TACE

Q-site Finder generated ten clusters based on probe and protein interactions after submitting the pre-processed structure of TACE. Individual probe site associate preferentially to the best-suited binding sites on the protein surface and these are the positions where a tentative ligand could interact and optimize its van der Waals interaction energy (Laurie 1908). The obtained results included nearly 27 residues. All these residues are covered in the grid while performing docking. The active cleft of TACE has a catalytic zinc residue at its center pentacoordinated by the three-imidazole N2 atoms of His 405, His 409, His 415. Along with these residues Glu 406, Met 345, Pro 437, Trp 312, Asp 344

around the zinc atom constitute the active site. The docked complex was forming a hydrogen bond with Gly 346 and showing hydrophobic interactions with many of the active site residues. Fig. 7.6 is the ligplot showing hydrogen and hydrophobic bonds between ligand and macromolecule TACE. Fig. 7.7 depicts the stigmasterol and TACE complex after molecular docking. A schematic of detailed ligand atom interactions with the protein residues before MD simulations is shown here (Fig. 7.8).

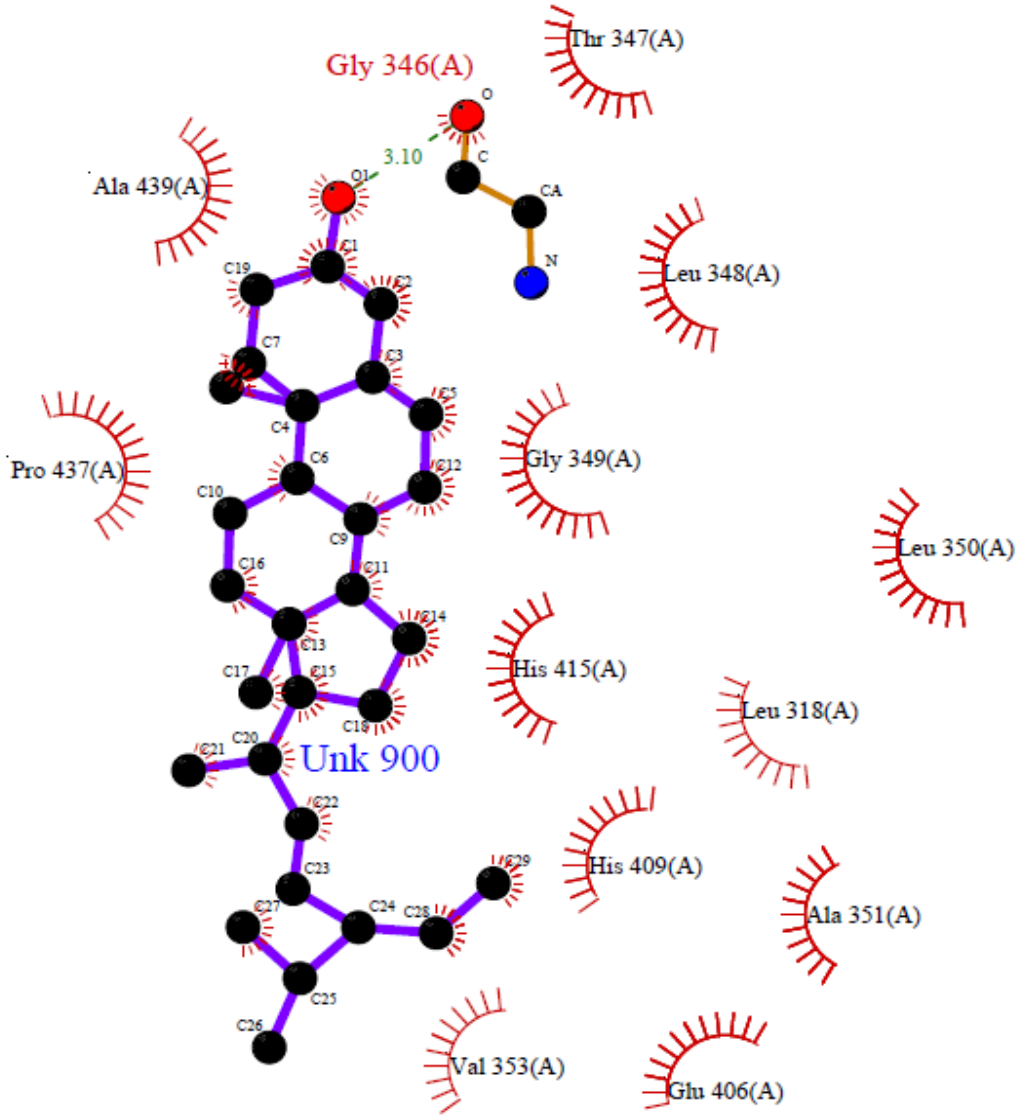


Fig. 7.6: Ligplot showing hydrogen and hydrophobic bonds between stigmasterol and TACE

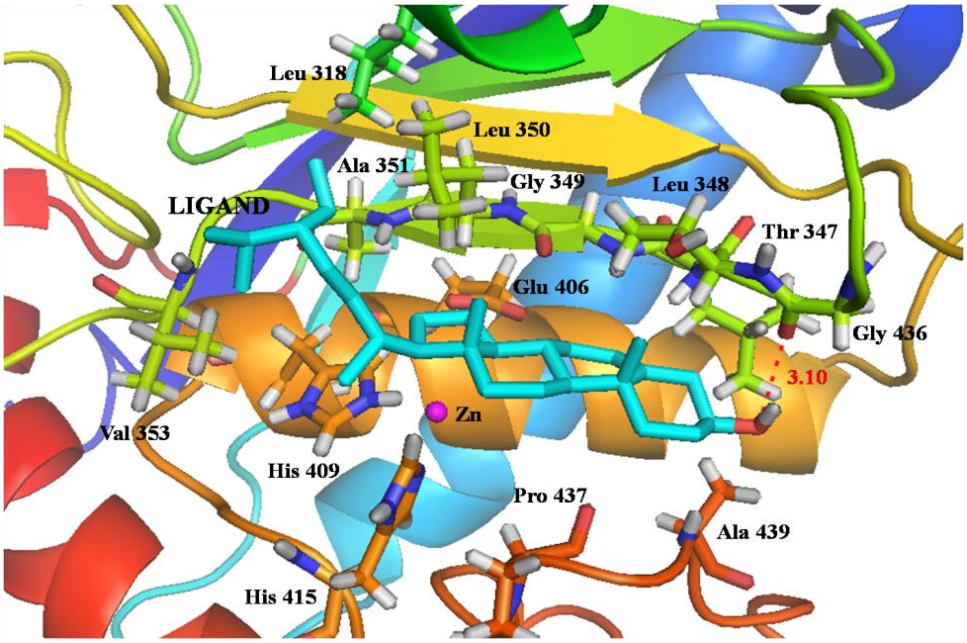


Fig. 7.7: Stigmasterol and TACE Complex after docking

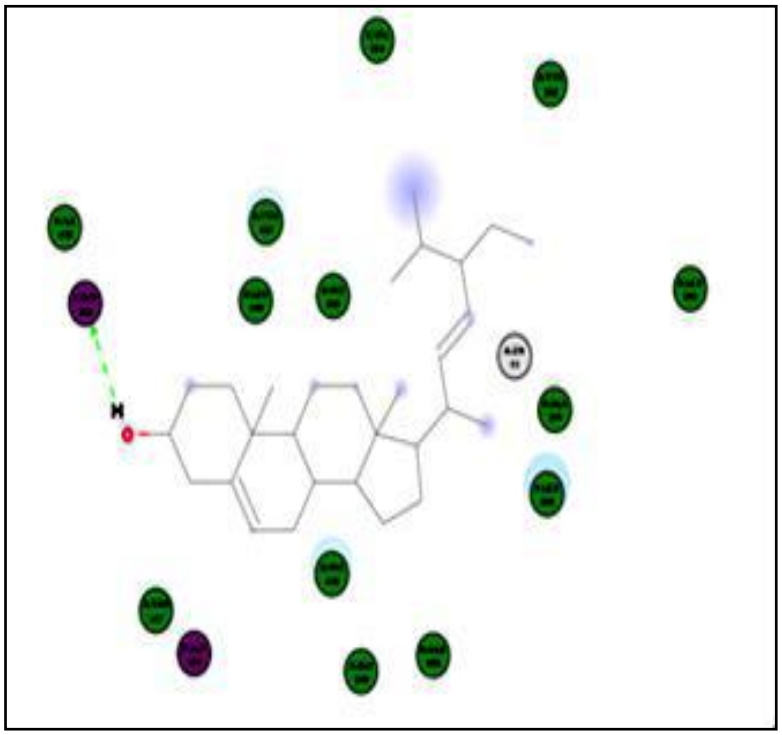


Fig 7.8: A schematic representation of ligand interaction with the protein residues before performing MD simulations

ii. Molecular docking simulation

We have investigated molecular docking and MD simulations on stigmasterol and TACE complex for the stability in the bodily conditions. CPK model of the complex is shown in Fig. 7.9. Computed Root mean square deviation (RSMD) (Fig. 7.10) suggests that docked complex is stable. Root mean square deviation was calculated by least square fitting of CA from the initial structure and found to be stable around 2A during simulations; suggesting that the docked complex is stable.

Ligand and the active site residues in the receptor domain within 8A distance were considered for the interaction studies. The docked complex was forming a hydrogen bond with Gly 346 and within the hydrophobic interaction range with many of the active site residues. Hydrogen bond distance between ligand and receptor is shown in Fig 7.11.

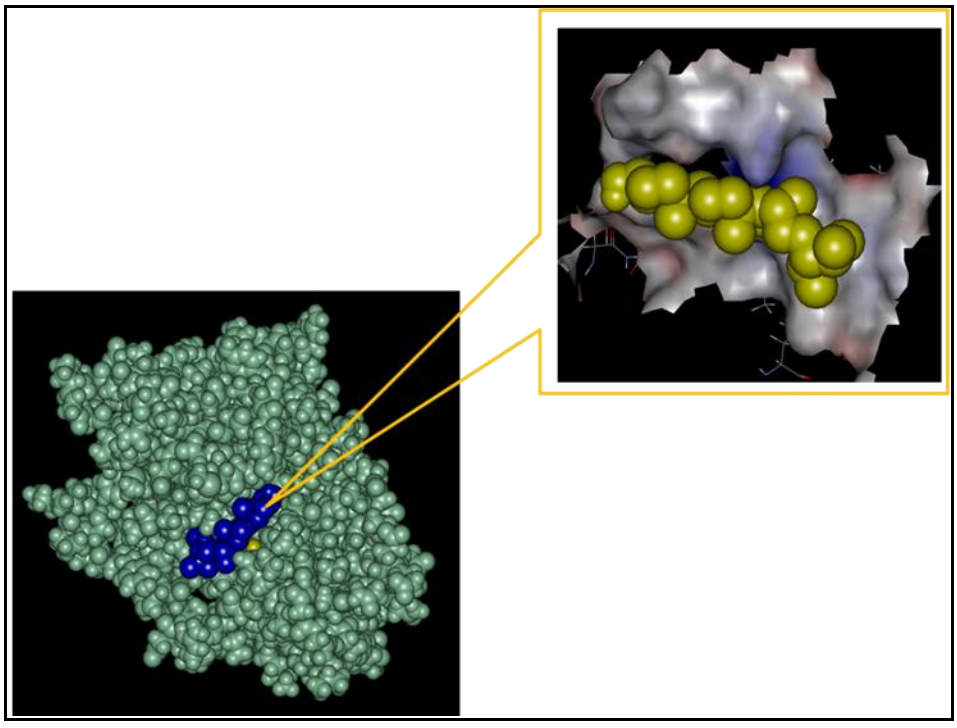


Fig. 7.9: Stigmasterol and TACE complex rendered in cpk

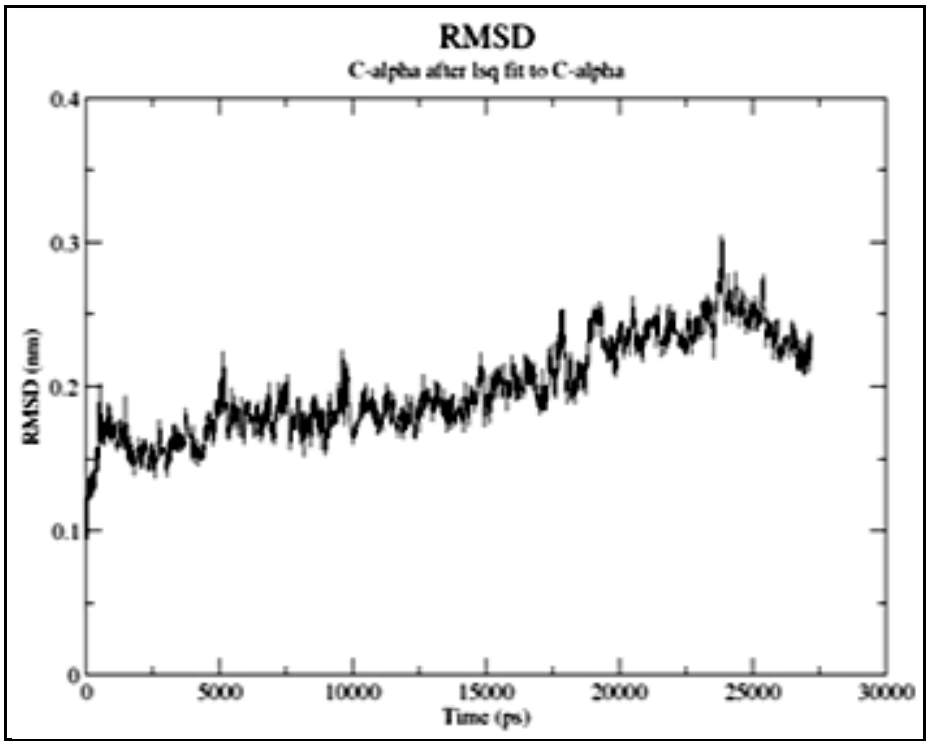


Fig. 7.10: RMSD plot

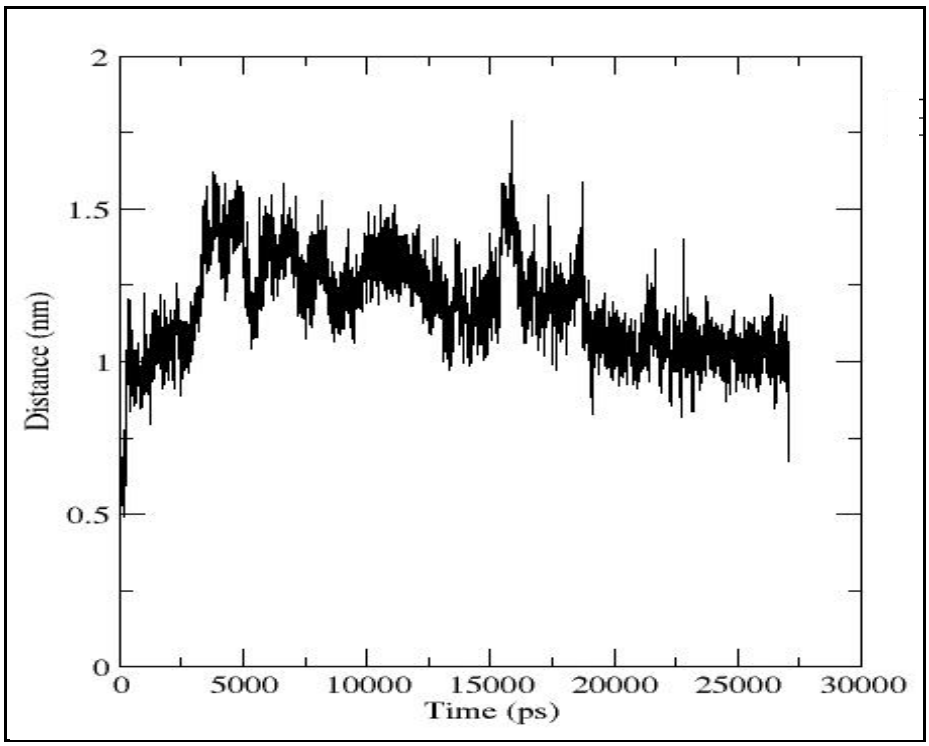


Fig. 7.11: Hydrogen bonding pattern between ligand and receptor indicating formation of strong hydrogen bonding during simulations

Hydrogen bonding pattern between ligand and receptor is indicating the formation of strong hydrogen bond during simulations. Hydrogen bond existence map for various amino acids interacting with ligand is given in Fig. 7.12. Cyan implies the presence of a hydrogen bond and Red implies the absence of one. The *y*-coordinate shows the hydrogen-bond index.

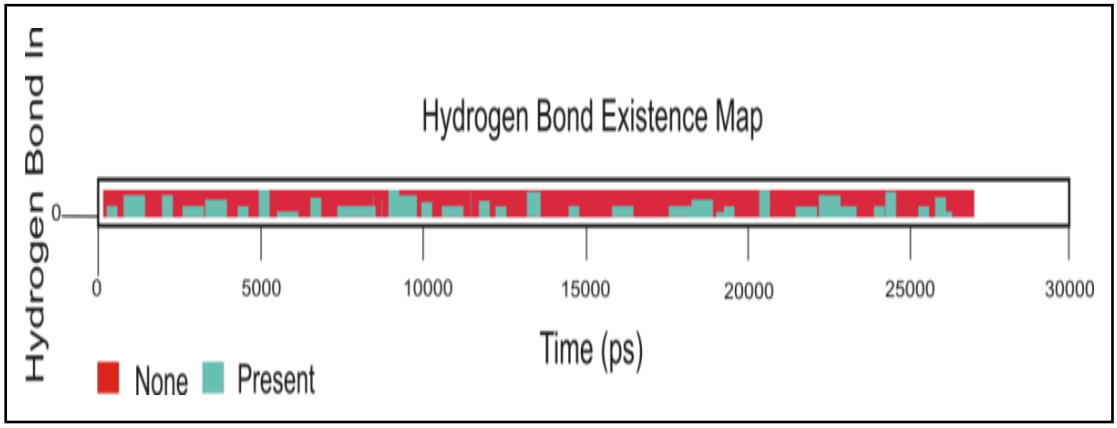


Fig. 7.12: Hydrogen bond existence map

In the present study, we examined the molecular mechanism of the stigmasterol on TACE that helps in understanding of the cancer cell characteristics. We observed that stigmasterol docked onto active site of TACE. The active site of TACE comprises zinc atom coordinated by a conserved zinc-binding domain (405-HexGHxxGxxH-415) (Maskos et al. 1998). It is also determined by the three-dimensional structure of TACE through Q-site Finder server. The ligand is interacting with key amino acids Leu 348 and Gly 349 present in the binding pocket of the enzyme (Elumalai et al. 2012). The binding pocket also co-occurred with the binding site of stigmasterol acquainted in the co-crystallized structure acquired from PDB. Molecular docking score of the 3D structure of stigmasterol with the above-described active site of TACE was - 10.04

predicting good binding affinity of TACE with stigmasterol. Docked complex exhibited molecular interaction with glutamate and histidine residues that play a key function during the proteolytic reaction procedure. Short hairpin RNA silencing TACE (shTACE) prevented and efficiently treated acute and chronic ulcerative colitis by decreasing TNF- α level (Song et al. 2016). The active site of TACE was blocked with highly selective new non-hydroxamate sulfonamide TACE inhibitors, considering as therapeutic targets for the treatment of TNF-dependent pathologies (Sarkate et al. 2015). A quinazoline derivative therapeutically improved arthritis through suppressing production of TNF- α mediated by TACE (Pu et al. 2015). Another study on TACE inhibition revealed that TACE activation by non-receptor tyrosine kinase Src in mechanically stressed cardiomyocytes is harmful as inhibition of TNF- α through this process can result to heart failure (Niu et al. 2015). Considering above facts, the interaction of TACE and stigmasterol were anticipated to intervene with the interaction of substrate for the binding site of TACE, hence strengthening the idea of stigmasterol as TACE inhibitor. Molecular dynamics simulation is an authentic and modern way for the prevention of several diseases (Gajula et al. 2013). Molecular dynamics simulations are useful in studying the conformational dynamics of biological macromolecules (Borovykh et al. 2006). Molecular dynamics of the docked complex were examined to find out its stableness in bodily conditions. A simulation run time was 30ns, which was sufficient for the rearrangement of a ligand-bound protein molecule to acquire a stable binding manner. Root mean square deviation was calculated by least square fitting of CA from the initial structure and found to be stable around 2A during simulations;

suggesting that the docked complex is stable. A structure showing the stationary phase was considered to study the molecular interaction pattern in the docked complex. H-bond, hydrophobic interaction, and van der Waals interaction were the factors that play an important role to make the stigmasterol and TACE a stable interaction. The dynamic stableness of stigmasterol during simulation runtime and its interaction with the important residues present at the binding site of the enzyme possibly consolidate the mode of action of stigmasterol on inhibition of TACE, which is responsible for the release of the soluble form of TNF-α.

7.4. Drug-likeliness

The successful evaluation has been done for drug-likeness based on Lipinski's "Rule of Five", while 46 computed physicochemical properties or molecular descriptors were used to predict ADMET (absorption, distribution, metabolism, elimination and toxicity) of the compound.

7.5. Conclusion

The tested methanolic extract showed moderate inhibitory activity for AChE achieving values over 62%. Molecular dynamics simulation studies revealed binding pattern of probable herbal drug bacoside A onto structure of acetyl cholinesterase. Our docking simulation outcome shows high binding affinity of bacoside A to AChE. Molecular dynamics simulations defend the hypothesis bacoside A is a probable ligand to inhibit AChE. Findings of the present study are valuable for further analysis of bacosideA/AChE complex as a probable drug of AD.

Molecular docking and simulation study revealed that presence of a ligand in the active site of TACE, interacting with the key residues consolidated the idea that stigmasterol is a potential inhibitor of TACE. Furthermore, this study is a stepping-stone in order understands the prevention mechanism of TNF- α, a high concentration of which is responsible for colon cancer. Hence, it can be used for further studies as a natural anticancer drug.

The study of stigmasterol has been published in "Advance Material proceedings" entitled "**Anticancer property of green material through computational approach".**

CHAPTER 8

SUMMARY AND CONCLUSIONS

Medicinal plants are the vital source of pharmacologically active compounds to cure various ailments. Many medicinal plants are at the verge of extinction mainly due to overexploitation via pharmaceutical industries and urbanization. Need of the hour is to conserve of the plant species through plant tissue culture techniques. Plant tissue culture techniques like *in vitro* propagation has been proved a pivotal for producing large number of genetically similar plants in very less time and cryopreservation for medium to long time.

In addition, *in vitro* propagation is a modern tool that unfolds a vital production link between mass propagation and conservation via optimizing plant growth medium and plant growth hormones. Use of nodal explants culture and callus production for *B. monnieri* and shoot base culture for *C. borivilianum* are remarkable for *in vitro* propagation as it produces large quantity planting material in less time and eliminates the possibility of endogenous contamination in the studied accessions.

Environmental conditions effects the therapeutic potential of the species. Therefore, different accessions belonging to different agro-climatic zones contain variable concentrations of pharmaceutically active metabolites. Accession-based study of *in vitro* plant cultures based on growth parameters, presence of therapeutically important secondary metabolites and antioxidant potential has identified accessions IC 554588, IC 344312 and IC 554585 of *B. monnieri* and accession IC 558379 of *C. borivilianum* as elite accession.

Pharmaceutical requirement of therapeutically useful secondary metabolites such as bacosides and stigmasterol has increased significantly during last two decades. Therefore, to enhance the secondary metabolite production and consequently pharmacological effects including the antioxidant potential of the species, it is important to incorporate elicitors on *in vitro* cultures of *B. monnieri* and *C. borivilianum*. A study on elicitors revealed that plant cultures treated with elicitors resulted with increase in increased yield of major secondary metabolites such as amount of total bacosides and stigmasterol. Elicitors enhanced the therapeutic potential of both the medicinal plants.

Genetic diversity is the basis of species diversity. It plays an important role of precursor in the study of plant species because the extent of genetic diversity has an effect on evolutionary potential of the species. Application of molecular markers on 19 accessions of *B. monnieri* for the assessment of genetic diversity exhibited wide variation in genetic relation using SCoT and CBDP markers, the gene-targeted markers. Plant breeders can utilize these genetically diverse accessions for producing superior quality germplasm in terms of having higher pharmacological properties.

Conventional methods of drug development are time consuming and costly. However, modern bioinformatics techniques combined with several software utilize phytocompound as lead molecule against the disease to be cured. By computational approach, we demonstrated a precise interaction model in which bacoside and stigmasterol were bound to a particular region of acetylcholine esterase and tumor necrosis factor-α converting enzyme respectively. The docking and MD simulation data of these interactions revealed that bacoside can be effective for Alzheimer's disease and stigmasterol is having anticancer activity against colon cancer.

CHAPTER 9
FUTURE PROSPECTS

'Medicinal plants as herbal material' is now becoming a major area of interest to the scientific society across the globe for drug development for safe and effective drug discovery. Many species of important medicinal plants are at the verge of extinction. Therefore, conservation and sustainable use of therapeutically valuable medicinal plants is the need of an hour. Though, remarkable efforts have been done by many researchers for large-scale production and conservation of important species, still, there is a gap between demand and supply of herbal plants.

Accession-based study has the potential to identify superior accession using molecular marker technique, which has the capacity to explore accessions of various agro-climatic zones. Call for the present time is to save the superior accessions and develop superior varieties through breeding programs. In future, biotechnological approaches and metabolic engineering can be used to meet the increasing demand of pharmacologically active and commercially profitable secondary metabolites.

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ANNEXURE 1

Media Composition

1- Murashige and Skoog (MS) Medium

Preparation of stock solution of macronutrients, micronutrients and vitamins. Stocks are stored at 4˚C. The appropriate amount of stock solution is taken to prepare 1 L of MS medium.

Major Salts

Major salts	mg/L	500 ml stock (20X)
Ammonium nitrate	1650	16.5 gm
Potessium nitrate	1900	19 gm
Calcium chloride.$2H_2O$	440	4.4 gm
Magnicium sulphate.$7H_2O$	370	3.7 gm
Monopotassium phosphate	170	1.7 gm

Minor Salts

Minor salts	mg/L	500 ml stock (200X)
Boric acid	6.2	620 mg
Manganese sulphate.$4H_2O$	22.3	2230 mg
Zinc sulphate.$4H_2O$	8.6	860 mg
Potassium iodide	0.83	83 mg
5. Sodium molybdate.$2H_2O$	0.25	25 mg
Cobalt chloride.$6H_2O$	0.025	2.5 mg
Copper sulphate.$5H_2O$	0.025	2.5 mg

Vitamins

Vitamins	mg/L	500 ml stock (200X)
Thiamine (HCl)	0.1	10 mg
Niacine	0.5	50 mg
Glycine	2.0	200 mg
Pyrodoxine (HCl)	0.5	50 mg

Iron 500ml stock (200X)

3.725 g of N_{a2}EDTA (Ethylenediaminetetra acetic acid) is dissolved in 250 ml distill water. 2.785 g of $FeSO_4.H_2O$ is dissolved in 250 ml distill water. Boil N_{a2}EDTA solution and add $FeSO_4$ gently by stirring. Mix 5 ml of Iron (200 stock) for 1 L of medium.

Carbohydrate source

Sucrose - 30 g/L

pH – 5.8

Agar – 0.8%

2- B5 (Gamborg) Medium

Major Salts

Major Salts	**mg/L**
Ammonium sulphate	134.000
Calcium chloride	113.230
Magnesium sulphate	122.090
Potassium nitrate	2500.000
Sodium phosphate monobasic	130.420

Minor Salts

Minor Salts	**mg/L**
Boric acid	3.000
Cobalt chloride hexahydrate	0.025
Copper sulphate pentahydrate	0.025
EDTA disodium salt dihydrate	37.300
Ferrous sulphate heptahydrate	27.800
Manganese sulphate monohydrate	10.000
Molybdic acid (sodium salt)	0.213
Potassium Iodide	0.750
Zinc sulphate heptahydrate	2.000

Vitamins

Vitamins	mg/L
Myo-Inositol	100.000
Nicotinic acid (free acid)	1.000
Pyridoxine HCl	1.000
Thiamine hydrochloride	10.000

Carbohydrate Source

Sucrose – 20 g/L

pH – 3.5 to 4.5

Agar – 0.8%

3 Nitsch Medium

Major Salts

Major Salts	**mg/L**
Potassium nitrate	950.000
Ammonium nitrate	720.000
Magnesium sulphate anhydrous	90.340
Potassium phosphate monobasic	68.000
Manganese sulphate.H2O	18.940

Minor Salts

Minor Salts	**mg/L**
Boric acid	10.000
Molybdic acid (sodium salt).2H2O	0.250
Zinc sulphate.7H2O	10.000
Copper sulphate.5H2O	0.0250
Ferrous sulphate.7H2O	27.850

Vitamins

Vitamins	mg/L
Myo – Inositol	100.000
Thiamine hydrochloride	0.500
Pyridoxine hydrochloride	0.500
Nicotinic acid (Free acid)	5.000
Folic acid	0.500
Biotin	0.050
Glycine (Free base)	2.000

Carbohydrate Source

Sucrose – 20 g/L

pH – 3.8

Agar – 0.8%

4-Schenk and Hilderbrandt

Major Salts

Major Salts	**mg/L**
Potassium nitrate	2500.000
Potassium phosphate monobasic	300.000
Calcium chloride.$2H_2O$	200.340
Magnesium sulphate	195.340
Manganese sulphate.H_2O	10.000

Minor Salts

Minor Salts	**mg/L**
Boric acid	5.000
Potassium iodide	1.000
Molybdic acid (sodium salt).2H2O	0.010
Zinc sulphate.7H2O	1.000
Copper sulphate.5H2O	0.200
Cobalt chloride.6H2O	0.1000
Ferrous sulphate.7H2O	15.000
EDTA disodium salt.2H2O	20.000

Vitamins

Vitamins	mg/L
Myo – Inositol	1000.000
Nicotinic acid (free acid)	5.000
Pyridoxine HCl	0.500
Thiamine hydrochloride	5.000

Carbohydrate Source

Sucrose – 30 g/L

pH – 4.0

Agar – 0.8%

5-White Media

Major Salts

Major Salts	mg/L
Potassium nitrate	80.000
Calcium nitrate	221.960
Magnesium sulphate	360.000
Sodium phosphate monobasic	18.980
Potassium chloride	65.000
Sodium sulphate	200.000
Manganese sulphate.H2O	5.040

Minor Salts

Minor Salts	mg/L
Boric acid	1.500
Potassium iodide	0.750
Molybdenum trioxide	0.001
Zinc sulphate.7H2O	2.670
Copper sulphate.5H2O	0.010
Ferrous sulphate.7H2O	2.500

Vitamins

Vitamins	mg/L
Myo – Inositol	100.000
Nicotinic acid (free acid)	0.500
Pyridoxine HCl	0.100
Thiamine hydrochloride	0.100
Glycine (Free base)	3.000

Carbohydrate Source

Sucrose – 20 g/L

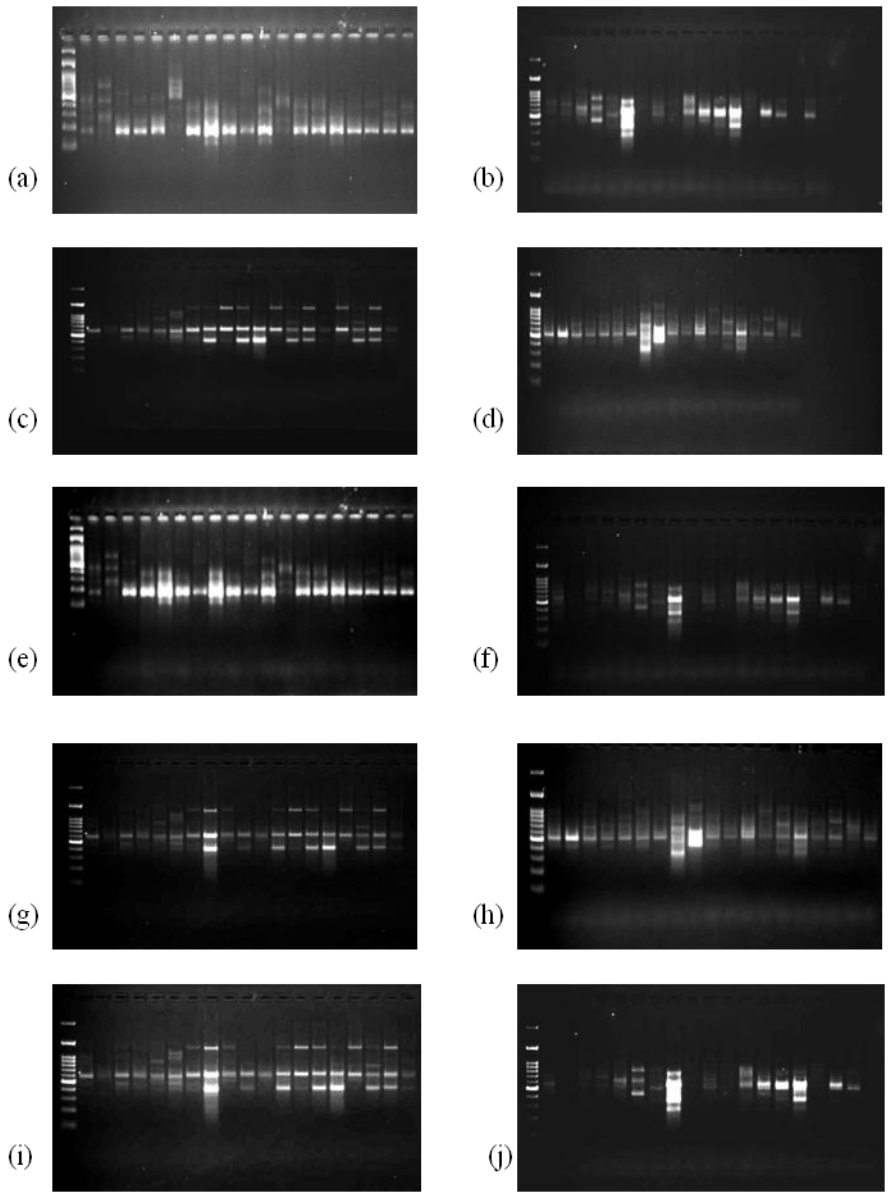
pH – 4.7

Agar – 0.8%

ANNEXURE 2

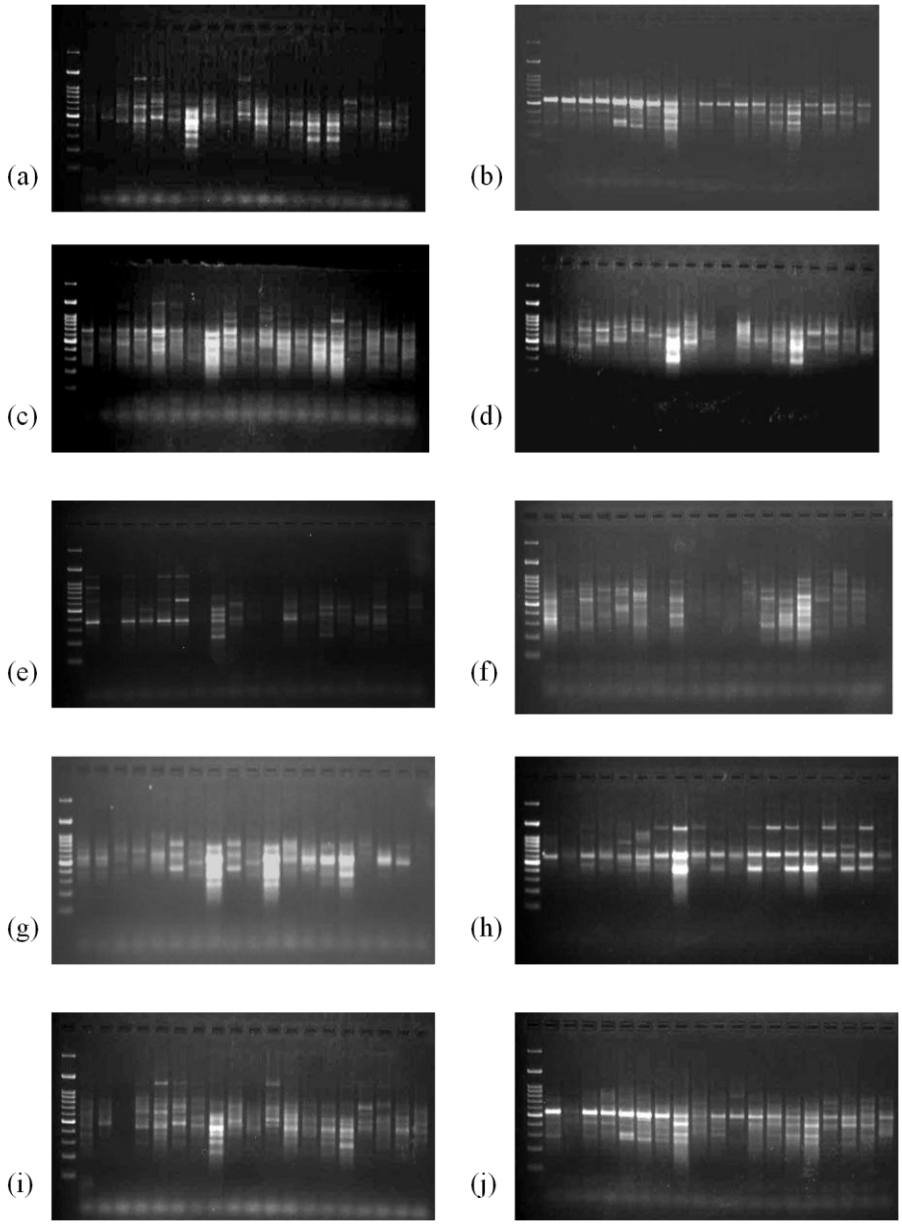
PCR amplified products of 19 accessions of *B. monnieri* CBDP with 1 Kb ladder (M)

Photographs (a) to (j) denotes the amplified products of primer 1 to primer 10



PCR amplified product of 19 accessions of *B. monnieri* SCoT with 1 Kb ladder (M)

Photographs (a) to (j) denotes the amplified products of primer 1 to primer 10



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Bulletin of Environmental Contamination and Toxicology

ISSN 0007-4861
Volume 99
Number 5

Bull Environ Contam Toxicol (2017)
99:614-618
DOI 10.1007/s00128-017-2183-6

Springer

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Uptake of Heavy Metals from Industrial Wastewater Using In Vitro Plant Cultures

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Received: 7 April 2017 / Accepted: 22 September 2017 / Published online: 30 September 2017

Abstract The plant species *Bacopa monnieri* has been observed to reduce the heavy metal concentrations in its vicinity. The present study is a comparison of in vitro culture and soil-grown plants of *B. monnieri* to remove Cr and Cd, from synthetic solution and effluent obtained from industrial area. Results were obtained at every half hour interval upto 180 min. Samples were observed for light absorption using UV–Visible spectrophotometer. Statistically, both systems reclaimed Cr and Cd from polluted water. In vitro cultures showed 67% and 93% removal of Cr and Cd from industrial wastewater whereas soil-grown plants showed 64% and 83% Cr and Cd removal. However, reduction rate was significantly higher for in vitro culture as compared to soil-grown plants. Besides other advantages, in vitro plant cultures proved to be more potent to detoxify pollutants in less time. This approach can be used for the removal of heavy metals at large scale.

Keywords *Bacopa monnieri* · Bioactive compound · Ecology · Phytoremediation · Water pollution

Industrial development, especially in developing countries deals with new challenges to the biosphere. Among the available resources, water is a precious commodity that requires special attention. Majority of water near industrial areas is highly polluted thereby not suitable for human consumption and agriculture (Hettick et al. 2016). The world is heading towards water crisis (Rosegrant and Cai 2001) due to the burgeoning population which is exhausting the available fresh water resources. Improvement of living conditions often comes at the cost of the environment. Heavy metal contaminated water from industries is added to rivers making it unfit for human consumption. Hazardous heavy metals found in industrial wastewater are Cr, Cd, Cu, Ni, Pb, As and Zn. A density of more than 5 g per cubic centimeter is generally considered as heavy metal determining factor. Ingestion of heavy metals beyond the permitted concentration, can adversely affect the human health (Chang et al. 1996; Wang and Shi 2001; Beyersmann and Hartwig 2008). One of the toxic heavy metal present in the contaminated water is chromium (Cr) which exists in two forms—trivalent [Cr (III)] or hexavalent [Cr (VI)]. Hexavalent Cr occurs in the form of chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) which has more toxicity than other valency states. Trivalent chromium is non-toxic and absorbed less readily than hexavalent chromium (Sharma and Forster 1995). High exposure to Cr (VI) can lead to cancer in the digestive tract and lungs, gastric pain, nausea, vomiting, severe diarrhea, fatigue, irritability, hemorrhage, and damage to nervous system (Rosegrant and Cai 2001; Singh et al. 2011). Cd is commonly used in industrial practices as an anticorrosive agent, a stabilizer in polyvinyl chloride products, as a color pigment and in the fabrication of Ni–Cd batteries (Godt et al. 2006). Continued consumption of food or water, containing even low levels of Cd may result in renal failure (CSEM 2008).

Conventional methods for removing heavy metal ions from industrial wastewater demands high investments and operational costs (Rich and Cherry 1987) and therefore, not suitable for small scale industries. On the contrary, in vitro technique like adsorption through activated carbon (AC) is an effective and economical substitute to remediate

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wastewater from toxic heavy metals. Used contaminated biomaterial can be disposed off or processed for further use without harming the environment depending on the contaminant characteristics (Kim and Qi 1995; Wilbur et al. 2012). Environment friendly technique of utilizing in vitro plant cultures for the uptake of heavy metals has an advantage in metabolic studies like the ease of adding inhibitors and inducers in the culture medium (Doran 2009), which can be helpful to enhance the remediation efficiency of in vitro plant cultures. However, not much has been explored so far about the metabolic pathways associated in the transformation of xenobiotic compounds and capacity of certain plants to bear, detoxify and gather a significant amount of heavy metals (Couselo et al.2012), moreover dissimilarity in xenobiotic metabolism between in vitro culture and soil-grown plants was noticed (Doran 2009). In a greenhouse pot experiment, a biomaterial biochar decreased the bioavailability of Cd, Cr and Zn with the promotion in the growth of *Machilus pauhai* plant (Guo et al. 2017). As the in vitro cultures are maintained in microbial free environment, they can be exploited to distinguish the responses and metabolic capabilities of tissue-culture plants from those encountered with microorganisms in their natural environment (Chaudhry et al. 2005; Lebeau et al. 2008). In vitro technique namely hairy root culture was found to be particularly noteworthy in understanding the enzymatic process involved in phytoremediation of phenols and thereby exploring a cost effective system to remediate the polluted environments (Paola et al. 2006). Waoo et al. (2013) standardized an in vitro technique to propagate large number of *Lantana camera* for the removal of heavy metals from the environment. The small amount of biomaterial generated by the plant species that have inherent competence to accumulate or metabolize various pollutants could be enhanced by in vitro culture material without sacrificing the natural habitat. In addition, soil-grown plants have a limited lifespan whereas in vitro plant cultures can be supplied on demand without disturbing the natural habitat. Therefore, use of plant tissue culture has tremendous scope in phytoremediation research as model plant system to discover the biochemical response of plant cells to environmental pollutants. The plant species *Bacopa monnieri* (Brahmi), family Scrophulariaceae, is a weak wetland plant known as memory enhancer in *Ayurvedic* systems of medicine. Various studies on uptake of single or mixed metal removal by *B. monnieri* through in vitro techniques have been successfully done by various scientists till date (Sinha et al. 1996; Sinha 1999; Shukla et al. 2007). Acknowledging above facts, the present study is focused on removal of heavy metals like Cr (VI) and Cd by in vitro plant cultures and soil-grown plants of *B. monnieri* from synthetic solution as well as industrial wastewater with respect to time. Multilinear regression (MLR) has been used for statistical analysis.

Materials and Methods

Plant samples of *B. monnieri* obtained from National Bureau of Plant Genetic Resources (ICAR-NBPGR, Delhi)- were maintained at Delhi Technological University, Delhi. Green parts of the plants were air-dried and crushed using mortar and pestle. AC was prepared by the method used by Singanan and Singana (2007). To the powdered samples, concentrated sulphuric acid was added in the ratio of 1:1.8. Samples were then heated in a hot-air oven at 160°C for 6 h, to release black, coarse AC. After cooling, the AC was washed with double distilled water by repeated centrifugation at 5000 rpm (Medline Scientific). Once pH 7.0–7.5 was attained, the samples were dried in the hot air oven at 105°C for 2–3 h until moisture was completely removed. The dried samples were sieved through 90 µm sieve to obtain only fine AC particles. The prepared biomaterial i.e. AC samples were weighed, characterized and stored at room temperature. The characteristics of AC derived from in vitro propagated and agro net grown plant of *B. monnieri* is represented in Table 1.

Synthetic wastewater containing Cr and Cd was prepared by using analytical grade $K_2Cr_2O_7$ and $CdCl_2$ (Sigma-Aldrich), at concentration of 100–500 and of 1000–5000-ppm respectively. Wastewater containing effluents from metal industries in the Bawana Industrial Area, Delhi, India, was collected from the Western Yamuna Canal, which flows through that area. Wastewater was diluted with double distilled water to a dilution factor of 10 for the study. Samples of Cr and Cd were observed for light absorption using spectrophotometer (UV/Vis PerkinElmer LEMBDA 950) at absorption maxima 350 and 283 nm respectively. Characteristics of industrial wastewater used in the experiment are shown in Table 2. Batch mode adsorption experiment was carried out by taking 60 mL of each test solution with 1 mg/mL AC in 150 ml conical flask. The conical flasks were then agitated at 100 rpm using a mechanical shaker (REMI

Table 1 Characteristics of AC prepared in vitro culture and soil-grown plants of *B. monnieri*

Serial no.	Parameters	Value	
		In vitro	Soil-grown
1	Moisture (%)	0.18	0.15
2	Ash (%)	0.37	0.41
3	pH	7.2	7.4
4	Particle size	≤90 µm	≤90 µm
5	Iodine number	720 mg/g	690 mg/g

Table 2 Characteristics of industrial wastewater

Sl. no	Parameters	Value
1	pH	7.9
2	Electrical conductivity	368 µS/cm
3	Total dissolved solids	328-ppm
4	Cr concentration	225-ppm
5	Cd concentration	1950-ppm

Orbital Shaker). Periodic observation was taken from 30 to 180 min until there was little or negligible decrease in concentration of Cr and Cd. Percent removal of Cr and Cd on the adsorbents was calculated as:

Removal $\% = 100 \times (C - C_f)/C_o$, where C_o is the initial concentration and C_f is the final concentration in ppm.

Before starting the experiment, the instrument was calibrated with optimum wavelength. Presence of any target metal in the blank solution was checked for the accuracy of the results. Limit of detection and limit of quantification for Cr was 20 and 60 ppm whereas for Cd it was 1000 and 3000 ppm respectively. Detection of Cr and Cd was performed by the method used by Parks et al. (2004) and Gavris et al. (2013) respectively.

Results and Discussion

Two standard curves corresponding to the concentration for Cr and Cd in the synthetic water sample have been shown in the Fig. 1a and b respectively.

In vitro plant cultures adsorbed significantly higher amount of metal ion as compared to soil-grown plants. Noticeable effect of in vitro culture and soil-grown plants on synthetic Cr and Cd removal with respect to time was observed (Table 3). Soil grown plants showed 55%–100% removal whereas in vitro plant cultures removed 86%–100% of Cr. Cd removal from in vitro plant cultures and soil grown plants was 54% and 55% respectivelyIncrease in the concentrations of metal ions adversely affects the adsorption. Maximum amount of Cr and Cd removal was noticed up to 120 min. Rate of removal was significantly higher up to 90 min, which reduced afterwards. Equilibrium was attained at 180 min for all the concentrations studied. According to statistical analysis (MLR) of synthetic Cr, percentage removal of Cr is highly correlated with time and concentration as R-Sq(adj) is 87.2% and 93.5% for in vitro plant cultures and soil-grown plants respectively. Significant differences in the removal of Cr using in vitro plant cultures and soil-grown plants could be justified by the value of F being greater than $F_{critical}$ (ANOVA). Further AC obtained from in vitro plant cultures removes higher amount of Cr as compared to soil-grown plants. For synthetic Cd, percentage removal is highly correlated with time and concentration as R-Sq(adj) is 92.9% and 92.2% for in vitro plant cultures and soil-grown plants respectively. Since $F < F_{critical}$, it can be concluded that the removal of synthetic Cd using in vitro and soil-grown plants is almost equal. Similar study was conducted by Singanan and Singanan (2007) using AC from *Tridax procumbens* to remove Cr (95%–97%) from the tannery water after 180 min of treatment. Absorption of hexavalent Cr by AC prepared from bamboo waste (*Oxytenanthera*

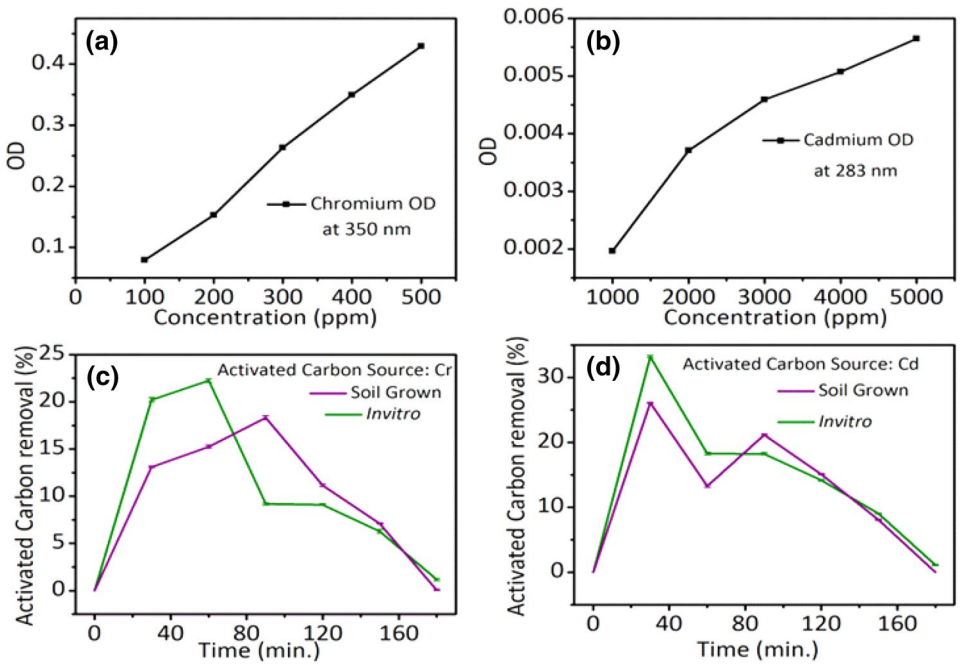

Fig. 1 Standard curve **a** Cr and **b** Cd. Comparison of rate of removal **c** Cr **d** Cd with in vitro culture and soil-grown plants in industrial wastewater

Table 3 Effect of in vitro culture and soil-grown plants on the removal of synthetic Cr and Cd with respect to time

Time (min)	Concentration of Cr and Cd (ppm)																			
	IV		SG		IV		SG		IV		SG		IV		SG		IV		SG	
	100	1000	100	1000	200	2000	200	2000	300	3000	300	3000	400	4000	400	4000	500	5000	500	5000
	Cr	Cd	Cr	Cd	Cr	Cd	Cr	Cd	Cr	Cd	Cr	Cd	Cr	Cd	Cr	Cd	Cr	Cd	Cr	Cd
0	100	1000	100	1000	200	2000	200	2000	300	3000	300	3000	400	4000	400	4000	500	5000	500	5000
30	70	300	90	400	80	1400	150	1200	130	2300	265	2200	200	3750	340	2900	250	4500	430	4100
60	30	200	65	100	60	1100	95	1050	85	1850	230	1650	145	2950	285	2400	210	3900	370	3800
90	10	50	30	50	20	600	65	500	50	1250	70	1400	100	2500	210	2000	130	3300	300	3150
120	0	0	0	0	0	400	40	200	18	930	163	1750	64	2060	200	1660	85	2800	250	2650
150	0	0	0	0	0	280	24	100	0	620	85	1120	40	1790	195	1460	70	2400	225	2300
180	0	0	0	0	0	280	16	100	0	590	59	1120	40	1770	195	1460	70	2300	225	2250

abyssinica) was reported by Dula et al. (2014). AC from eucalyptus seeds exhibited toxic metal ion (zinc) adsorption from the contaminated water (Senthil et al. 2016). Khoramnejadian and Saeb (2017) reported metal (Cr, Cd Cu and Ni) quenching ability of *Amaranthus retroflexus* from contaminated soil after 120 days of cultivation.

After attaining equilibrium at 180 min, metal ion was removed from industrial wastewater. However, in vitro culture rapidly removes toxic metals as compared to soil-grown plants. Effect of in vitro culture and soil-grown plants on industrial wastewater is shown in Table 4. According to statistical analysis, $F < F_{critical}$, it can be concluded that there was not much difference in the removal of Cr and Cd from industrial wastewater using in vitro and soil-grown plants.

Comparison of rate of removal of Cr and Cd with in vitro culture and soil-grown plants in industrial wastewater is shown in Fig. 1c and d respectively. Rate of reduction in metal ion concentration was higher with in vitro plant cultures as compared to soil-grown plants.

Many researchers have proved that in vitro cultures are reliable source for toxicity and tolerant studies (Mumma and Davidonis 1983; Azevedo et al. 2005). Singh et al. (2017) reported the potential of in vitro cultured *Vetiveria zizanoids* for Arsenic removal. In vitro cultures are also appropriate for applying inhibitors and chemical effectors as several metabolic inhibitors and agents helped in revealing this mechanism (Doran 2009). In vitro cultures are suitable to meet the maximum contaminant level (MCL) standard 0.005 mg/L (Agency for Toxic Substances and Disease 1999) and 0.1 mg/L (EPA 2008) for Cd and Cr respectively. In addition, in vitro plant cultures own the exact genomic information of their soil-grown parent plant and could be used in –omic research aimed at uptake of heavy metals like genomic, transcriptomic, proteomic and metabolic (Doran 2009). Application of this methodology need preliminary treatment, like suspended solid removal from polluted water but it can be applied for industrial wastewater treatment.

Present study concludes that in vitro plant cultures of *B. monnieri* are more competent over soil-grown plants for reclamation of polluted water containing Cr and Cd and for large-scale remediation process for the betterment of mankind, agriculture and environment. It has been proven statistically also that both reclaimed Cr and Cd from synthetic solutions as well as from industrial wastewater. However, rate of reduction was significantly higher with in vitro plant cultures as compared to soil-grown plants. In vitro plant

Table 4 Effect of in vitro culture and soil-grown plants on Cr and Cd concentration in industrial wastewater

Time (min)	Activated carbon source			
	In vitro grown plants against Cr	Soil-grown plants against Cr	In vitro grown plants against Cd	Soil-grown plants against Cd
0	225	225	1950	1950
30	180	195	1300	1450
60	130	160	950	1200
90	110	120	600	800
120	89	96	325	515
150	76	81	156	360
180	74	81	136	351

cultures offer microbial free environment with no translocation barrier to determine intrinsic capacity of plant cells for remediation of contaminants. Technical applicability like cost-effectiveness, easily manageable biomaterial, rapid and eco-friendly set up are the key factors that play an important role in the selection of in vitro cultures for detoxification of pollutants from the wastewater.

Acknowledgements The authors wish to express thanks to the Vice Chancellor of Delhi Technological University and Head of Department of Biotechnology for providing the facility and support to conduct the research work.

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Anticancer property of green material through computational approach

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Received: 31 March 2016, Revised: 30 September 2016 and Accepted: 18 April 2017

DOI: 10.5185/amp.2017/606
www.vbripress.com/amp

Abstracts

The purpose of research: Colorectal cancer is third most prevalent cancer in developed countries with increasing cases in developing countries. Studies show that the chronic intestinal inflammation is associated with increased risk of developing colorectal cancer. Inflammation is an immunological response to external damaging stimuli and is govern by an endogenous pyrogen and pleiotropic pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α). TNF-α plays an important role in the development of humoral immune response. Production of TNF-α has been implicated in various other pathologies including diabetes, osteoporosis, multiple sclerosis and inflammatory bowel diseases also. Several studies have shown that anti-inflammatory effect of stigmasterol, a phytosterol of an endangered medicinal plant *Chlorophytum borivilianum*, is mediated by suppression of TNF-α. The latter is synthesized as a membrane-anchored precursor. The soluble form of TNF-α is released into extracellular space by tumor necrosis factor alpha converting enzyme (TACE), a multidomain metalloproteinase.

Principal results: We have investigated the anti-cancer effect of the green material against colon cancer by using computational molecular docking and molecular dynamics simulations approach. With this study, we tried to explore stigmasterol as a potential inhibitor of TACE. The active cleft of TACE has a catalytic zinc residue at its centerpentacoordinate by the three-imidazole N_2 atoms of His 405, His 409, His 415. Along with these residues Glu 406, Met 345, Pro 437, Trp 312, Asp 344 around the zinc atom constitute the active site. The docked complex formed a hydrogen bond with Gly 346 and showing hydrophobic interactions with other active site residues. Molecular dynamics simulations confirmed that hydrogen-bond connectivity has not been lost throughout our simulations of 30ns. Computed RSMD suggests that docked complex is stable.

Major conclusions: Therapeutic blockade of TACE could be highly beneficial in case of chronic inflammatory conditions including colon cancer. In the present study, we conclude that stigmasterol, a green material may be considered as a possible therapeutic agent in the treatment of colon cancer and the same can certainly be confirmed through *in vivo* studies and clinical trials to confirm their effectiveness in patients.

Keywords: Colon cancer, molecular docking, stigmasterol, TACE, TNF-α.

Introduction

Colorectal cancer is one of the third commonly diagnosed cancers. This non-skin cancer is one of the major causes responsible for death in both men and women in developed countries. According to American Cancer Society, Cancer Facts & Figures (2014), there are 1,36,830 estimated new cases of colon cancer and 50,310 estimated deaths in both the sexes [**1**]. Colon is the part of the digestive system or gastrointestinal system. Most colorectal cancers originate from adenomatous polyp. Polyps are the growth that begins in the inner lining of the colon and moves toward the center. Most of the polyps are noncancerous. Only a few types of polyps can become cancer known as adenomas. Colon cancer grows slowly which takes 10-15 years [**2**]. Risk factors for this disease include physical inactivity, obesity, and high consumption of meat, smoking, and heavy alcohol intake. A family background of colon cancer or adenomatous polyps and personal history of inflammatory bowel disease are the other risk factors associated with colon cancer. Inflammation and tumorogenesis is a grievous combination, well supported by various researchers in last decade.

Inflammation is a potential source of sporadic and genetic colon cancer.

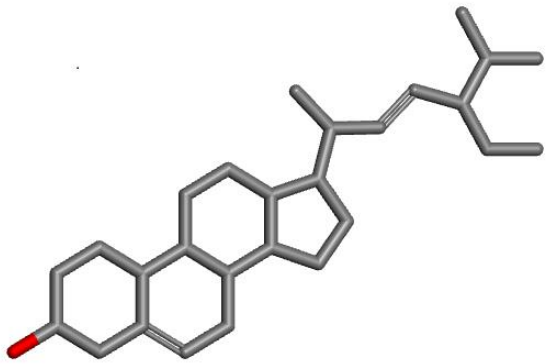

Fig. Structure of Stigmasterol.

Chronic inflammatory bowel disease ulcerative colitis is an important risk factor for the development of colon cancer [**3**]. Treatment of colon cancer with the oral drugs is possible only when diagnosed at the early stage of cancer development. Some FDA approved drugs for colon cancer are Avastin, Bevacizumab, Camptosar, Cetuximab, 5FU (Fluorouracil Injection). Surgery, radiotherapy, and chemotherapy are advised at the latter stage of colon cancer, which are associated with a reoccurrence of the disease and side effects like fatigue, constipation or diarrhea, a temporary or permanent colostomy, bladder irritation etc. Apart from the continuous development of new approaches to combat this baneful disease, not all the approved drugs can be prescribed for continuous long-term use due to drawing some adverse effects. Few advanced imaging techniques enabled the progression of treatment approach in diagnosis of hepatic metastatic disease and rectal cancer with the limitations like technical, economical and logistic challenges [**4**]. Besides the advancement of therapeutic strategies; treatment of colorectal cancer need to be more specific and effective [**5**]. On the contrary, medicinal plants or green material has a great potential to develop herbal drugs with very low or no adverse effects.

'Medicinal plants as Green material' is now becoming a major area of interest to the scientific society across the globe for drug development. Medicinal plants have the capacity to lower down the effects of cholesterol due to the presence of phytosterols. The most important phytosterols are sitosterol, campesterol, and stigmasterol. *Chlorophytum borivilianum*, 'Safed Musli', is one of the important medicinal plants having stigmasterol as major secondary metabolite with high medicinal value. *C. borivilianum*, also known as "the golden root", is an endangered herb that belongs to family 'Liliaceae'. Tuberous roots of this herb contain immunomodulatory and adaptogenic properties and are used to treat impotency, sterility and enhance male potency [**6**]. It is being used to cure physical illness and weakness, diabetes, arthritis, natal and postnatal problems, rheumatism and joint pains. This herb is an antimicrobial, anti-inflammatory and antitumor agent, also used to medicate diarrhea, dysentery, gonorrhea and leucorrhea. Medicinal properties and various biological activities viz. aphrodisiac, anti-oxidant, anti-cancer and immune booster are attributed by its major metabolite stigmasterol. Experimental studies suggest that due to having an anti-inflammatory effect, stigmasterol may offer protection from the most common cancers such as colon, breast, and prostate cancer. The chemopreventive effect of stigmasterol on *in vivo* cancer model was explored [**7**]. 5.34µg/mL stigmasterol was required to inhibit the activity of TNF-α [**8**]. TNF-α is a major immune-modulatory and proinflammatory cytokine that is synthesized as a membrane-anchored precursor. It supports the previous studies indicating the anti-inflammatory properties of stigmasterol, with low concentrations of tumor necrosis factor-alpha (TNF-α). Monocytes and macrophages are responsible for releasing TNF-α, involved in the host immune response [**9**]. Pro-inflammatory mediators such as TNF- α, IL-6, and nitric oxide are crucial for the uninterrupted functioning of immune system especially in the presence of any infection. On the contrary, it can be the reason for the development of tissue and organ injury when overproduced [**10**]. Increased concentration of TNF-α is responsible for the pathogenic process of infectious as well as autoimmune diseases [**11**]. TNF- α is the mediator of tumor-associated inflammation and tumorigenesis [**12**]. It was observed as an important mediator of initiation and advancement of colitis-associated colon carcinogenesis [**3**]. Colon inflammation was found to be responsible for the promotion colon TNF- α which is linked to increased chances of colon pathogenesis [**13**].

Various studies associate tumor necrosis factor alpha converting enzyme (TACE) with colon cancer [**9**]. TACE is the member of A disintegrin and a metalloproteinase-containing enzyme (ADME). It is responsible for producing the soluble form of TNF-α from its membrane-bound precursor i.e. pro TNF-α. An experimental study conducted on teleost proved TNFα-converting activity of TACE [**14**]. Various other studies proved the involvement of TACE in the suppression of colon cancer [**9**]. It is well known that high concentration of TNF-α is found in the cases of colon cancer. Inhibition of TNF-α production can suppress the signals that are responsible for releasing the mature TNF-α. Although a number of proteases have been shown to process pro-TNF-alpha, the most efficient are TNF-α converting enzyme (TACE). Since the enzyme plays an important role in converting TNF-α in soluble form, targeting the enzyme could be a potential therapeutic strategy counteracting the increase in TNF-α concentration,

which has been seen in many cases of colon cancer. The present study explored a novel therapeutic perspective of stigmasterol for colon cancer in which overexpression of TNF-α was suppressed by stigmasterol through inhibition of TACE. Molecular docking studies have been used to identify the binding modes. A Molecular Dynamics (MD) simulation was carried out for the docked complex to analyze its stability inside the bodily conditions by using Gromacs simulation suite.

Experimental

Materials

Protein and ligand preparation

The crystal structure of TACE [PDB: 1BKC] was retrieved from Protein Data Bank. Structure of ligand molecule stigmasterol (CID: 5280794) was obtained from NCBI – PubChem Compound Database.

Prediction of active site

Understanding the reaction of catalytic residues is necessary in order to understand the accurate function of an enzyme. Although active site information of TACE was partially reported [**15**] earlier, current study presents more details on co-crystallized structure with its inhibitor and further validation has been done by *in silico* analysis. A most probable active site with amino acid residue information was obtained from Q-site Finder web server.

Molecular docking

Molecular docking was performed with AutoDock4 [16]. AutoDock4 is a one of the widely-used docking tool having an efficiency of predicting rapid and exact bound confirmations of ligand and their potential targets. AutoDock docking models are often consistent with X-ray crystal structures [**17, 18**].

MD simulation in water

A Molecular Dynamics (MD) simulation was carried out for the docked complex to analyze its stability inside the bodily conditions by using Gromacs simulation suite [**19**].

Methods

AutoDock uses a grid-based method for quick evaluation of binding energy of test confirmations. AutoDock starts with preprocessing of ligand by removal of coordinates from the PDB file and water molecules. AutoDock allows the target to be embedded in the grid while the investigating atom is consecutively directed to each grid point. Interaction energy of investigating atom and target molecule is calculated and is secured in the grid, which is then referred during docking simulation. Lamarckian genetic algorithm is used for conformational searching [**16**]. AutoDock4 uses a semi empirical free energy force field for the prediction of binding free energies of ligand to macromolecular targets. Q-site Finder anticipates the information active site residues by using energy criteria and computes van der Waals interactions of methyl probe with the target molecule. The clusters of favorable energies are graded according to the total interaction energies and the cluster of maximum energy filled the first rank [**20**]. Polar hydrogen was added to the protein structure to provide accurate ionization. The ligand was attributed to gasteiger charges and rigid roots. 13 Rotatable bonds were adjusted. Key residues were embedded in energy-container grid of 60 Å × 60 Å × 60 Å (x, y, z). The best confirmation was obtained through default Lamarckian genetic algorithm as a search protocol. The output from AutoDock4 was analyzed with Viewerlite 5.0. Ligplot was generated using PDBsum determined the number and length of H- bonds between the ligand and the receptor. The application of MD simulations to study the protein dynamics is well described in many previous studies [**21, 22, 23, 24**]. Here, we performed MD simulations for 30ns and studied the stability of protein as well as the ligand as a complex.

Result and Discussion

Identification of Active Catalytic site in TACE

Q-site Finder generated ten clusters based on probe and protein interactions after submitting the pre-processed structure of TACE. Individual probe site associate preferentially to the best-suited binding sites on the protein surface and these are the positions where a tentative ligand could interact and optimize its van der Waals interaction energy [20]. The obtained results included nearly 27 residues. All these residues are covered in the grid while performing docking. The active cleft of TACE has a catalytic zinc residue at its center pentacoordinated by the three-imidazole N_2 atoms of His 405, His 409, His 415. Along with these residues Glu 406, Met 345, Pro 437, Trp 312, Asp 344 around the zinc atom constitute the active site. The docked complex was forming a hydrogen bond with Gly 346 and showing hydrophobic interactions with many of the active site residues. **Fig. 1(a)** depicts the stigmasterol and TACE complex after molecular docking while the **Fig. 1(b)** is the ligplot showing hydrogen and hydrophobic bonds between ligand and macromolecule TACE.

Molecular docking simulation

We have investigated molecular docking and MD simulations on stigmasterol and TACE complex for the stability in the bodily conditions. CPK model is shown in **Fig. 2**. Computed Root mean square deviation (RSMD) (**Fig. 3**) suggests that docked complex is stable. Root mean square deviation was calculated by least square fitting of CA from the initial structure and found to be stable around 2A

during simulations; suggesting that the docked complex is stable.

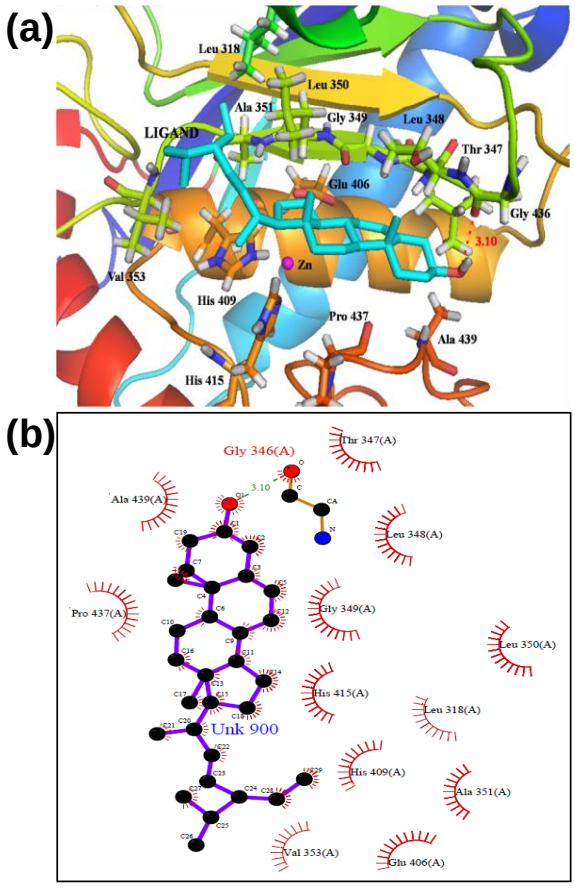

Fig. 1. (a)Stigmasterol and TACE Complex, (b) Ligplot showing hydrogen and hydrophobic bonds between ligand and macromolecule TACE.

Ligand and the active site residues in the receptor domain within 8A distance were considered for the interaction studies. A schematic of detailed ligand atom interactions with the protein residues before MD simulations is shown here (**Fig. 4**). The docked complex was forming a hydrogen bond with Gly 346 and within the hydrophobic interaction range with many of the active site residues. Hydrogen bond distance between ligand and receptor is shown in **Fig. 5** (**a**). Hydrogen bonding pattern between ligand and receptor is indicating the formation of strong hydrogen bond during simulations. Hydrogen bond existence map for various amino acids interacting with ligand is given in **Fig. 5** (**b**). Cyan implies the presence of a hydrogen bond and Red implies the absence of one. The *y*-coordinate shows the hydrogen-bond index.

In the present study, we examined the molecular mechanism of the stigmasterol on TACE that helps in understanding of the cancer cell characteristics. We observed that stigmasterol docked onto active site of TACE. The active site of TACE comprises zinc atom coordinated by a conserved zinc-binding domain (405-HexGHxxGxxH-415) [**15**]. It is also determined by the three-dimensional structure of TACE through Q-site Finder server. The ligand is interacting with key amino acids Leu 348 and Gly 349 present in the binding pocket of the enzyme [**25**].

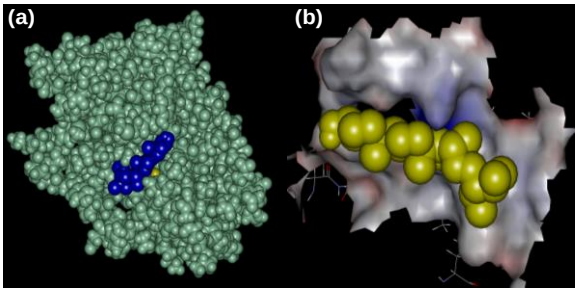

Fig. 2. complex1_tace rendered in cpk.

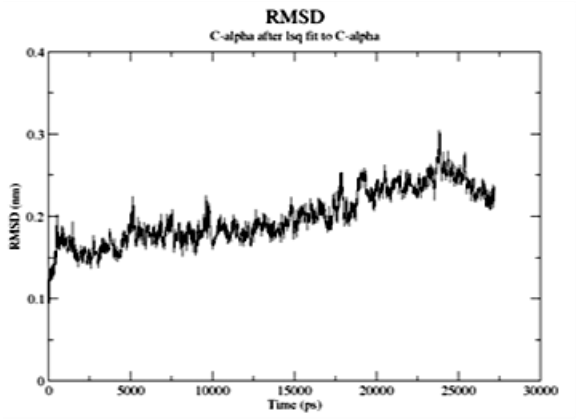

Fig. 3. Root mean square deviation.

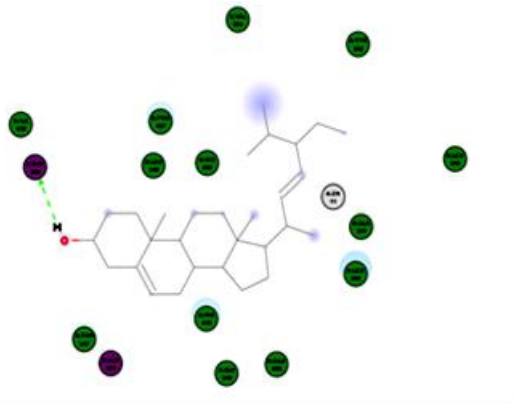

Fig. 4. A schematic representation of ligand interaction with the protein residues before performing MD simulations.

The binding pocket also co-occurred with the binding site of stigmasterol acquainted in the co-crystallized structure acquired from PDB. Molecular docking score of the 3D structure of stigmasterol with the above-described active site of TACE was -10.04 predicting good binding affinity of TACE with stigmasterol. Docked complex exhibited molecular interaction with glutamate and histidine residues that play a key function during the proteolytic reaction procedure. Short hairpin RNA silencing TACE (shTACE) prevented and efficiently treated acute and

chronic ulcerative colitis by decreasing TNF- α level [**26**]. The active site of TACE was blocked with highly selective new non-hydroxamate sulfonamide TACE inhibitors, considering as therapeutic targets for the treatment of TNF-dependent pathologies [**27**]. A quinazoline derivative therapeutically improved arthritis through suppressing production of TNF- α mediated by TACE [**28**]. Another study on TACE inhibition revealed TACE activation by non-receptor tyrosine kinase Src in mechanically stressed cardiomyocytes. This process could be harmful to specific blockade of TNF- α secretion, which in turn leads to congestive heart failure [**29**]. Considering above facts, the interaction of TACE and stigmasterol were anticipated to intervene with the interaction of substrate for the binding site of TACE, hence strengthening the idea of stigmasterol as TACE inhibitor.

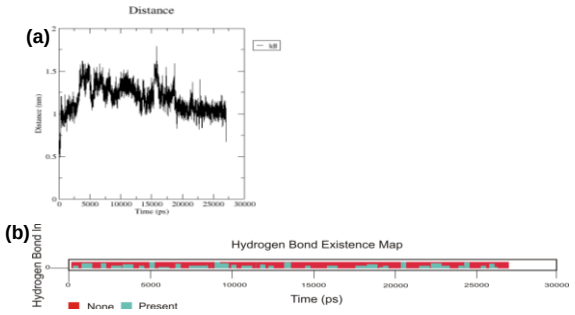

Fig. 5. (a): Hydrogen bond distance between ligand and receptor, (b) Hydrogen bond existence map.

Molecular dynamics simulation is a reliable and new way for the prevention of many diseases [**30**]. Molecular dynamics simulations are a widely used computer simulations technique to study the conformational dynamics of biological macromolecules such as proteins [**31**]. Molecular dynamics of the docked complex were examined to find out its stableness in bodily conditions. A simulation run time was 30ns, which was sufficient for the rearrangement of a ligand-bound protein molecule to acquire a stable binding manner. Root mean square deviation was calculated by least square fitting of CA from the initial structure and found to be stable around 2A during simulations; suggesting that the docked complex is stable. A structure showing the stationary phase was considered to study the molecular interaction pattern in the docked complex. H-bond, hydrophobic interaction, and van der Waals interaction were the factors that play an important role to make the stigmasterol and TACE a stable interaction. The dynamic stableness of stigmasterol during simulation runtime and its interaction with the important residues present at the binding site of the enzyme possibly consolidate the mode of action of stigmasterol on inhibition of TACE, which is responsible for the release of the soluble form of TNF-α.

Drug-likeliness

The successful evaluation has been done for drug-likeness based on Lipinski's "Rule of Five", while 46 computed physicochemical properties or molecular descriptors were used to predict ADMET (absorption, distribution, metabolism, elimination and toxicity) of the compound.

Conclusion

Molecular docking and simulation study revealed that presence of a ligand in the active site of TACE, interacting with the key residues consolidated the idea that stigmasterol is a potential inhibitor of TACE. Furthermore, this study is a stepping-stone in order understands the prevention mechanism of TNF-α, a high concentration of which is responsible for colon cancer. Hence, it can be used for further studies as a natural anti-cancer drug.

Acknowledgements

We would like to thank Vice Chancellor of Delhi Technological University, Head of Department of Department of Biotechnology and Institute of Biotechnology for providing the facility and support to conduct the research work.

Author's contributions

Conceived the plan: N.J., N.B., N.S.; Performed the experiments: N. J., N.S.; Data analysis: N.J., G.M., N.S., N.B; Wrote the paper: N.J., N.S., N.B. Authors have no competing financial interests.

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Cite this article
Jauhari N, Raina H, Soni G, Chadha N and Bharadvaja N (2018)
Mechanistic insights into the anticancer mode of action of an herbal drug. *Bioinspired, Biomimetic and Nanobiomaterials* **7(1)**: 20–26, https://doi.org/10.1680/jbibn.17.00003

Research Article
Paper 1700003
Received 18/01/2017; Accepted 31/05/2017
Published online 25/08/2017

Keywords: computational studies/ natural materials/protein

Bioinspired, Biomimetic and Nanobiomaterials

ice publishing

Mechanistic insights into the anticancer mode of action of an herbal drug

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Medicinal plants are a vast repository of natural compounds with therapeutic effects against various ailments. Bioactive compounds of these plants have shown to possess anticancer activities. Cancer is one of the fatal diseases causing premature deaths across the world. Two important metabolites, serpentine, a major secondary metabolite of *Rauwolfia serpentina*, and amarogentin, isolated from *Swertia chirata*, are found to possess anticancer properties. A comparable in silico analysis of the two anticancer agents serpentine and amarogentin has been done to evaluate their ability to inhibit two potential molecular targets for cancer, nuclear factor-κB (NF-κB) and cyclo-oxygenase-2 (COX-2). The least binding energies of amarogentin with NF-κB and COX-2 are –7·173 and –7·649, respectively, which are better than that of serpentine. The molecular simulation of amarogentin and serpentine suggests that amarogentin has better binding affinities with both cancer targets. Amarogentin is thermodynamically more stable with COX-2 than with NF-κB. Amarogentin is a potent anticancer agent as evidenced by the inhibition of COX-2. This finding would be beneficial to people with cancer.

1. Introduction

The pharmacological journey of treating many life-threatening diseases such as cancer is successful only when it is diagnosed at early phases. Most of the world today is widely affected by cancer, as it has become the leading cause of death today. People's ever-changing lifestyle has enormously contributed to the rising number of cancer cases in society.[1] Conventional treatments for cancer are associated with many side-effects, and in addition, they present inadequate anticancer activity.[2] This necessitates the discovery of more persuasive, less toxic and highly specific anticancer agents as a more feasible treatment option for cancer.[3] This is how the role of medicinal plants comes into play, as a healthy substitute for synthetic drugs. The exploitation of medicinal plants as a potential therapeutic alternative for cancer is already well known.[4,5] More than 3000 plants have been known to possess anticancer properties.[6] Further, the relevance of plant-based drugs for the treatment of cancer is rising at a rate of 10–40% globally.[5,7,8] Out of the available medications for cancer, approximately 60% are significantly attributed to plant sources.[9]

Two important metabolites, serpentine from *Rauwolfia serpentina*[10] and amarogentin from *Swertia chirata*,[11] are found to have potential anticancer properties.[12,13] The use of plant metabolites with anticancer properties to inhibit molecular targets for cancer that include proteins (transcription factors, inflammatory mediators, enzymes) or signaling pathways that regulate the mechanism of cell proliferation and related events is the preferred choice of cancer therapy.[14]

An important and age-old medicinal plant *S. chirata*, commonly known as 'Chirata', belongs to the family Gentianaceae and is utilized for the treatment of multifarious disorders.[15] This medicinally important family contains many plants famous for their bitter taste and renowned as conventional medicines for appetite improvement and fever. It has great importance in the Indian system of medicine (Ayurveda) as antipyretic, antihelminthic, antiperiodic and as laxative, and is being used for the cure of asthma and leucorrhoea. Pharmacological properties of this herb are still at experimental stage but are well known to possess a number of useful metabolites with anti-inflammatory and hepatoprotective action.[16,17] Crude extracts of *S. chirata* showed the presence of three main phytochemicals, namely, mangiferin, swertiamarin and amarogentin, and their derivatives, the presence of which was confirmed through high-performance liquid chromatography.[18] Amarogentin, the most acerbic substance, is a secoiridoid glycoside with a bitter taste even at a dilution of 1:58 000 000[19] and is proven to inhibit cell proliferation as well as induce apoptosis in epithelium during carcinogenesis. It has been explained that the extract of *S. chirayita* controls programmed cell death and, at the same time, suppresses cell proliferation at the target site.[12] 7,12-Dimethylbenz [*a*]anthracene (DMBA)-induced carcinogenesis model of mice was taken for cytotoxic study, and cytotoxicity test revealed IC50 value of amarogentin is 0·5 mg. The extract of *S. chirayita*, rich in amarogentin, downregulates cyclo-oxygenase-2 (COX-2) protein expression in DMBA-induced skin lesion, while amarogentin-induced caspase-3 maturation was noticed, which is responsible for the initiation of apoptosis.[12] A group of researchers delivered novel research on the chemopreventive role of amarogentin in liver

carcinogenesis by way of modulation of cell cycle and apoptosis on carbon tetrachloride (CCl_4)/*N*-nitrosodiethylamine-induced liver carcinogenesis mouse model system.[20] Amarogentin inhibits platelet activation by the suppression of phospholipase C (PLC) γ2, protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) pathway.[21] Thus, amarogentin possesses the capability to prevent or treat thromboembolic disorders as well as carcinogenic activities. Indian snakeroot (*Rauvolfia serpentine*), an important medicinal plant, belongs to the family Apocynaceae. The root extract of this plant contains alkaloid and is used to produce antihypertensive drugs worldwide.[22] The antitumor activity of *R. serpentine* in different mouse sarcomas in vivo was noticed.[23,24] A stable cell line F-Habp07 has been designed by overexpressing human hyaluronan binding protein 1 (Habp1) in murine fibroblasts, and its accumulation in the mitochondria is responsible for excess reactive oxygen species (ROS) generation without any external stimuli.[13] A reduction in ROS generation, inhibition of p65 subunit of nuclear factor-κB (NF-κB) nuclear translocation and enhancement in glutathione were noticed when experimented with serpentine. They revealed a novel non-cytotoxic antioxidant serpentine efficacy, which is found to be comparable with other well-known antioxidants.

The present scenario demands more efficient targeted therapies for cancer that are particularly aimed at cancerous cells or the processes involved in their metastasis to other parts of the body and survival. A number of molecular targets for cancer have been recently identified. One of the potent targets to inhibit various diseases is NF-κB, a ubiquitous transcription factor. NF-κB was first identified in 1986.[25] It is well known for its function in regulating cell-signaling responses. As the name indicates, it is a nuclear factor bound to an enhancer component of the immunoglobulin kappa light chain gene in B cells. NF-κB controls over 500 different gene products and is a known mediator of inflammation. It comprises a Rel homology domain (deoxyribonucleic acid (DNA)-binding domain/dimerization domain) on a nuclear localization sequence; these sequences are maintained from *Drosophila* to humans. It is also involved in the regulation of cell signaling responses. The abnormal activation and expression of NF-κB are responsible for various ailments, including cancers, diabetes mellitus, cardiovascular diseases, autoimmune diseases, viral replication, septic shock, neurodegenerative disorders, ataxia telangiectasia, arthritis, asthma, inflammatory bowel disease and several other inflammatory conditions. The regulation of cellular proliferation and differentiation, immune system response and the process of programmed cell death or apoptosis are widely attributed to this transcription factor. Therefore, targeted therapies aimed at inhibiting NF-κB-associated signaling holds a great potential to combat numerous ailments.[26] A total of five genes, NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), c-Rel and RelB, constitute the assembly of NF-κB. The NF-κB proteins are classified into two categories.[27] The ones that are synthesized in their mature form and the others in their precursor form. In the normal state, the NF-κB dimer remains inactively present in the cytoplasm due to their interaction with NF-κB inhibitors. In response to a variety of cytokines, growth factors (epidermal growth factor receptor and insulin growth factor receptor) and tyrosine kinases, NF-κB is activated. In addition, signaling pathways, such as Ras/MAPK and PI3K/Akt, are responsible for the activation of NF-κB. The induced NF-κB activation is a highly regulated process. However, the molecular alterations of cancer cells may cause impaired NF-κB activation. The unregulated NF-κB activation may lead to the upregulation or downregulation of genes involved in the regulation of apoptosis, cell cycle control, adhesion, or migration. These altered processes play a major task in the development and progression of cancer and clearly suggest the role of NF-κB in cancer.[28] Various experimental studies proved NF-κB as a potential target against many ailments. Inhibition in tumor formation and growth of natural killer/T-cell lymphoma cell lines induced by the Epstein–Barr virus through the NF-κB and PI3K/Akt pathways was explored.[29] Paris saponin II (PSII), an active component of the Chinese herbal medicine *Rhizoma paridis*, reduces angiogenesis and the development of human ovarian cancer mouse model by suppressing NF-κB signaling.[30] Thus, the inhibition of NF-κB signaling pathway can reduce the menace of many chronic ailments.

Another potent molecular target for cancer therapy is COX-2, which is also formally called prostaglandin H2 synthase-2. This enzyme, with two known isoforms COX-2 and COX-1, has a well-established role in the formation of prostanoids from arachidonic acid. The two isoforms are distinct in the manner that COX-1 is native to most types of cells and is a housekeeping enzyme with almost constant amounts under either physiological or pathological condition. On the other hand, COX-2 is rapidly present in diseased situations and often absent in normal state. The induction of COX-2 has been first done by a viral oncogene[31] or by a tumor promoter.[32] Experimental studies have also justified its role in a variety of growth factors and mitogens for the induction of COX-2.[33,34] Thus, COX-2 has a strong relevance to cellular proliferation and cancer.[35] The development of COX-2 inhibitors can be the potential therapeutic approach for cancer. The prevention of colon cancer with the use of COX-2 has already been reported in several animal models.[36] As the mechanism of action of anti-inflammatory agents is well studied in clinical research, there is a strong background to develop potent COX-2 inhibitors for human trials.[37] A team of researchers have examined four genes, RassF1A, APC, Rar-beta and prostaglandin-endoperoxide synthase 2 (PTGS2), in placental samples and compared them with maternal white blood cells. PTGS2 was found to be hypomethylated in the placentas compared to the maternal cells, which has an important clinical implication to, for example, placental abnormalities.[38] Normal mammary gland lymphangiogenesis, mammary tumor-associated lymphangiogenesis, tumor cell invasion into lymphatics and metastasis were found to be reduced in rodent models of postpartum breast cancer through COX-2 inhibition during the involution window.[39] The involvement of genotypic

polymorphisms in COX-2 to increase breast cancer risk in Taiwanese females was also examined.[40] Their findings implied that the C allele of G-765C, polymorphic variants of COX-2, was linked with a reduced risk of breast cancer and could be considered as an early detection and prognostic marker for breast cancer. Liver cancer is one of the most common malignancies and the leading cause of death worldwide. Hepatocellular carcinoma, liver cirrhosis, hepatitis and several liver diseases can be the result of the invasion and migration of hepatitis C virus (HCV). The invasion of HCV is enhanced by HCV non-structural protein 3, while the matrix metalloproteinase-9 and COX-2 are responsible for the migration of HCV.[41] Therefore, COX-2 is an experimentally proven target for many diseases, including cancer.

In the present study, a comparable analysis of two anticancer agents, serpentine, a major secondary metabolite of *R. serpentine*, and amarogentin, isolated from *S. chirata*, has been explored for their ability to inhibit potential molecular targets for cancer, NF-κB, and COX-2. In silico approach comprising molecular modeling, docking and simulation strategy is hereby analyzed to find the significant outcome in support of the proposed idea of NF-κB activation suppression and COX-2 inhibition by plant secondary metabolites that possess anticancer properties.

2. Results and discussion

2.1 Homology modeling

The protein sequence of COX-2 (604 AA, UniProtKB accession number: P35354) was retrieved from Uniprot. After using the Basic Local Alignment Search Tool (Blast), the structure of COX-2 from *Mus musculus* (PDB ID: 1PXX) was selected as best template based on their sequence percentage identity.

The automated homology modeling software Prime, version 3.1 (Schrödinger, LLC, New York, 2012 (license: Institute of Nuclear Medicine and Allied Sciences, INMAS)) on Linux operating environment was used to check the three-dimensional (3D) model of the enzyme.

2.2 Molecular docking

The results in Table 1 indicate the docking score, which clearly suggests that amarogentin could be a potential inhibitor of both COX-2 and NF-κB compared to serpentine. The two-dimensional representation of this best-fit receptor–ligand interaction is shown in Figure 1, which clearly illustrates the participation of interacting amino acid residues.

The residues shown in Figure 1 are present in the binding cavity of COX-2 and directly participate in several bond formations with amarogentin. These are present in the ligand binding site responsible for the function of protein.

In the process of molecular docking, the docking score is used to predict the strength of the binding affinity of the two docked

Table 1. Docking results ranked based on XP Glide score

Ligand/protein	COX-2	NF-κB
Amarogentin	–7·649	–7·173
Serpentine	–4·064	–4·687

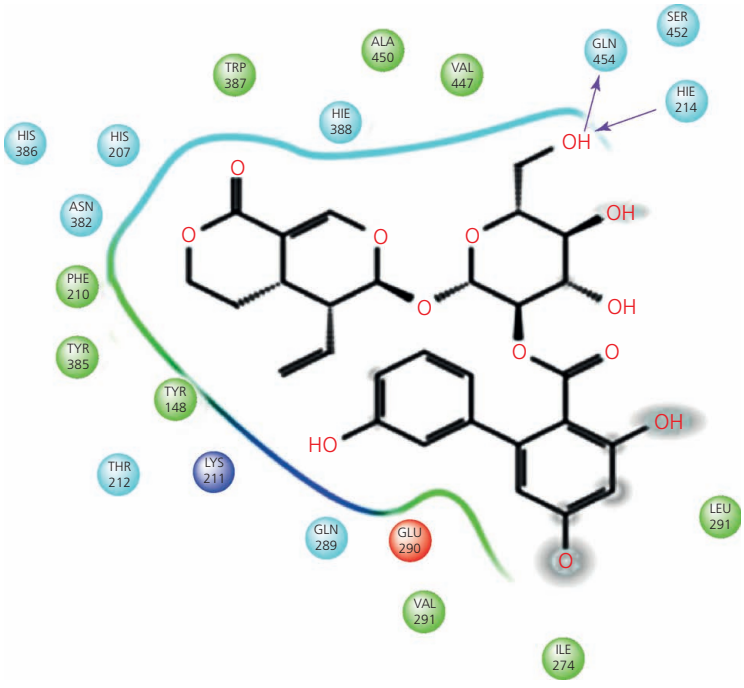

Figure 1. Two-dimensional representation of the docked complex

molecules, which is necessary for further study of the establishment of the probable anticancer drug. The binding score represents better protein–ligand binding interaction. In the present study, serpentine and amarogentin are the small organic compounds (probable drug) which are docked to target protein NF-κB and COX-2. Abnormal activation and expression of NF-κB are responsible for various ailments, including cancers. The regulation of cellular proliferation and differentiation, immune system response and process of programmed cell death or apoptosis are widely attributed to this transcription factor. Therefore, targeted therapies aimed at inhibiting NF-κB-associated signaling hold a great potential to combat numerous ailments like cancer. On the other hand, COX-2 is rapidly present in diseased situations and often absent in normal state. Experimental studies have also justified its role in a variety of growth factors and mitogens for the induction of COX-2. Thus, COX-2 has a strong relevance to cellular proliferation and cancer. The development of NF-κB and COX-2 inhibitors can be the potential therapeutic approach for cancer. The two anticancer agents serpentine and amarogentin are docked to target protein – that is, NF-κB and COX-2 with a good binding score represent them as the probable anticancer drug.

2.3 Molecular dynamic simulation

The molecular dynamic (MD) simulation result file was analyzed using the analysis options in the Desmond Molecular Dynamics System, version 3.1 (D. E. Shaw Research, New York, 2012; Schrödinger (license: Inmas)) program. The pressure value widely fluctuated over the course of the 1000 ps simulation run, but this behavior is not unexpected. The running average of these data shows that the average value of the pressure is 1·05 bar and the case is the same with the volume (Figure 2).

In order to analyze MD simulation, the calculation of root-mean-square deviation (RMSD) for the trajectories of COX-2 docked with amarogentin was correspondingly done using the initial complex structure (zero frames of the trajectory) as a reference. Figure 3 clearly illustrates that the complex has attained stability as the RMSD is less than 2·5 Å for the entire simulation span. The duration of simulation taken in the study permits rearrangement of side-chains of the drug–protein complex to find their most stable binding modes. To conclude the result of this simulation, the study proposes amarogentin as a probable and a valuable natural ligand for cancer target COX-2.

The role of NF-κB is prominent in the activation of proinflammatory genes, including cytokines, chemokines and adhesion molecules and could be a potent therapeutic target for anti-inflammatory drugs. Various experimental studies proved NF-κB and COX-2 as the potential targets of many disorders. The inhibition of NF-κB and COX-2 by *Operculina turpathum* extract to suppress oral squamous cell carcinoma cell lines was explored.[42] NF-κB acts as the inducer for large B-cell lymphomas, which can be blocked by inhibiting NF-κB.[43] Another study revealed that NF-κB regulates a set of genes that increases murine fibroblasts.[44] An

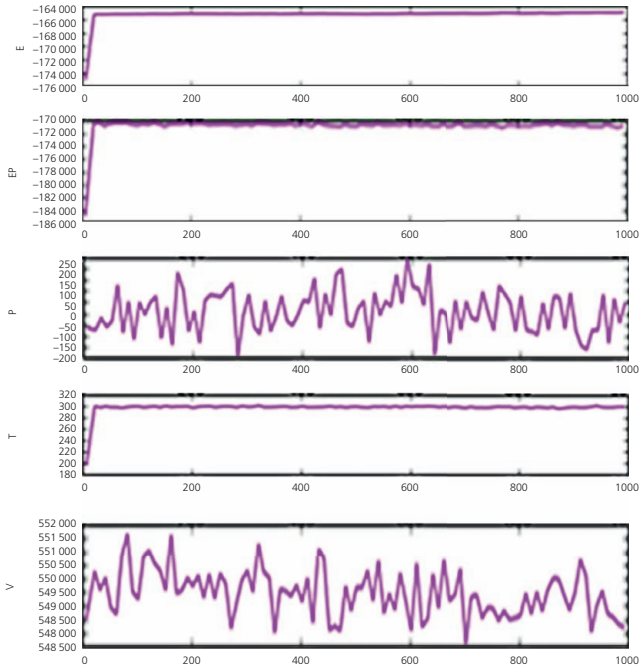

Figure 2. Simulation quality analysis

enormous amount of data exhibits the involvement of transcription factor NF-κB in the progression of cancer, which advocates NF-κB as a potent therapeutic target. Current studies revealed that COX-2 has been found to be involved in apoptosis resistance, angiogenesis and tumor progression, and inhibitors exclusively used for COX-2 can prevent the progression of cancer.[45] The anticancer potential of amarogentin was revealed by in vivo inhibition of cancer cell proliferation in mice xenograft model, and amarogentin was shown to cause apoptosis of cancer cells.[46] Amarogentin-rich purified fraction was observed to suppress cell proliferation and cause apoptosis at the target site.[47] Another experimental study proved that amarogentin-rich methanolic extract of *S. chirata* suppressed COX-2 protein expression in vivo, which leads to the pathway through which amarogentin controls the carcinogen-induced abnormally high rate of proliferation of cells.[12] The similar study proved a reduction in skin carcinogenesis in a mouse model by the downregulation of COX-2 expression through amarogentin. Further, in the same study, amarogentin–COX-2 complex was found to be more stable compared to the amarogentin–COX-1 complex in molecular dynamics simulation.[48] COX-2 is responsible for the conversion of arachidonic acid (AA) to prostaglandins by way of two-step catalytic mechanism. The process begins with the oxidization of AA to intermediate prostaglandin, hydroperoxy endoperoxide (PGG2), known as the cyclo-oxygenation step, and later, PGG2 is reduced into hydroxyl endoperoxide (PGH2), which has a vital role as the precursor for several prostanoids, identified as peroxidation step.[49] The catalytic domain is one of the three functional domains of COX-2, which possesses the active site for ligand binding.[50] Amarogentin specifically binds at the catalytic site inside the hydrophobic channel. The analysis of two ligand–protein

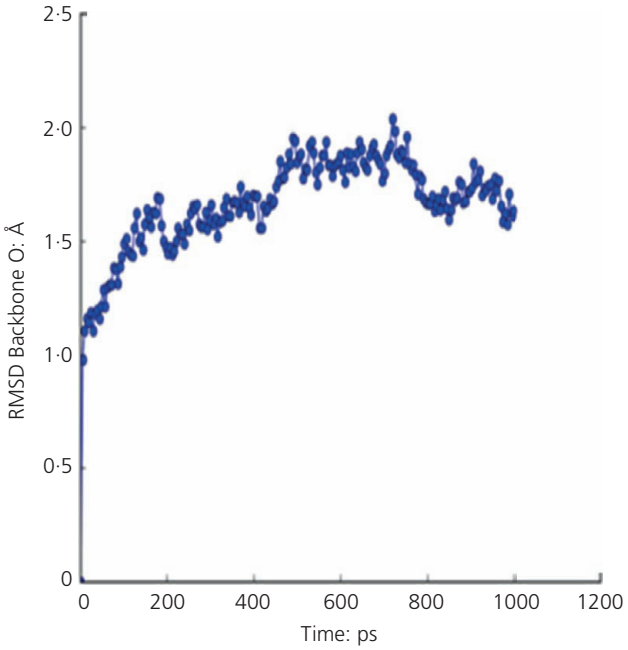

Figure 3. Plot showing RMSD of the backbone of COX-2/ Amarogentin (complex)

complexes revealed that both interactions are reasonably stable, with amarogentin proving more well fixed inside the binding cavity of COX-2. Therefore, the complex was subjected to MD simulation analysis to evaluate the stability of the complex in bodily conditions. The enzyme COX-2 significantly encourages tissue growth by inhibiting apoptosis, prominently at the tissue damage repair site,[51] and increases DNA synthesis mechanism by enhancing cell proliferation and cell mobility.[52] It can also be induced in the different tissues of the body in the inflammatory condition and tumorigenic signals. Amarogentin is well studied for its anticancer properties, suppression of cell proliferation and induction of apoptosis at the target site. In the present study, molecular docking complex showed the participation of interacting amino acid, which is a response to good docking score. Hydrophobic and hydrogen-bond interactions strengthen the amarogentin–COX-2 complex. To explore the behavior of amarogentin–COX-2 in close vicinity, MD simulation was performed. The analysis of MD, like RMSD, remained stable throughout the MD run, confirming the stable binding of the complex. The docked complex of the ligand target and the protein provides a detailed picture of the key binding site, and thus, it gives prospects to explore further the interplay of the binding site residues at the molecular level and will help in the in vitro exploration of the mechanism involved in natural anticancer agents. The overall stability of the simulated complex of COX-2 and amarogentin can help explore the observed results to further levels of study and investigation. Cancer is a well-known life-threatening disease, prevalent in society nowadays. Natural agents from medicinal plants have been proven to have great potential in cancer therapeutics. Computational analysis of these agents can provide a rational approach to discover naturally occurring potent antitumor agents. In the present study, molecular modeling, docking and dynamic simulation studies were used as the basic approach in evaluating the comparative inhibition capability of two potential anticancer agents against cancer targets. Docking studies have been done to compare the binding of serpentine and amarogentin with both the potent targets NF-κB and COX-2. The least binding energies of COX-2 with amarogentin and serpentine are −7·649 and −4·064, respectively, while the values are −7·173 and −4·687, respectively, in the case of NF-κB with both ligands. These energy values show that amarogentin and COX-2 have a better binding affinity than other docked complexes. Amino acid residues Gln, Van, Ala, Trp, His, Asp, Phe, Tyr, Thr, Lys and Glu are present in the binding cavity of the COX-2, and docking studies explored their bond formation with amarogentin. These results support the hypothesis that amarogentin has the potential to disrupt the activity of COX-2, being accounted by hydrophobic and hydrogen (H)-bond interactions. This method of computational analysis has thus provided a rational approach to propose that *S. chirata*-derived amarogentin can serve as a potent anticancer agent through its ability to mediate the inhibition of cancer targets like COX-2 and NF-κB. RMSD of COX-2 and amarogentin complex has attained a stationary phase during the later stage of the simulation and that is always 2·5 Å for the entire simulation length, suggesting the stability of the complex. The simulation length applied in the complete study was long enough to permit the rearrangement of side-chains of the indigenous as well as the drug-complexed protein to get their best stable binding mode. In conclusion, the present MD simulations made the dynamic structural stability of COX-2 with the drug amarogentin clear, together with the inhibitory mechanism. Thus, the result of the study proposes that amarogentin is a better potential natural anticancer agent compared to serpentine and can be explored further as a potent inhibitor of other molecular targets of cancer as well.

3. Experimental

3.1 Target structure prediction

Three-dimensional structure of the p65 subunit of NF-κB (PDB ID: 1NFI) was available in protein data bank (PDB). The 3D structure of COX-2 was generated by the homology modeling method using Prime, version 3.1 (Schrödinger, LLC, New York, 2012 (license: Inmas)). A Blastp search was made against the PDB database to identify the maximum matching template for modeling. The best model was validated the Structural Analysis and Verification Server and Verify_3D.[53]

3.2 Ligand preparation

Three-dimensional coordinates of amarogentin (CID: 115149) and serpentine (CID: 73391) were retrieved in .sdf format from the PubChem database of the National Center for Biotechnology Information. These .sdf files were converted to .pdb format by the Open Babel tool. Ligand coordinates were prepared and processed using Ligprep, version 2.5 (Schrödinger, LLC, New York, 2012 (license: Inmas)) module.

3.3 Active site prediction

Estimated binding pockets of both receptors were defined using Sitemap, version 2.6 (Schrödinger, LLC, New York, 2012 (license Inmas)) application of Maestro 9.3.5 (license: Inmas).

3.4 Molecular docking

The receptor structures were prepared and minimized within the protein preparation wizard of Maestro 9.3.5 (license: Inmas). Grids for each site were generated. The docking study of modeled COX-2 and 1NFI was done using Glide, version 5.8 (Schrödinger, LLC, New York, 2012 (license: Inmas)).[54] Docking calculations were performed using the XP (Extra Precision Mode) scoring function and with the OPLS 2005 force field.

3.5 Molecular dynamic simulation

The MD simulation study of the best-docked complex was done with the help of Desmond Molecular Dynamics System, version 3.1 (D. E. Shaw Research, New York, 2012; Schrödinger (license: Inmas)) as implemented in the Schrödinger package. TIP3 model was used to describe the water molecule. The final system was composed of approximately 50 000 atoms. For 1 ns of the production runs, a time step of 5 ps with NPT (Constant Number, Pressure, Temperature) ensemble at 310 K was used to simulate the docked complex.

4. Conclusions

COX-2 is one of the most striking topics in modern biological, biochemical and pharmacological research, and in the recent years, numerous studies shedding light on its inhibition/regulation have increased manifold. Natural ligands are the center of interest in this context due to negligible harmful effects. A comparative computational analysis on amarogentin and serpentine has been done with the two potent anticancer targets COX-2 and NF-κB. The result of the study proposes that amarogentin is a better potential natural anticancer agent compared to serpentine. In silico analysis offered a rationalization of the power of naturally occurring amarogentin to inhibit COX-2. The prominent value of the binding energy involved in the binding of amarogentin to COX-2 consolidates the thermodynamic stability of the binding. Conclusively, the results strongly suggest that amarogentin is a potent anticancer agent as evidenced by the inhibition of COX-2.

Acknowledgements

The authors would like to thank the vice chancellor of Delhi Technological University and the head of the Department of Biotechnology and Institute of Nuclear Medicine and Allied Sciences, Delhi, for providing the facility and support to conduct the research work.

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TÜBİTAK

Turkish Journal of Botany

http://journals.tubitak.gov.tr/botany/

Review Article

Turk J Bot
(2014) 38: 1027-1035

doi:10.3906/bot-1405-93

Phytochemical importance of medicinal plants as potential sources of anticancer agents

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Received: 30.05.2014 • **Accepted:** 16.10.2014 • **Published Online:** 17.11.2014 • **Printed:** 28.11.2014

Abstract: The diverse and magnificent plant kingdom of the world is widely known for its medicinal importance. The potential medicinal properties of plant species have contributed significantly in the development of various herbal therapies for a number of diseases across the globe. The benefits of herbal medicine over allopathic medicine have helped medicinal plants to regain their importance in the field of health and medicine. Cancer is one of the major health problems that have widely affected the world's population. There is a great need to combat this disease with better and more effective medication as compared to existing therapies. A vast number of medicinal plants are known to have biochemical constituents with anticancer properties. The chemical metabolites of natural origin that possess anticancer properties can serve as potential lead compounds in drug designing. This association of medicinal plants and cancer needs further research and experimentation in order to develop and design anticancer drugs. The present review is an effort to compile information on some of the geographically diverse and important medicinal plants that possess anticancer activity.

Key words: Biochemical constituent, anticancer properties, medicinal plants, drug designing, antiinflammatory, antiviral, antitumor, antimalarial, analgesic

1. Introduction

Medicinal plants are considered a repository of numerous types of bioactive compounds possessing varied therapeutic properties. The therapeutic potential of plants has been well explored over a very long time period. The vast array of therapeutic effects associated with medicinal plants includes antiinflammatory, antiviral, antitumor, antimalarial, and analgesic. Cancer is one of the major obstacles to human health around the world. Among all epidemic diseases, cancer holds the first place as a death-causing disease. The main reason behind the growing number of cancer cases is the changing lifestyle of the population across the globe. Keeping in view the statistical data, the most prevalent cancer among females is breast cancer, accounting for about 23% of total cancer cases; in males, the most prevalent is lung cancer, which accounts for 17% of total cancer cases (Jemal et al., 2011). Poor survival rate of cancer patients in developing countries is attributed to the lack of timely diagnosis and limited treatment facilities. There is a great need to address this epidemic disease with more effective therapeutic and preventive strategies, which could be possible with the use of natural compounds.

Recently the scientific world has experienced an upsurge of interest in the therapeutic potential of medicinal plants as a source of promising anticancer agents. However, the application of plant-based compounds for the treatment of cancer can be traced back to 1950s. Some of the very first anticancer agents derived from plants are vinca alkaloids, vinblastine, vincristine, and cytotoxic podophyllotoxins. Statistical data suggest that 16 plant-derived anticancer drugs have been subjected to clinical trials thus far (Belayachi et al., 2013). Landmarks of these clinical trials are flavopiridol, isolated from the Indian tree *Dysoxylum binectariferum*, and meisoindigo, isolated from the Chinese plant *Indigofera tinctoria*, which have been documented to have less toxicity than conventional chemotherapeutic anticancer drugs (Saklani and Kutty, 2008). These discoveries have propelled the scientific interest of various research groups in the discovery of new anticancer agents from all-natural product sources, inclusive of plant secondary metabolites. The emerging importance of natural anticancer agents demands more research and experimentation in order to develop successful natural therapeutic options for this disease. This review focuses on the phytochemical aspect of some of the potential anticancer medicinal plants with data gathered from the scientific literature of the PubMed database. Thus, the present review aims to assemble information on some of the medicinal plants that possess anticancer properties and thus great potential for cancer treatment.

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2. Medicinal plants as the best choice for cancer treatment

The chemical components of medicinal plants mainly possess antioxidant properties that contribute to their anticancer potential. Flavones, isoflavones, flavonoids, anthocyanins, coumarins, lignans, catechins, and isocatechins are the major classes of bioactive constituents responsible for the antioxidant action (Nema et al., 2013). The great potential of plant-based compounds for the treatment and prevention of cancer is attributed to their safety, low cost, and oral bioavailability. However, a few plant-based compounds induce some side effects. These side effects can be overcome by dose-dependent administration and usage, and do not in any case make them unsuitable for phytochemical research. The already available expensive conventional therapies for cancer like chemotherapy and radiotherapy have a number of side effects such as myelosuppression and neurological, cardiac, pulmonary, and renal toxicity, which pose serious harm to the quality of life (Alonso-Castro et al., 2011). Therefore, there is a need to develop treatment options that include more potent and less toxic anticancer drugs as compared to existing drugs. The market statistics mark the availability of approximately 60% plant-based anticancer drugs (Gordaliza, 2007). Medicinal plants constitute a common alternative to cancer treatment in many countries of the world (Gerson-Cwilich et al., 2006; Tascilar et al., 2006). Cytotoxic screening of a number of plants has been done to correlate their anticancer activity and further expand their scope for drug development (Akter et al., 2014). Owing to potential benefits of plant-based drugs for cancer treatment, their use is increasingly growing from 10% to 40% across the globe; specifically, on the Asian continent, it has reached 50% (Cassileth and Deng, 2004; Molassiotis et al., 2006). Anticancer benefits associated with natural plant derivatives demand extensive scientific screening and clinical experimentations for the development of improved drugs.

3. Medicinal plants with anticancer properties

3.1. *Actaea racemosa* L.

Actaea racemosa belongs to the family Ranunculaceae and has its origin in eastern North America. Its common names are 'black cohosh' and 'black snakeroot'. The main characteristic chemical compounds present in this plant are cycloartenol-type triterpenoids, cimicifugoside, and cinnamic acid derivatives. The plant is well known for its use in conditions such as chronic ovaritis and amenorrhea (Mahady et al., 2002). An active metabolite of this plant, actein has been shown to inhibit the proliferation of human breast cancer cells and human liver cancer cells (HepG2) and thus possess antitumor activity. Actein alters the expression of cholesterol and fatty acid biosynthetic genes, p53 pathway genes, CCND1, and ID3. Actein-induced inhibition of growth of human HepG2 liver cancer cells is because of the reduced free fatty acid and cholesterol levels in the liver (Einbonda et al., 2009).

3.2. *Allium sativum* L.

Allium sativum is commonly known as garlic and belongs to the family Liliaceae/Alliaceae. It contains sulfur compounds, numerous enzymes, 17 amino acids, and minerals like selenium. Garlic contains much bioavailable selenium, which is an antioxidant and could be chemopreventive (Ip and Lisk, 1996). It is used in the treatment of earaches, deafness, leprosy, severe diarrhea, fever, and stomachaches. One of its chief therapeutic effects is to treat cardiovascular diseases by lowering blood pressure as well as cholesterol. It also acts as an antimicrobial and chemopreventive agent. S-Allylmercaptocysteine is the most important antitumor constituent of aged garlic extract. The antiproliferative effect of thioallyl compounds has been studied in a number of cell lines and the results have shown sensitivity of these compounds to breast and prostate cell lines (Sigounas et al., 1997).

3.3. *Andrographis paniculata* Wall. ex Nees

Andrographis paniculata is a herbaceous plant that belongs to the family Acanthaceae. It is extensively grown on the Asian continent. It is commonly known by the names 'king of bitters' and 'creat'. Four lactones, chuanxinlian A (deoxyandrographolide), B (andrographolide), C (neoandrographolide), and D (4-deoxy-11,12-didehydroandrographolide), constitute its important secondary metabolites. The plant possesses antibacterial, antifungal, antiviral, choleretic, hypoglycemic, hypocholesterolemic, and adaptogenic effects (Bhatnagar et al., 1961). Its blood-purifying property makes it a choice for the treatment of a number of disease conditions such as boils, skin eruptions, chronic undetermined fevers, and scabies. The plant also contains an anticancer agent called andrographolide, which is an antiinflammatory diterpenoid lactone that inhibits interleukin-6 (IL-6) expression, suppresses IL-6-mediated signals, and induces cell apoptosis by the activation of apoptosis-related proteins/mitogen-activated protein kinases, and thus plays an important role as a cytotoxic agent in the case of liver cancer (Ji et al., 2007).

3.4. *Ardisia crenata* Roxb.

Ardisia crenata is a member of the family Myrsinaceae, which is mostly found in the warm climates of tropical and subtropical regions. This plant is also known by the names 'coral bush', 'coralberry', 'hen's eyes', 'spiceberry', and 'red berries'. Numerous chemical constituents that have been extracted and characterized from this plant include cyclic depsipeptide, peptide, alkenylphenol, and triterpenoid saponins (Horgen et al., 1997). It has been

widely used as traditional medicine to cure diseases such as pulmonary tuberculosis, hepatitis, chronic bronchitis, and irregular menstruation. Its cytotoxic effect is attributed to ardisiacrispin, which is a mixture of 2 triterpenoid saponins, ardisiacrispins A and B. Experimental studies have shown that the mixture of triterpenoid saponins inhibits the uncontrolled proliferation of the Bel-7402 liver cancer cell line by inducing proapoptotic and microtubule disruptive activities (Li et al., 2008). Microtubules play an important role in mitosis, and so targeting them yields antiproliferative effects in cancer cells.

3.5. *Boswellia serrata* Roxb.

Boswellia serrata belongs to the family Burseraceae and is extensively found in India, North Africa, and the Middle East. Its common names include 'Indian olibanum tree', 'olibanum', 'luban', and 'gond'. It contains an array of chemical constituents like oils, terpenoids, sugars, and volatile oils. B-Boswellic acid is the major constituent among 4 pentacyclic triterpene acids of this plant (Krieglstein et. al., 2001). The plant's gummy exudates are associated with therapeutic effects, including astringent, antiarthritic, expectorant, stimulant, and antiseptic effects. Acetyl-11-keto-β-boswellic acid (AKBA), an active component from this medicinal plant, has the ability to strongly inhibit tumor angiogenesis induced through vascular endothelial growth factor (VEGF) signaling. It also inhibits multiple steps of VEGF-induced cell proliferation, migration, invasion, and tube formation. Studies have shown that AKBA suppressed tumor growth in human prostate tumor xenograft mice treated daily with a dose of 10 mg/kg after solid tumors reached ~100 mm^3 (n = 5). Thus, the compound AKBA is antitumorous in nature (Pang et al., 2009).

3.6. *Catharanthus roseus* (L.) G.Don

Catharanthus roseus belongs to the family Apocynaceae and is commonly known as Madagascar periwinkle. It is originally native to the island of Madagascar. Alkaloids, its main chemical constituents, are employed in the treatment of circulatory diseases, and specifically in the relief of obstruction of normal cerebral blood flow. The plant also possesses medicinal activities like astringent, diuretic, and antidiabetic effects and can serve as a cold remedy to ease lung congestion and inflammation. Two pharmacologically active alkaloids, vinblastine and vincristine, are well known for their significant curative effects against human neoplasms. Vinblastine sulfate (sold as Velban) is also employed in therapy for lymphosarcoma, choriocarcinoma, neuroblastoma, and carcinoma of the breasts, lungs and other organs in acute and chronic leukemia. Vincristine sulfate (sold as Oncovin) is known to arrest mitosis and is thus used as a treatment for acute leukemia in children and lymphocytic leukemia. Vincristine sulfate is also used as therapy for Hodgkin disease, Wilkins tumor, neuroblastoma, and reticulum cell sarcoma (Noble, 1990).

3.7. *Centella asiatica* L.

Centella asiatica is a small herbaceous perennial plant and a member of the family Apiaceae. Its common names are 'Asiatic pennywort' and 'gotu kola'. The plant is native to India, China, Indonesia, Australia, the South Pacific, Madagascar, and South and Central Africa. Phytochemical studies have shown the presence of glycoside asiaticoside and asiatic and madecassic acid. Its medicinal importance in chronic diseases has already been mentioned in the Ayurvedic system of medicine, where it is known as a 'brain tonic' for various mental disorders. It is being employed for the treatment of heatstroke, diarrhea, ulcerations, eczema, and traumatic diseases. The presence of asiatic acid, a pentacyclic triterpene, in this plant has added to its medicinal importance as an anticancer agent. The cytotoxic effect of asiatic acid has been investigated and it has been shown to decrease the viability of HepG2 cells in the case of liver cancer. The decrease in cell viability is due to increased expression of a tumor-suppressor p53 gene mediated by increased levels of intracellular calcium (Lee et al., 2002).

3.8. *Curcuma longa* L.

Curcuma longa belongs to the family Zingiberaceae and is widely grown in Asiatic countries, mainly in India and China. It is commonly known as turmeric. Biliary disorders, anorexia, coryza, cough, diabetic wounds, hepatic disorder, rheumatism, and sinusitis are some of the conditions for which the plant has shown its medicinal properties (Ammon et al., 1992). An array of pharmacological activities of this plant includes antiinflammatory, antihuman immunodeficiency virus, antibacterial, and antioxidant effects and nematicidal activities. Its major chemical constituent is curcumin and it exhibits numerous biological actions. The curcumin compound also exhibits antitumor potential via suppression of the various events involved in multiple steps of carcinogenesis such as transcription factor, NF-κB, AP-1, and STAT-3 and the ability to repress proinflammatory pathways of COX-2 and iNOS (Plengsuriyakarn et al., 2012).

3.9. *Fagara zanthoxyloides* L.

Fagara zanthoxyloides belongs to the family Rutaceae, widely distributed in Uganda and other African countries. It is commonly known by the name 'candlewood'. Root-bark extract is used in treating elephantiasis, toothache, sexual impotence, gonorrhea, malaria, dysmenorrhea, and abdominal pain. The benzophenanthridine alkaloid fagaronine exhibits antitumor activity against P388 and L1210 murine leukemic cell lines in vivo. Fagaronine inhibits the DNA replication of rapidly growing cancer cells by inhibiting DNA and RNA polymerase activities

and protein synthesis. Fagaronine leads to the disruption of replication in rapidly growing cancer, thus affecting their growth. Reverse transcriptases are also inhibited by fagaronine (Fleury et al., 2000).

3.10. *Glycyrrhiza glabra* L.

Glycyrrhiza glabra is commonly known by the name 'sweetwood' and belongs to family Fabaceae. The Mediterranean and certain areas of Asia are its habitat. It contains an active chemical complex composed of triterpene saponins, flavonoids, polysaccharides, pectins, simple sugars, amino acids, mineral salts, and various other substances (Obolentseva et al., 1999). Important secondary metabolites glabridin and hispaglabridins A and B are associated with significant antioxidant activity, and estrogen-like activity is contributed by glabridin and glabrene. It has been used against the human immunodeficiency virus (Hattori et al., 1989), cytomegalovirus, and herpes simplex virus. The antitumor activity of the plant is attributed to the metabolite isoliquiritigenin (2′,4′,4-trihydroxychalcone), which has shown potential chemopreventive activity through various mechanisms like induction of phase II enzymes such as quinone reductase 1, increased expression of glutathione peroxidase 5, and downregulation of several cytochrome P450 genes (Cuendet et al., 2010).

3.11. *Indigofera tinctoria* L.

Indigofera tinctoria belongs to the family Papilionaceae and is found in most countries of Africa, Australia, and Asia. It is commonly known as indigo. Phytochemical screening has shown that the plant contains flavonoids, terpenoids, alkaloids, and glycosides as its chemical constituents (Verma and Suresh, 2002). Medicinal benefits of the plant include treatment of chronic bronchitis, asthma, ulcers, skin diseases, gastropathy, and epilepsy; it also contributes to growth of hair and acts as an antidepressant. The contribution of this plant towards potential antitumor activity is because of its metabolite indirubin, which has the ability to inhibit Lewis lung carcinoma (LLC) in mice. Indirubin is also a potent inhibitor of cyclin-dependent kinases. Indirubin-3′-monoxime inhibits the proliferation of a large range of cells, mainly through arresting the cells in the G2/M phase of the cell cycle. The inhibition of DNA polymerase I activity and subsequently DNA synthesis contributes to antitumor activity. Experimental studies in several cell lines, in cell-free assay, and in rats with Walker 256 sarcoma have shown that indirubin significantly inhibits DNA synthesis (Hoessel et al., 1999).

3.12. *Mangifera indica* L.

Mangifera indica belongs to the family Anacardiaceae and is commonly known as mango. *M. indica* has its origins in India and Myanmar and it is the most cultivated *Mangifera* species. It has applications as a medication for gastrointestinal disorders, bilious disorders, blood disorders, scurvy, and vitamin A deficiencies (night blindness). Fresh mango leaves have also been used for the treatment of diabetes (Shah et al., 2010). In addition to the above medicinal importance, a recent study reported the presence of a triterpene called lupeol in mango fruit that possesses cytotoxic effect against skin cancer, as it has been shown to induce apoptosis in human epidermoid carcinoma A431 cells. Apoptosis is induced in a dose-dependent manner in association with the caspase-dependent mitochondrial cell death pathway. It also inhibits the Akt/PKB signaling pathway by inhibition of Bad (Ser136) phosphorylation. Thus, lupeol is anticancerous in its ability to inhibit several molecular targets involved in cancer (Prasad et al., 2009).

3.13. *Morinda citrifolia* L.

Morinda citrifolia is commercially known by the name 'noni' and it is a member of family Rubiaceae. It is native from Southeast Asia (Indonesia) to Australia. The major secondary metabolites of this plant are lignans, oligo- and polysaccharides, iridoids, fatty acids, scopoletin, flavonoids, catechin, β-sitosterol, damnacanthal, and alkaloids (Wang et al., 2002). Its pharmacological benefits include use in treatment for malaria, jaundice, hypertension, boils, carbuncles, stomach ulcers, stomachache, fractures, diabetes, loss of appetite, urinary tract ailments, abdominal swelling, hernias, and human vitamin A deficiency, as well as use as a general febrifuge and for analgesic effect. Two unique glycosides, 6-O-(β-D-glucopyranosyl)-1-O-octanoyl-β-D-glucopyranose and asperulosidic acid, have been isolated from the noni fruit's juice and were found to be highly efficient in inhibiting TPA- or EGF-promoted cell transformation as well as related AP-1 activity in the mouse epidermal JB6 cell line (Liu et al., 2001). Increased AP-1 activity is associated with malignant transformation and cancer-promoting agents. Thus, the ability to inhibit AP-1 activity marks the importance of these glycosides as antitumor agents.

3.14. *Newbouldia laevis* Seem.

Newbouldia laevis is commonly known as the African Border tree and belongs to the family Bignoniaceae. It is used as a therapy against many diseases. Its potential applications are as a remedy for epilepsy and convulsions in children, in treatment of rheumatism, and as a febrifuge. Its extracts have also been shown to exhibit antimicrobial activity (Kuete et al., 2007) and antimalarial properties (Eyong et al., 2006). The cytotoxic effect of the plant is attributed to its compound 2-acetylfuro-1,4-naphthoquinone, which induces apoptosis, even without caspase 3/7 activation. In pancreatic cancer cell lines, it is known to inhibit the formation of blood capillaries (Kuete et al., 2011).

3.15. *Nigella sativa* L.

Nigella sativa is commonly called 'black caraway', 'black cumin', or 'black seed' and belongs to the family Ranunculaceae. It is well distributed in Central Asia. Phytochemical studies have reported the presence of a number of chemical constituents like nigellicine, nigellidine, nigellimine-N-oxide, thymoquinone, dithymoquinone, thymohydroquinone, nigellone, thymol, arvacrol, oxy-coumarin, 6-methoxycoumarin and 7-hydroxycoumarin, α-hedrin, and steryl glucoside, as well as rich amounts of flavinoids, tannins, essential fatty acids, essential amino acids, ascorbic acid, iron, and calcium. Its medicinal values include analgesic, antiinflammatory, antihistaminic, antiallergic, antioxidant, anticancer, immune stimulation, antiasthmatic, antihypertensive, hypoglycemic, antibacterial, antifungal, antiviral, and antiparasitic effects (Ali and Bluden, 2003). Thymoquinone, a secondary metabolite of this plant, has cytotoxic effects as it induces apoptosis in tumor cells by suppressing NF-κB, Akt activation, and extracellular signal-regulated kinase signaling pathways; it also inhibits tumor angiogenesis (Plengsuriyakarn et al., 2012).

3.16. *Panax ginseng* C.A.Mey.

Panax ginseng is a prevalent perennial herb that belongs to the family Araliaceae and is commonly known as ginseng. It is widely found in China, Korea, Japan, Russia, and the United States. The active chemical components include a diverse group of steroidal saponins that are known as ginsenosides (Attele, 1999). It exhibits a multitude of pharmacological effects including immunomodulatory and antiinflammatory, and it helps in improvement of physical stamina, stimulation of the appetite, and enhancing of learning, memory, and behavior (Sun, 2004). It is also well known for its adaptogenic effects that help to improve resistance to stress. Its polyphenol compounds and saponins contribute to its antitumor potential. The cytotoxic nature of ginsenosides is clearly shown by their potential to induce cell death (such as apoptosis and necrosis), and by their association with antiproliferation, antiinvasion, and antiangiogenesis properties. Thus, ginseng is anticancerous in nature (Yue et al., 2007).

3.17. *Plumbago zeylanica* L.

Plumbago zeylanica is a member of the family Plumbaginaceae and originated from Southeast Asia. Its common names are 'white leadwort', 'Ceylon leadwort', 'plumbago', and 'chitrak'. Chemical screening studies have shown the presence of a number of secondary metabolites: plumbagin, coumarins seselin and suberosin, 2,2-dimethyl-5-hydroxy-6-acetylchromene, plumbagin acid, β-sitosteryl-glucoside, bakuchiol, 12-hydroxyisobakuchiol, saponaretin, isoorientin, isoaffinetin, and psoralen. Its wide-ranging medicinal applications include its use in treatment of fever and malaria, diarrhea, piles, dyspepsia, skin diseases (leprotic lesions) (Gupta et al., 1993), gastrointestinal complaints (Giday et al., 2006), ulcers, and scabies. Plumbagin, a quinoid isolated from the plant's root, has been shown to possess antitumor activity by potential effects in the control of hormone-refractory invasive prostate cancer (PCa). The inhibitory effects of plumbagin against a number of molecular targets like protein kinase C epsilon (a PCa proliferative marker), STAT-3, AKT, and PI-3K result in the inhibition of growth and invasion of PCa. Plumbagin, in addition to inhibiting growth of cancer cells, also induces apoptosis in cancer cells (Aziz et al., 2008).

3.18. *Rhinacanthus nasutus* (L.) Kurz

Rhinacanthus nasutus belongs to the family Acanthaceae and is widely distributed in parts of subcontinental India and in Southeast Asia and China (Rao et al., 2010). The plant is commonly known by the name 'snake jasmine'. Naphthoquinone, rhinacanthins (A–D, G–Q), rhinacanthone, and lignan groups are the major phytochemical compounds of this plant (Thirumurugan et al., 2000). Pharmacological benefits attributed to its active constituents include treatment of eczema, pulmonary tuberculosis, herpes, hepatitis, diabetes, hypertension, and various skin diseases. Studies have suggested that rhinacanthins M, N, and Q and related naphthoquinone esters as well as synthetic compounds, 1,2-naphthoquinones and 1,4-naphthoquinones, are antitumorous as they selectively inhibit the growth of KB, HeLa, and HepG2 human cancer cells and normal Vero cells. The partial arrest of cells at the G2/M phase in rhinacanthin N treatment has been observed experimentally, which helped to prevent further damage and gave cells the time to repair the defect or undergo apoptosis (Siripong et al., 2006).

3.19. *Scutellaria baicalensis* Georgi

Scutellaria baicalensis belongs to the family Lamiaceae. It is native to eastern Asia and has major applications in traditional Chinese medicine. Its common names are 'Chinese skullcap', 'baikal', 'scute', and 'scutellaria'. Root extracts of this plant contain almost 70 flavonoids, anthocyanidins, chalcones, flavanonols, flavonols, flavanones, and flavones. The pharmacological effects of this plant include antidiabetic, antiinflammatory, antioxidative, hepatoprotective, antiviral, antianxiety, antitumor, and antihypertensive effects (Bhandari et al., 2010). Its antitumor activity is attributed to baicalein, wogonin, wogonoside, and skullcapflavone II (neobaicalein). All these metabolites at micromolar concentrations have shown inhibitory effects against the proliferation of human tumor cell lines LXFL and 529L (large-cell lung carcinomas). Baicalein inhibits 12-lipoxygenase activity and contributes to anticancer potential against a number of other cancers, also. The inhibition of 12-lipoxygenase

activity acts as interference in the signaling mechanism needed for tumor growth (Zhou et al., 2008).

3.20. *Solanum incanum* L.

The geographical habitat of *Solanum incanum*, a member of the family Solanaceae (nightshade plants), is the temperate and tropical regions of the world. The plant is commonly known by the names 'bitter apple' and 'thorn apple'. The main chemical constituents of the plant are steroid glycosides, which are known to possess defensive role against pathogens and predators of the plant. Solanin and solasonine are steroid alkaloids, which have applications against cutaneous mycotic infections and other infectious conditions (Al-Fatimi et al., 2007). Another metabolite, solamargine, is known to have cytotoxic activity towards normal skin fibroblasts by its ability to induce cell apoptosis. The cytotoxic effects of solamargine have been experimentally studied in 4 human lung cancer cell lines. The molecular effects of this metabolite that clearly validate its potential as an anticancer agent for tumor necrosis factors and Bcl-2-related resistance of human lung cancer cells include the release of cytochrome c, downregulation of antiapoptotic Bcl-2 and Bcl-xL, increase of caspase-3 activity (important for apoptosis), and DNA fragmentation (Liu et al., 2004).

3.21. *Vismia laurentii* De Wild.

Vismia laurentii belongs to the family Guttiferae and is widely distributed in the tropical and subtropical regions of the world. There is no English common name for this plant. Chemical studies have reported the presence of xanthones, anthraquinones, and prenylated anthrones in this plant. Recently, in a systematic search for new bioactive lead structures, 1 new xanthone, laurentixanthone C, and 3 known compounds identified as vismiaquinone, bisvismiaquinone, and dammaradienol were isolated from *V. laurentii* (Hussain et al., 2012). It is used as a remedy for the treatment of skin diseases (such as dermatitis, leprosy, scabies, and eczema) and wounds. Experimental studies suggesting the anticancer potential of xanthone V1 have already been done in a number of cancer cell lines. The effects of xanthone V1 on the cell cycle distribution, apoptosis induction, and caspase-3/7 activity have been investigated in the CCRF-CEM cell line and show evidence for its anticancer potential as a cytotoxic agent. Xanthone V1 leads to activation of caspase-3/7 enzymes, which play important roles as apoptosis inducers (Kuete et al., 2011).

3.22. *Withania somnifera* (L.) Dunal

Withania somnifera is a minor, timbered shrub in the family Solanaceae that is commonly known as ashwaganda. It is found in Africa, the Mediterranean, and India. The major biochemical constituents are steroidal alkaloids and steroidal lactones, and as a class of constituents, they are called withanolides. Therapeutic values associated with this plant are adaptogen, aphrodisiac, antiinflammatory, deobstruent, antibiotic, diuretic, narcotic, sedative, abortifacient, immune system-stimulating, astringent, and antioxidant effects (Singh et al., 2010). The antitumor property of the plant is well defined by the fact that withaferin A, a chemical constituent of this medicinal plant, inhibits growth of MDA-MB-231 and MCF-7 human breast cancer cells and MDA-MB-231 xenografts in vivo, in association with apoptosis induction mediated by reactive oxygen species production due to the inhibition of mitochondrial respiration (Hahm et al., 2011).

3.23. *Xanthium strumarium* L.

The genus *Xanthium* is a member of the family Asteraceae, comprising 25 species of American origin (Oudhia, 2001). *X. strumarium* is commonly known as 'cocklebur' or 'burweed'. In India it is well known for its use as a remedy in hemicrania diseases (Kamboj and Saluja, 2010). Its health-promoting benefits include antitumor, antibacterial, antifungal, antiinflammatory, antinociceptive, antitussive, hypoglycemic, antimitotic, antitrypanosomal, antimalarial, diuretic, antioxidant, analgesic, repellent, and insecticidal activities. Its metabolite, 8-epi-xanthatin, and its epoxide show strong evidence of being antitumorous as they significantly inhibit the proliferation of cultured human tumor cell lines. 8-epi-Xanthatin acts by farnesyltransferase inhibitory effect and also inhibits microtubule-interfering agents. These inhibitions contribute to the anticancer activity of 8-epi-xanthatin (Kim et al., 2003).

3.24. *Zanthoxylum nitidum* (Roxb.)

Zanthoxylum nitidum belongs to the family Rutaceae and is widely distributed in Southeast Asian countries and Australia (Hu et al., 2007). It is commonly known by the name 'prickly ash'. The presence of true alkaloids, carbohydrates, flavonoids, and amino acids has been revealed by phytochemical studies (Bhattacharya and Zaman, 2009). Nitidine chloride, oxynitidine, dihydronitidine, 6-methoxy-5,6-dihydrochelerythrine, α-allocryptopine, and skimmianine are the main alkaloids isolated from its roots. The medical application of the plant includes the cure of toothache, stomachache, fever, rheumatism, paresis, boils, cough, colic, vomiting, diarrhea, and cholera. The anticancer potential of the plant is attributed to the chemical constituent nitidine, known for its ability to exhibit cytotoxic activity against LLC. It is a DNA intercalator that is generally classified as an inhibitor of topoisomerases I and II. The inhibition of these enzymes leads to subsequent apoptosis in cancer cells (Fang et al., 1993).

4. Discussion

Medicinal plants have contributed richly to the health of human beings. The vast potential of plant-based medicines in the treatment of numerous diseases has

always contributed to the value of the plant community as a major area of research and development. Chemotherapy is an important option in modern cancer treatment, and plant-derived chemotherapeutic agents have contributed greatly to the progress of oncology/chemotherapy development and to clinical practice. The need to cure cancer and find better ways to combat this disease has raised the need to find anticancer compounds in the plant kingdom. Plant extracts and the bioactive compounds present in them, which are responsible for anticancer activity, have to be screened for valuable information. Innovations in multidisciplinary investigative methods offer great promise for plant-derived drug discovery and development. This review includes medicinal plants with chemical constituents that possess anticancer properties. Most of the plant compounds described in this review are antitumorous, as explained by in vitro studies. Some of their metabolites are cytotoxic in nature, with the ability to induce apoptosis in cancer cells; this includes *Andrographis paniculata*, *Centella asiatica*, *Newbouldia laevis*, *Nigella sativa*, *Panax ginseng*, *Plumbago zeylanica*, *Solanum incanum*, and *Vismia laurentii*. According to the International Union for the Conservation of Nature's Red List of Threatened Species (www.iucnredlist.org.), the most comprehensive inventory for the conservation status of biological species, most of the above-mentioned plants have not yet been evaluated for their conservation status, except for *C. asiatica* and *M. indica*, whose status is "Least Concern" and "Data Deficient", respectively. In addition, the medicinal plants mentioned here are not confined to a particular geographical region; rather, they are diversely present across various regions of the world. In vitro studies have revealed the anticancer potential of the metabolites discussed in this review, so they hold scope for further experimentations. The plant metabolites mentioned in this review possess varying mechanisms of action that contribute to their anticancer nature. Some metabolites work by selectively killing the rapidly dividing cancer cells, while others target molecular factors (mostly proteins) that are abnormally expressed in cancer cells. The cytotoxicity of cancer cells is induced by impairing the cell cycle (mitosis) at certain stages and also by promoting molecular factors responsible for apoptosis (increase of caspase-3/7, down regulation of Bcl-2, etc.). The antitumor effect of certain metabolites is observed in their ability to inhibit abnormally expressed growth factors (mostly protein tyrosine kinase) and thus inhibit the growth of cancer cells. The anticancer metabolites mentioned in this review can be further researched based on prior toxicological investigations to develop them as anticancer drugs. In the process of drug development, they can be subjected to clinical trials to account for their safety and effectiveness. Therefore, in the present review, an effort has been made to compile information about some of the plants possessing anticancer activity for various types of cancer. This review can help others to explore herbs to further extend and promote the use of medicinal plants as potential tools to treat cancer.

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PS-IV-48

In-vitro propagation of endangered medicinal herb *Chlorophytum borivilianum* Sant and Fern.

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Abstract

Safed musli, 'a golden root' is a unique gift of nature to mankind since time immemorial as an alternative to chemical aphrodisiac. National Medicinal Plants Board (NMPB) New Delhi has recognized safed musli as the sixth important herb among 28 medicinal plants to be protected, promoted and preserved. The fleshy roots of Safed musli have medicinal properties and are used to prepare the herbal drug which is considered as a valuable nervine and general tonic for strength and vigor. Recently the phytocompound present in the plant is utilized in cancer studies as it possesses the anti-tumor properties. This plant is endangered because of the difficulties in cultivating the roots and its long dormancy period. Safed musli commands great demand and it has exorbitant price for its processed products both in indigenous and global markets. In the present study we attempt *in vitro* tuberization with different concentrations of auxins and cytokines on the basal media in order to get higher number of roots. It is an important way to conserve and commercially utilize plant resource for the welfare of human health.

Key words: Safed musli, endangered, aphrodisiac, phytocompound, anti-tumor

Safed musli, botanically known as *Chlorophytum borivilianum* Sant and Fern., belongs to family Liliaceae. National Medicinal Plants Board (NMPB), New Delhi, has recognized Safed musli as the sixth important herb among 28 medicinal plants to be protected, promoted and preserved [1]. Among the medicinal plants, safed musli is a potential endangered with unparallel medicinal properties. Only the fleshy roots, which have medicinal properties, are used to prepare the herbal drug which is considered as a valuable nervine and general tonic for strength and vigor. There is a lot of demand for its processed products but the production is far behind the demand due to slow propagation rate in nature. There is a great demand for fleshy root of safed musti.

Greater emphasis needs to be given for *in-situ* conservation, where the plant species are adapted to the specialized environment. Amongst *ex-situ* methods, storage of seeds in seed gene banks the most popular and conventional strategy has limited application in this texa. In view of this, there is an urgent need to apply in vitro culture methods for the micropropagation and conservation of this valuable threatened plant species. *Chlorophytum* is threatened with extinction in India due to indiscriminate collection and over exploitation of natural resources for commercial purposes to meet the requirements of the pharmaceuticals industries, coupled with limited cultivation [2]. In the view of this there is an urgent need to apply in vitro culture methods for the micropropagation and

conservation of this valuable threatened plant species. There are a few reports in literature regarding micropropagation of the taxa. In vitro multiplication of *C. borivilianum* was achieved on MS medium supplemented with 2 mg l^{-1} BA. Among the different combinations involving auxins (IBA/NAA) tested for rooting, IBA (3 mg l^{-1}) was relatively better [3]. Our emphasis was to obtain higher number of shoots and shoot length in less time as compared to wild plant growing under natural habitat so as to conserve and commercially utilize plant resource for the welfare of human health.

The plant material was collected from Central Institute of Medicinal and Aromatic Plants, Lucknow and maintained in the agro net of the Delhi Technological University, Delhi. Healthy young plants taken from the net house were thoroughly washed under running water after soaking in detergents like teepol or tween-20 for 10 minutes. Explants were excised and surface sterilization was carried out with 0.1% (w/v) mercuric chloride ($HgCl_2$) and 0.2% streptomycin for 6-8 min. after treating with 0.1% (w/v) bavistin. Finally, treated explants were washed with sterilized distilled water for 3-4 times before inoculation. Explants were cultured on MS medium supplemented with different growth regulators. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C and 1.06 kg cm-2 for 15 min. The cultures were kept at 25±2°C under 16/8h photoperiod (30 ìmol m^{-2} s^{-1}) light/dark provided by cool white fluorescent lamps.

Various concentrations of BA (1mg l^{-1} –5mg l^{-1}) were tested for its effect on multiplication of shoots. Shoot number and shoot length were recorded after 8 weeks of culture. Twelve explants were used per treatment and the experiments were repeated twice. Shoots were transferred to rooting media containing various concentrations of auxins. The rooted shoots, after 8 weeks of rooting, were transferred to pots for hardening. Agar was thoroughly washed under running tap water. Plantlets were transferred to planton (Tissue culture box, Tarson) containing autoclaved mixture of soil, sand and manure, and covered with perforated polythene bags having whole. Plants were watered every 2-3 days with MS broth (pH 5.8) for 2-3 weeks. Polythene bags were removed after 3 weeks and the plants transferred to earthen pots containing garden soil.

Attempts were made to standardize a procedure of *in-vitro* establishment and shoot multiplication. It is known that the plants growing in field become more prone to fungal and bacterial attack. Parts of these plants are difficult to sterilize. Effective sterilization procedure for aseptic establishment of cultures was standardized. Of the various media tested, MS containing different concentrations of BA was the most suitable for culture establishment. Shoot emergence was observed within 2 weeks of culture and by 4 weeks, good healthy shoots were formed in all the media. The difference in response to various treatments became noticeable by 8 weeks. Maximum shoot length and higher number of shoots was observed in 3 mg l^{-1} BA. Shoot length and number of shoots were reduced as the concentration of BA was increased further.

MS Supplemented with BA had enhancing effect on the number of shoots with 3 mg l^{-1} BA. The present study clearly demonstrates successful *in-vitro* multiplication with different concentration of BA. Significant rooting was observed with the addition of auxins viz. NAA (Naphthalene acetic acid) and IAA (Indole-3-acetic acid) in MS medium. However, type of rooting is different in the media consisting of NAA alone and in combination of NAA and IAA. The root proliferation was obtained on full strength of MS medium supplemented with increasing concentration for IAA at 1mg l^{-1} and NAA at 3mg l^{-1} [5]. The finding is significant in terms of the fact that *in vitro* clonal multiplication of Safed musli, a rare Indian medicinal herb, has been achieved on MS medium supplemented with 3mg l^{-1} BA using young shoot bases as explants. Significant success has been achieved in number of medicinal plants regarding standardization of various stages of *in vitro* propagation which is a prerequisite for applying *in-vitro* techniques for germplasm conservation. Rapid clonal multiplication leading to regeneration of plantlets through tissue culture is an important pre requisite for *in-vitro* conservation. Results of the present study compare well with the earlier reports and revealed that elite collection of *C.borivilianum* could be established and multiplied *in-vitro*. Thus generated *in-vitro* shoots will serve as source material for further *in-vitro* conservation studies.

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PS-IV-49

Effect of salicyclic acid and proline on seed germination of aged and fresh seeds of radish (*Raphanus sativus*)

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Abstract

Seed treatment of naturally aged and fresh seeds of radish with water, salicyclic acid and proline showed improved percent germination as compared to control. Seed germination is the growth of an embryonic plant within a seed and result in the formation of the seedling. During the ageing process, seeds become less viable due to oxidative damages. Under ambient seed storage conditions, radish seeds loose vigour and viability exhibiting signs of physical deterioration. Oxidative damage of membranes and of other cellular constituents has been invoked as a mechanism of seed ageing. Seed ageing mechanisms include bio-membrane degradation, protein denaturation, interference with DNA and protein synthesis, accumulation of toxic materials and destruction of electron transport system of oxidative phosphorylation. Proline accumulation has been reported to be important for tolerance of certain adverse environmental conditions [1]. Proline catabolism is an important regulator of cellular ROS balance and can influence numerous additional regulatory pathways. Another plant growth regulator that appears to be involved in the regulation of proline metabolism is salicyclic acid. Several recent studies reported a promoting effect of SA on proline accumulation even in the absence of stress [2]. The present investigation was planned with the objective to study the effect of water, salicyclic acid and proline on germination and vigour of naturally aged radish seeds.

Key words: Radish, salicyclic acid, proline and ageing

The seeds of radish (*Raphanus sativus)* cultivar Punjab Pasand were used for different seed treatments i.e water, salicyclic acid (50 mg/L and 100 mg/L) and proline (5 mM and 10 mM). The effect of these treatments on seed germination of fresh and aged seeds of radish was recorded at 25°C. Seed treatments replicated thrice each with fifty seeds were kept for 12 h and 24 h. After the seed treatment with different solutions, seedling were raised between the two layers of germination paper and kept in the seed germinator. The data on seed germination and vigour was recorded after 10 days of sowing.

The results exhibited that the fresh seeds of radish variety Punjab Pasand had more percent germination at 25°C than the aged seeds of radish. Hydration, Salicyclic acid and Proline treatments for 12 and 24 h increased the percent germination of seeds as compared to control . The hydration of seed for 12 h and 24 h had increased the germination in fresh as well as naturally aged seeds but the effect was more pronounced in fresh seeds of