Production of Bacoside A- A potent herbal drug for Alzheimer in callus cultures of *Bacopa monnieri*(L.) Pennell

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Production of Bacoside A- A potent herbal drug for Alzheimer in callus cultures of *Bacopa monnieri*(L.) Pennell

Dissertation submitted in partial fulfilment

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by

Leonard

(Roll Number: *214BM2018*)

based on research carried out

under the supervision of

Prof.Nivedita Patra

May, 2016

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May 23, 2016

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We the below signed, after checking the dissertation mentioned above and the official record book (s) of the student, hereby state our approval of the dissertation submitted in partial fulfilment of the requirements of the degree of Master of Technology in Biotechnology at National Institute of Technology Rourkela. We are satisfied with the volume, quality, correctness, and originality of the work

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Supervisor's Certificate

This is to certify that the work presented in this dissertation entitled 'Production of Bacoside A- A potent herbal drug for Alzheimer in callus cultures of *Bacopa monnieri*(L.) Pennell' by *Leonard*, Roll Number *214BM2018*, is a record of original research carried out by him under my supervision and guidance in partial fulfilment of the requirements of the degree of *Master of Technology* in *Biotechnology*. Neither this dissertation nor any part of it has been submitted for any degree or diploma to any institute or university in India or abroad.

Prof. Nivedita Patra

Supervisor

I dedicate this work to all my well-wishers and critics...

Leonard

Declaration of Originality

I, *Leonard*, Roll Number *214BM2018* hereby declare that this dissertation entitled : 'Production of Bacoside A- A potent herbal drug for Alzheimer in callus cultures of *Bacopa monnieri*(L.) Pennell' represents my original work carried out as a post graduate student of NIT Rourkela and, to the best of my knowledge; it contains no material previously published or written by another person, nor any material presented for the award of any other degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the sections "Reference" or "Bibliography". I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

I am fully aware that in case of any non-compliance detected in future, the Senate of NIT Rourkela may withdraw the degree awarded to me on the basis of the present dissertation.

May 23, 2016
NIT Rourkela

Leonard

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ABSTRACT

Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disease .An important approach for treating AD is the inhibition of acetylcholine esterase (AChE) and Monoacylglyceride Lipase (MAGL). Many of the medicinal plants have suggested for treatment of Alzheimer. But only few plants only proved for their Anti-Alzheimeric potential. One such herb is *Bacopa monnieri*. . Bacosides are most active constituents of *Bacopa monnieri*. Bacoside A , mostly reported herbal drug- a mixture of four triglycosidic saponins, namely bacoside A3, bacopaside II, bacopasaponin C and the jujubogenin isomer. Its inhibiting potential were analysed by molecular docking. It is having very low binding energy of -20.1 kcal/mol and -12.7 kcal/mol on the targets AChE and MAGL respectively. The druglikeness and toxicity were predicted with ADMET predictor tools namely molinspiration and protox respectively. Bacoside comes under class IV toxicity. This project related to the production of Bacoside A in Callus Cultures of *Bacopa monnieri* and its enhancements through Elicitation. Elicitor may be defined as a substance which, when introduced in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds. Callus of *Bacopa monnieri* was efficiently induced by 0.5mg/l of NAA. supplement of Auxins IAA was not much efficient in callus induction. Cytokinin BAP was used to inhibit the root formation. The developed fried callus was used for suspension culture under optimised parameters. The suspension cultures were treated under the influence of the Elicitors. HPLC was used for estimation of Bacoside A.

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List of Abbreviations

2,4 D	2,4-Dichlorophenoxyacetic acid
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's Disease
ADMET	Adsorption, Distribution, Metabolism, Excretion and Toxicity
APP	Amyloid Precursor Protein
BAP	Benzyl Amino Purine
$CuSO_4$	Copper Sulphate
DW	Dry weight
GABA	gamma-Aminobutyric acid
HP	*Hypericum perforatum*
HPLC	High Performance Liquid Chromatography
IAA	Indole Acetic Acid
IBA	Indole Butyric Acid
Kn	Kinetin
MAGL	Mono Acyl glyceride Lipase
MJ	Methyl Jasmonate
MO	*Mellisa officinalis*
MS	Murashige Skoog
NAA	Napthalic Acetic Acid
NH_4	Ammonium
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NO_3	Nitrate
PDB	Protein Data Bank
SA	Salicylic Acid
TDZ	Thidiazuron
$ZnSO_4$	Zinc Sulphate

List of Symbols

%	Percentage
°C	Degree celcius
μM	Micro molar
G	Gram
Kcal/mol	Kilo calorie per mole
Kg	Kilogram
L	Litre
Mg	Milligram
mM	Milli molar
Nm	Nanometer
rpm	Revolution per minute
α	Alpha
β	Beta
γ	Gama

CHAPTER 1

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease associated with memory loss, and thinking impairment (Johnson *et al*., 2000). The Characteristics of Alzheimer include alterations in the brain, such as the accumulation of β-amyloid plaques and neurofibrillary tangles. An important strategy to treat AD is the inhibition of acetylcholine esterase (AChE) and Monoacyl Glyceride Lipase (MAGL). Memory enhancement and reduction of beta amyloids are the other factors for treatment of AD. AChE and Monoacyl Glyceride lipase are being the targets for the drugs for Alzheimer.

Acetylcholinesterase (AChE) is the enzyme which catalyses the breakdown of Acetylcholine which is an important neurotransmitter. The enzyme AChE needs to be inhibited to prolong the occurrence of Acetylcholine. Monoacyl Glyceride Lipase is the enzyme which hydrolysis the triglycerides to fatty acids and glycerol. MAGL needs to be inhibited to reduce the accumulation of amyloid tangles and neurofibrils.

A number of scientific investigations have been carried out by researchers to find out potent drug from medicinal herbs to treat neurological disorders. Generally Herbs which have anti-inflammatory and anti-oxidant activities may be used in the treatment of AD. *Bacopa monnieri* has exhibited better potential biological activities against Alzheimer than many of the suggested herbs.

Bacopa monnieri is widely known as water hyssop or jalbrahmi ,indigenous to the wetlands of southern india *Bacopa monnieri* was previously mentioned in the Ayurvedic literatures such as Athar-Ved, Charaka Samhita and Susrutu samhita as Medhya rasayana to sharpen the intellect and mental deficits attenuation(Aguiar *et al*., 2013) It has been used as medicine for attenuation of cognitive decrements in ayurvedha. The active constituents of *Bacopa monnieri* are steroidal saponins namely bacoside A and B. But the identity of bacoside B remains unclear. Bacoside A is most Studied Constituent. The IUPAC name of Bacoside A is 3-(a-L-arabinopyranosyl)-O-b-D-glucopyranoside- 10, 20-dihydroxy-16-keto-dammar-24-ene.

Table 1.1: Taxonomical Classification of Brahmi

Kingdom	Plantae
Order	Lamiales
Family	Plantaginaceae
Genus	Bacopa
Species	B. monnieri
Bionomial name	*Bacopa monnieri*(L.) Pennell

Bacoside A is a mixture of four triglycosidic saponins, namely bacoside A3, bacopaside II, bacopasaponin C and the jujubogenin isomer of bacosaponin C (bacopaside X) (Deepak *et al*., 2005). The structure of Bacoside A has been shown in figure 1.2 Bacoside A, have been considered to be the main bioactive constituents responsible for the cognitive effects of *B. monnieri*.

Fig 1.1: *Bacopa monnieri*
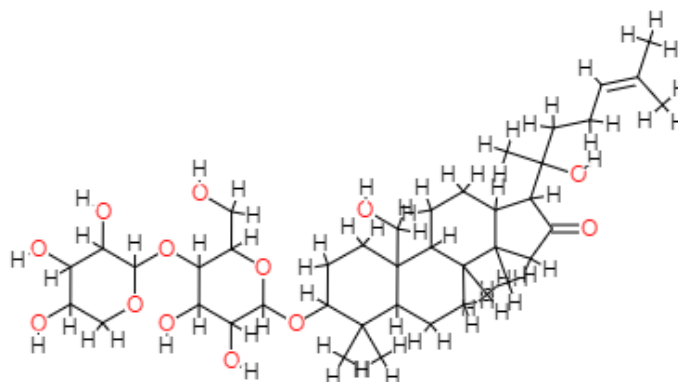
Fig 1.2: Structure of Bacoside A

The amount of Bacoside A present in the plant is only 0.2 %. But the importance of the Bacoside A is high. Plant tissue culture techniques would be useful to enhance the herbal drug production. (Tejavathi and Shailaja.,1999). Elicitation is a process by which the production of important metabolites could be stimulated by the treatment of small amounts of chemicals or biological molecules (Brooks and Watson., 1986). Elicitors are stimuli from external source which capable of prompting the immune response of the plant which favours the accumulation of specific Secondary metabolite.

This project deals with the *in silico* analysis for drug like activities of Bacoside A and its enhanced production in suspension cultures of *Bacopa monnieri* by the influence of elicitors. The drug like activities were analysed by molecular docking and ADMET predictor.Plant calluses were induced with the different concentration of Auxins and Cytokinins namely IAA, NAA & BAP. Induced calluses were inoculated in suspension cultures under optimised condition for mass production. The suspension cultures of *B.monnieri*were induced with the different elicitor for the production the Bacoside A. The antioxidant potential of the callus was analysed by *in vitro* analyses.

CHAPTER 2

OBJECTIVES

- Selection of Plants and In Silico analyses of the Phytodrugs
- Standardisation of callus induction with varying concentrations of Auxins (NAA,IAA)and Cytokinins (BAP)
- Preparation of suspension culture and Elicitation
- Estimation of Bacoside A by HPLC

CHAPTER 3

LITERATURE REVIEW

3.1 ALZHEIMER DISEASE

Alzheimer's disease (AD) is a neurological disorder which destroys the memory and thinking skills gradually. Alzheimer's disease (AD) is named after German physician Aloes Alzheimer, who first described it in 1906 (Alzheimer's disease fact sheet). It is the most common form of dementia in older people. The severity of Alzheimer has been linked with the memory loss which is a cholinergic shortage (Bierer *et al.*, 1995; Collerton., 1986; Giacobini., 1990).

Approaches to enhance cognitive function in Alzheimer have included elongating the availability of acetylcholine (ACh) by the inhibition of ACh hydrolysis by acetylcholinesterase (AChE) enzyme., and reduction of Amyloid fibrillary tangles by inhibiting the enzyme MAGL.

Monoacylglycerol lipase (MAGL) is the enzyme which metabolizing the endocannabinoid 2-arachidonoylglycerol to Arachidonic acid (AA) in the brain. Chen *et al.*,(2012) reported that the inactivation of MAGL strongly suppressed production and accumulation of beta amyloid (Ab) allied with reduced expression of BACE1 enzyme.

The synthetic drugs such as Donepezil, Rivastigmine and Galantamine are used as AChE inhibitors in Europe (Howes *et al*. 2003).

An important approach for treating AD is the inhibition of acetylcholinesterase (AChE) and Monoacylglycerol lipase. A number of scientific research have been carried out on medicinal herbs. Herbs have anti-inflammatory and anti-oxidant activities that may be used in the treatment of AD.

3.2 SELECTION OF PLANT

3.2.1 *Centella asiatica* L.

The essential oil extracted from *C. asiatica* leaf comprises of monoterpenes , such as bornyl acetate, α-pinene, β-pinene and γ-terpinene which were reported to inhibit AChE

(Perry *et al*., 2000). These findings were suggesting that *C. asiatica* may be appropriate to treat Alzheimer and it may also influence cognitive function.

3.2.2 *Curcuma longa* **L.**

Many researches have concentrated on curcumin, a curcuminoid from rhizomes of *C.longa*. In vivo following oral administration of Curcumin against ethanol-induced brain injury was studied by Rajakrishnan *et al*., (1999). Results were showing that Curcumin could be neuroprotective which was related to a reduction in lipid peroxide levels and enhancement of glutathione in rat brain.

3.2.3 *Galanthus nivalis* **L.**

Galanthus nivalis was used conventionally in Bulgaria and Turkey for cognitive treatment. Galantamine, an Amaryllidaceae alkaloid from Galanthus nivalis L. is known for its potential against AChE (Mukherjee *et al*., 2007). Galantamine was reported for its inhibitory activity on AChE than butyrylcholinesterase. It is licensed for Alzheimer treatment in Eurpean countries (Lopez *et al*., 2002). It is modulating nicotinic receptors allosterically independent of inhibition of AChE. One of its derivative compounds was more active than galantamine on the inhibition of cholinesterase. Other Amaryllidaceae alkaloids such as assoanine, 11-hydroxygalantamine, oxoassoanine, sanguinine and epinorgalantamine have also been reported for their AChE inhibitory activity (Lopez *et al*., 2002).

3.2.4 *Huperzia serrata*

Huperzine A, is the compound isolated from *Huperzia serrata* from Lycopodiaceae family. Huperzine A and B are lycopodium alkaloids which could inhibit AChE in vitro and in vivo (Ashani *et al*., 1992; Laganie`re *et al*., 1991).These observations suggest Huperzine A has clinical potential in Alzheimer treatment. Huperzine A was found to be neuroprotective against Beta-amyloid fragment 25–53 (Xiao *et al*., 1999). It was also reported for oxygen-glucose deprivation by Zhou *et al*.,2001b and free radical induced cytotoxicity by Xiao *et al*. in 1999

3.2.5 *Salvia species*

The extracts of *Salvia officinalis* and *Salvia lavandulifolia* were reported to inhibit human AChE and butyrylcholinesterase *in vitro*. 1,8-Cineole was the most potent component in AChE inhibition (savelev *et al*.,2003).

3.2.6 *Hypericum perforatum*

HP showed inhibition of the neuronal reuptake of 5-HT, norepinephrine, GABA, and L-glutamate. It also exhibited enhanced neurotransmitter sensitivity (Butterweck., 2003).

3.2.7 *Polygala tenuifolia*

Root extracts of *P. tenuifolia* have been exhibited to reverse the effects of has been shown to be induced by Scopolamine in rats. It was reported as neuroprotective against toxic metabolites of APP *in vitro*. It showed dose dependent inhibition of AChE *in vitro* (Park *et al*.,2002). Sinapinic acid, a derivative of cinnamic acid, is found in root extract of *P.tenuifolia* and It has been showed to increase ChAT activity in brain-lesioned rats (Yabe *et al*., 1997).

3.2.8 *Ginko biloba*

EGb 761, the extract of *G. biloba* was showing effects against cognitive dysfunction. Desirable effects were observed on neuronal cell metabolism and cerebral circulation (Loffler *et al*., 2001). EGb 761 was found to be neuroprotective against beta-amyloid and toxicity induced by NO *in vitro* (Bastianetto *et al*., 2000a,b). The number of researches in vitro and in vivo showed that *G. biloba* having several activities related to Alzheimer treatment (Howes *et al*.,2003).

3.2.9 *Bacopa monnieri*

Bacopa monnieri is an indigenous plant of Indian subcontinent which has been used for weakening of cognitive decrements. Bacosides A and Bacosides B are the steroidal saponins which are the potent constituents of *B.monnieri*. The extracts of *B.monnieri* have reported to decrease beta amyloid levels in transgenic Alzheimer mice models (Holcomb *et al*., 2006). The extract of *Bacopa monnieri* at the dose of 40 mg/kg orally increased cholinergic neurons density significantly in hippocampus (Uabundit *et al*., 2010). The phytoconstituent Bacoside A and B of *Bacopa monneiri* have been found to show better docking potential on MAGL than many other suggested drugs with a highest docking score of -6.845 (Shrutika *et al*., 2013).

3.3 BIOLOGICAL ACTIVITIES OF *B.monnieri*

Table 3.1 Biological Activities of *B. monnieri*

Activity	Effects of *B.monnieri*	Reference
SOD and GSR enhancement	Bacoside A administration improved the activity of GSR and SOD in the brains of cigarette smoke exposed rats.it restored the levels of selenium and zinc in brain in which the both were reduced by cigarette snmoke.	Anbarasi *et al*,2006
Choline acetyl transferase Activation	Investigator studies the neuroprectection by Bacoside on Acetyl choline level. He hypothesised that the mechanism was not AChE inhibiton but the choline acetyl tranferase activation on maintaining Ach levels.	Rastogi *al*., 2012
Anti- Alzheimeric activity	The extracts of *B.monnieri* exhibited vital effects on β-amyloid inhibition and dissociation of preformed fibrils.	Mathew and Subramanian., 2012
AChE inhibitory activity	*B.monnieri* extracts showed β-amyloid neurotoxicity protection and potential inhibitory activity against AChE.	Limpeanchob *et al.*, 2008
CBF and Vasodilation	B.monnieri extract exhibited around 25% enhanced Cerebral Blood Flow in rat model	Kamkaew *et al.* 2013
Antioxidant activity	Brahmi extracted showed a significant antioxidant activity in many of the reported investigaytions	Sumathy *et al.*,2001; Russo *et al.*,2003

3.4 IN SILICO STUDIES ON *BACOPA MONNIERI*

Ramasamy *et al*, 2015 have reported an in silico study of Bacosides. They have used Discovery Studio 4.0 and ADMET predictor for the study of Druglikeness and toxicity. The reported the In vitro Assay for AChE inhibition Activity. The results

suggested the aglycones were having more binding potential than the Bacosides towards Target.

Ravel and Jency, 2013 reported docking studies of various phytochemicals suggested for the treatment of Alzheimer. They used MAGL (Monoacyl glyceride Lipase) as target. Their results showed that Bacosides were having high docking scores than other phytochemicals

3.5 IN VITRO CULTURES OF *BACOPA MONNIERI*

Talukdar (2014) reported the biosynthesis of bacoside A in the callus cultures of *Bacopa monnieri*. He has used the Auxins IAA , NAA, 2,4 D for the induction of callus. Lower concentrations of NAA yielded high callus induction than higher concentration. The best callus induction observed in high concentration of 2,4 D.

Vijayakumar *et al*., (2010) reported the in vitro propagation of *Bacopa monnieri*. Induction of callus was achieved in two weeks with NAA (0.5mg/l) and TDZ (0.25 mg/l). Further root induction and shoot induction was achieved by various combinations of Auxins and Cytokinins.

Using in vitro cell suspension cultures of B. monnieri, 5-6 fold well grown over 40 d, Rahman *et al*., (2002) have recorded up to 1 g/100 g dry weight of bacoside. An increase in total saponin bacoside content of 166% obtained from suspension culture established from callus biomass was reported by Monica *et al*. (2013).

Subashri *et al.,* (2014) reported the in vitro regeneration of *Bacopa monnieri*.The induction of multiple shoots from nodalsegments were highest in MS medium supplemented with 1.0 mg/l BA,1.0mg/l TDZ and 4.92mg/l 2ip.

Mehta *et al.,* (2012) reported the high frequency shoot regeneration and callus induction. Auxin 2,4 D and cytokinin BAP and Kinetin were used for both in this study.Leaf petiole explants were used forthe purpose of callus induction. Best growth was observed in MS medium supplemented with 0.25 mg/l 2, 4-D+ 0.5mg/l Kn and 0.25 mg/l 2,4-D+ 0.1mg/l BAP.

3.6 ELICITATION OF BACOSIDE A

An elicitor is a substance which, induce or improves the biological synthesis of specific compounds in the living cell while it was supplemented in minimal concentration. Elicitation is the stimulated or improved biosynthesis of metabolites due to addition of small amounts of elicitor molecules (Radman *et al*.,2003). Elicitors can be classified on the basis of their 'nature' like abiotic elicitors or biotic elicitors, or on the basis their 'origin' like exogenous elicitors and endogenous elicitors.

Abiotic elicitors are the substances of non-biological origin, predominantly inorganic salts, and physical factors. Biotic elicitors are constituents with biological source which include glycoproteins, chitin, glucans and polysaccharides from plant cell walls such as pectin and cellulose. Important parameters for elicitation are elicitor concentration, exposure time and age of culture (Namdeo., 2007).

Parale and Nikam (2009) reported the influence of biotic elicitors on the production of Bacoside A. Fungal elicitors *Pencilliumnotatum, Rhizopus Stolonifer, Coriolusvercicolor, Mucor sp.* and *Saccharomyces cerevisae* were used influence or this study in shoot cultures. The most significant elicitation was observed under the influence of *Saccharomyces cerevisae* and *Mucor* sp. Rest of the fungal elicitors did not make significant effects.

Shrama *et al.,* (2014) reported the influence of abiotic elictors for enhanced production of Bacoside A. this study reported the effect of $CuSO_4$, Jasmonic acid and Salicylic acid. Enhanced Bacoside production was observed at 45 mg/l $CuSO_4$ which yielded 1.42 fold higher than the control cultures in 9 days of exposure. Salicylic acid enhanced the production of Bacoside A to 1.32 fold higher than control at low concentration. Bacoside Accumulation decreased with the increase of Salicylic acid concentration. JA at a concentration of 1.0 mg/l significantly induced 3.08-fold higher yield of Bacoside A than control culture.

Largia *et al*., (2015) reported the influence of Methyl jasmonate and Salicylic acid synergism on Bacoside production in shoot cultures. Different concentrations of MJ , SA and MJ+SJ was used for elicitation. The highest enhancement was obtained at the synergistic effect of 25μM SA+ 25μM MJ. The mixture of elicitors influence more than the single elicitor.

Ahmed *et al* (2014) reported a novel elicitor. $ZnSO_4$ was supplemented with MS media for elicitation.   At 600μM of $ZnSO_4$ the elicitation observed qualitatively. The amount of Bacoside was not quantified.

3.7 ESTIMATION OF BACOSIDE A BY HPLC

HPLC conditions mentioned in Ayurvedic Pharmacopoeia of India for Bacoside estimation is slightly differing from British Pharmacopoeia. Researchers followed the both conditions. Mobile phase only differs.C_{18} column is widely used. The detection wavelength is 205 nm. Flow rate 1.5 ml/ min at temperature 30ºC.

API suggests the gradient of Acetonitrile and 0.05% ortho phosphoric acid (pH 2.8) at the ratio of 40:60 as mobile phase. Deepk *et al.,* (2005) followed this method.

BP suggests that the mixture of acetonitrile and 0.71% w/v anhydrous sodium sulphate, previously adjusted to pH 2.3 with sulphuric acid at the ratio of 31.5:68.5 as mobile phase. Murthy *et al.,* (2006) followed this protocol.

CHAPTER 4

MATERIALS AND METHODS

4.1 MOLECULAR DOCKING

AUTODOCK VINA 1.5.6 was the software used for molecular docking and PYMOL-v.1.8.2.0 was used for visualisation of Docking results. The enzymes Acetylcholinesterase and Monoacylglyceride Lipase were used as receptors for this study. Six phytochemicals namely Alpha solanin, Bacoside A, Galantamine, Huperzine A, Huperzine B and limonene and one synthetic drug donepezil were used as ligand for docking study.

Molecular docking procedure contains following steps.

Receptor preparation: The structures of AChE and MAGL were downloaded in .pdb format from PDB site. The receptor molecules were loaded in AUTODOCK vina Software. The water molecules were removed from receptor molecules and the polar Hydrogen molecules were added to receptors. Then the prepared receptors were saved in .pdbqt format. The GRID box was assigned to the receptor.

Ligand Preparation: Ligand molecule structures were downloaded from DRUGBANK and PubChem Sites. Ligands were loaded in AUTODOCK vina Software. The torsions were chosen for the ligand. And the output ligands were saved in the format of .pdbqt.

Docking of ligand and receptor: Configuration file was created with the information of Receptor , Ligand and Grid box dimensions. Docking of ligand and receptor was run through AUTODOCK vina by using the command "vina.exe –config conf.txt –log log.txt" in command panel. The results would be produced in terms of Binding energy. The lower value of binding energy is the positive indication of energy.

Visualisation of Docking results : Output files would be created in .pdbqt format. These files were visualised in PYMOL viewer. The receptor molecules were opened in PYMOL and the docking positions of ligands were visualised.

4.2 DRUG-LIKENESS AND TOXICITY

The chosen ligand molecule Bacoside A structure was downloaded from PubChem site and it was saved in the format of .mol. The molecular properties and the bioactivity scores were calculated by the online tool viz 'molinspiration'. The molecule in the format of SMILES was given as input in the 'molinspiration' tool. Then it was run for calcution and displayed the results of molecular properties and the bioactivity scores of Bacoside A.

PROTOX is the online toxicity predictor tool. The structure of Bacoside A was obtained from the PubChem which was linked with Ptotox toxicity predictor. The structure was given as input in the predictor and the calculated results were displayed. The toxicity results were saved and analysed for the properties of the molecule.

4.3 SELECTION OF PLANT AND COMPOUND

The plants with the potential for the treatment of Alzheimer were studied through literature. Many of the plants were reported for their ability to be a Alzheimer drug. But only few of the phytocompounds from some plants are proved that they are suitable drug for Alzheimer such as Bacoside A and Bacoside B from *Bacopa monnieri* and huperzine from *Huperzia serreta*. *Ginko biloba* is most widely reported plant with anti alzheimeric potential. *Ginko biloba* and *Huperzia serreta* are chinese herbs. *Bacopa monnieri* is Indian herband easily available.

Bacopa monnieri is plant which has been chosen for our research study. Bacoside A, a well-known herbal drug for Alzheimer is the compound to be produced in this project. Molecular docking results were the supporting documents behind the choice if Bacoside A.

4.3.1 COLLECTION OF PLANT

Bacopa monnieri is widely available in Rourkela. It was collected from Saya Pharma Chem . The high yielding variety CIM-Jagriti of Brahmi was procured from CSIR-CIMAP, Lucknow.

4.4 CALLUS INDUCTION

4.4.1 EXPLANT STERILISATION AND PREPARATION

The plant parts were washed in running tap water for 30 minutes to remove the muds and dirts.3-5 drops of tween 20 was added in 100ml of distilled water and it was shaken vigorously   The excised plant parts were soaked in the detergent for 15 minutes. After that it was washed thoroughly in tap water. After the removal of detergents the explants were washed with sterile distilled water for 3-4 times. The procedures after this step was done in sterile conditions. Then explants are dipped in 70% ethanol for 30 seconds. Then they immersed in 1.5% sodium hypochlorite solution for 5-8 minutes. Then it is washed with distilled water for 5 times. The washed explants were kept on sterile tissue paper to absorb the moisture. Sterilised plant parts are cut into small pieces in aseptic condition. The standard procedures were followed as mentioned by (De, 1997).

4.4.2 INDUCTION OF CALLUS

The MS medium solidified with agar was used for callus induction. The MS media is supplemented with different concentrations of Auxins namely IAA, NAA and Cytokinin BAP as mentioned in the table 5.3 at pH 5.8. Auxins only can induce the callus. Then the explants were inoculated in the sterilised MS solid media aseptically. The cultures were maintained at 24 ±2ºC. Friable callus is desirable for suspension cultures.

4.5 SUSPENSION CULTURE PREPARATION

The concentration of specific Auxin and Cytokinin which provide efficient callus induction is observed. 0.5 mg/l NAA and 0.1mg/l of BAP were supplemented in suspension cultures which is MS liquid media. Wet weight 500 µg of the friable calluses were inoculated in suspension culture of 100 ml. The culture is kept at 110 rpm in shaking incubator with 16 hours light and 8 hours dark condition for 20 days. Once the suspension culture was formed then it was sub cultured. 5 ml of Suspension culture was inoculated in 100 ml of sterilised MS liquid media.

The optimised parameters that influence the suspension culture are collected from literature. Most influencing parameter among the Sucrose, Phosphate, inoculum size and Nitrate to Ammonium ratio was determined by Plackett Burman Design. With the

optimised parameter such as carbon source, nitrogen source, temperature and pH were used for mass production and elicitation production in suspension cultures.

4.5.1 PLACKETT BURMAN DESIGN

Sucrose concentration, Phosphate concentration, Inoculum size and Nitrate to Ammonium ratio were the parameters which were chosen for Placket Burman Design. Each parameter was tested as two concentrations such as High and Low concentrations as mentioned in Table 4.1.

Table 4.1 Plackett Burman Factor value Assumptions

FACTOR	HIGH VALUE	LOW VALUE
Sucrose	60 g/l	30g/l
NO_3^-/NH_4^+ Ratio	5:1	2:1
Phosphate	2 mM	1.2 mM
Inoculum Size	1 g/l	0.2 g/l

Eight experiments were formulated and the Dry cell weight of the biomass was the response. The design Expert version 5.0.9 was the software used for the development of design. The cultures with test proportion of media were prepared for 50 ml and they were incubated at 27 ℃ in shaker incubator for 12 days at 110 rpm. Dry cell weight was estimated after12 days.

4.6 ELICITATION AND ESTIMATION OF BACOSIDE A

4.6.1 ELICITOR PREPARATION AND ELICITATION

Elicitors Chitosan and $CuSO_4$ were used for elicitation experiments. Chitosan is dissolved in diluted 1% acetic acid. $CuSO_4$ was dissolved in water. Casein hydrolysate was dissolved in distilled water. The three elicitors were used in the concentrations of 50 μg/ l, 100 μg/ l and 200 μg/ l. Different concentrations of these elicitors were supplemented with the 50 ml of Suspension culture. The culture is maintained with 16 hours light and 8 hours dark condition at 110 rpm. The exposure time for the Elicition process is 6-9 days. After the completion of elicitation period the influence of elicitor are analysed by the quantification of Bacoside A by HPLC. Accumulated biomass is collected by centrifugation. It was dried at 60℃ .Methanol was added to it and ultrasonicated for 8

minutes at 20kHz. The it was centrifuged and the collected supernatant was used for HPLC analysis.

4.6.2 ESTIMATION OF BACOSIDE A

Methanolic extracts of fresh plant, control callus and elicitor treated callus are used for the estimation of the amount of Bacoside A. Plants and callus were dried at 50°C. Then 500mg of dried plant and 200 mg of dried callus were finely grinded with mortar and pestle. 10 ml of methanol was added to each powdered samples and those were ultrasonicated for 8 minutes at 20kHz. The collected supernatant after the centrifugation was used for HPLC analyses. HPLC standard was prepared with methanol at the concentrations of 1 mg/ml. HPLC conditions are followed as the procedure mentioned in Indian Herbal Pharmacopoeia with slight modifications (Deepak *et al*., 2005). C_{18} column was used. The mobile phase used was the gradient of Acetonitrile and 0.05M Sodium phosphate whose pH was adjusted to 2.8 with ortho phosphoric acid as mentioned in Table 4.2. The flow rate is 1.0 ml/min. the detection wavelength is 205 nm.

Table 4.2 Mobile phase gradient ratio

TIME (minutes)	BUFFER at pH 2.8 (% V/V)	ACETONITRILE (% V/V)
0	70	30
25	60	40
35	40	60
36	70	30
45	70	30

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1 MOLECULAR DOCKING

The docking results were showed in table 5.1 in which Acetylcholine esterase was used as receptor. Table 5.2 shows the results of phytochemicals docked with the receptor Monoacyl Glyceride Lipase.

Table 5.1 Docking results for the target AChE

LIGAND	SOURCE	BINDING ENERGY (kcal/mol)
Alpha solanine	Solanum tuberosum	-16.1
Bacoside A	Bacopa monnieri	-20.1
Donepezil	Synthetic drug	-8.6
Galantamine	Galanthus nivalis L.	-9.3
Huperzine A	Huperzia serrate	-9.8
Huperzine B	Huperzia serrate	-9.7
Limonene	Citrus fruits	-11.8
Target : Acetylcholinesterase		

Table 5.2 Docking results for the target MAGL

LIGAND	SOURCE	BINDING ENERGY (kcal/mol)
Alpha solanine	Solanum tuberosum	-11.6
Bacoside A	Bacopa monnieri	-12.7
Donepezil	Synthetic drug	-6.6
Galantamine	Galanthus nivalis L.	-7.5
Huperzine A	Huperzia serrate	-7.7
Huperzine B	Huperzia serrate	-7.8
Limonene	Citrus fruits	-10.1
Target : Monoacyl Glyceride Lipase		

The figure 5.1 shows the docking position of ligand with receptor AChE at their best torsion whose RMSD value was minimum.
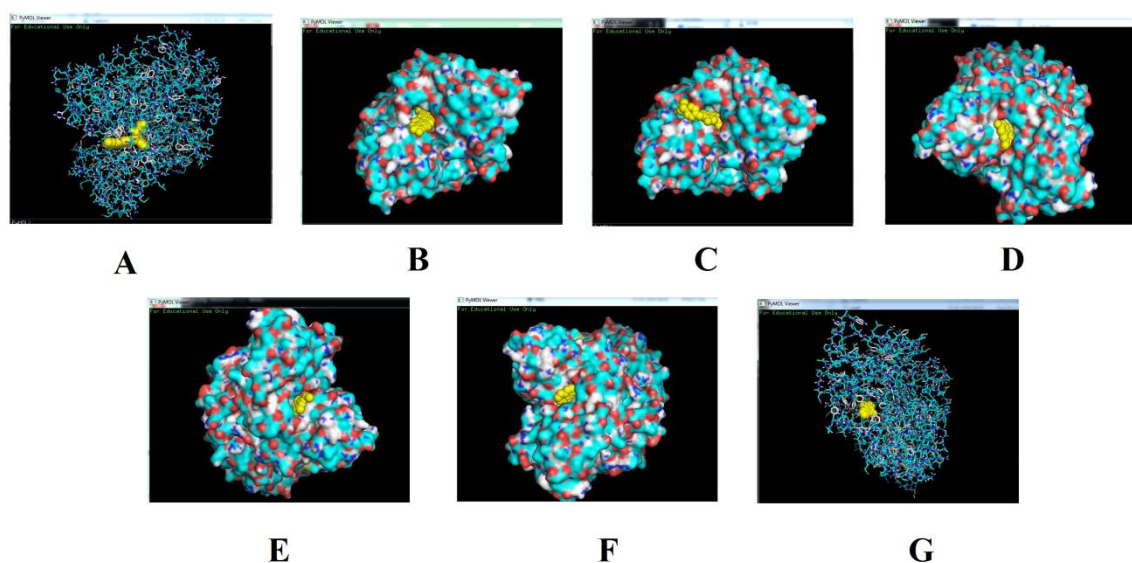

Fig 5.1 Docking of ligands with Target AChE (A-Alpha solanine, B-Bacoside A, C-Donepezil, D-Galantamine, E-Huperzine A, F-Huperzine B, G-Limonene)

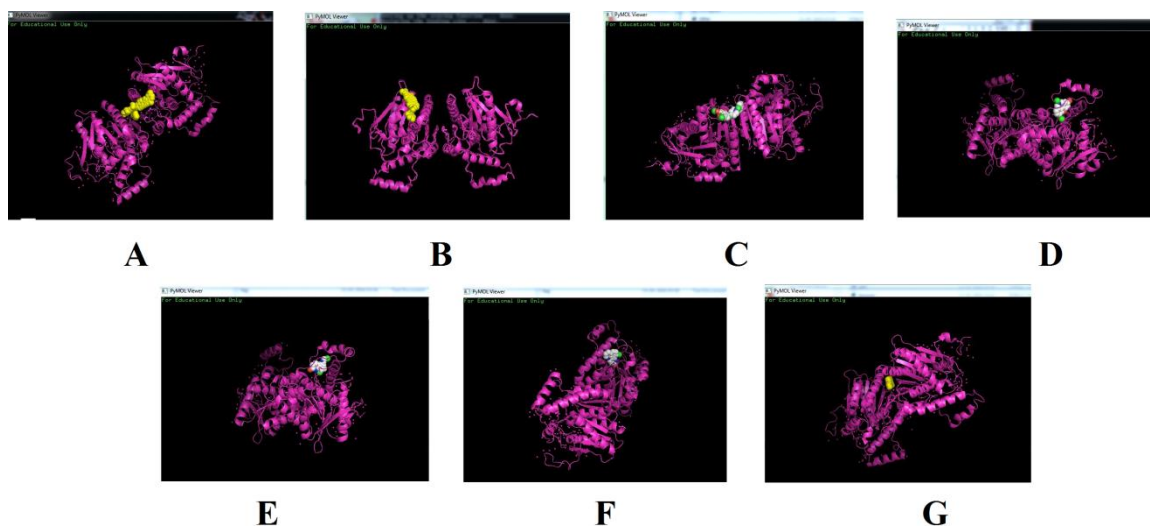Figure 5.2 shows the docking position of ligand at its best torsion with MAGL receptor

Fig 5.2 Docking of ligands with Target MAGL (A-Alpha solanine, B-Bacoside A, C-Donepezil, D-Galantamine, E-Huperzine A, F-Huperzine B, G-Limonene)

For the treatment of Alzheimer AChE and MAGL are the target receptor. Six phytochemicals were docked with these targets. A synthetic drug Donepezil also docked

with those receptor. Among these ligands Bacoside A is showing high affinity towards both the receptors since it is having very low binding energy. These results give us positive hope and are being a supporting document for the suggestion of Bacoside A to be Alzheimer drug.

5.2 DRUG-LIKENESS AND TOXICITY

The molecular properties and bioactivity scores of Bacoside A were calculated by the online predictor tool namely "molinspiration". The results have been showed in figure 5.3 and 5.4
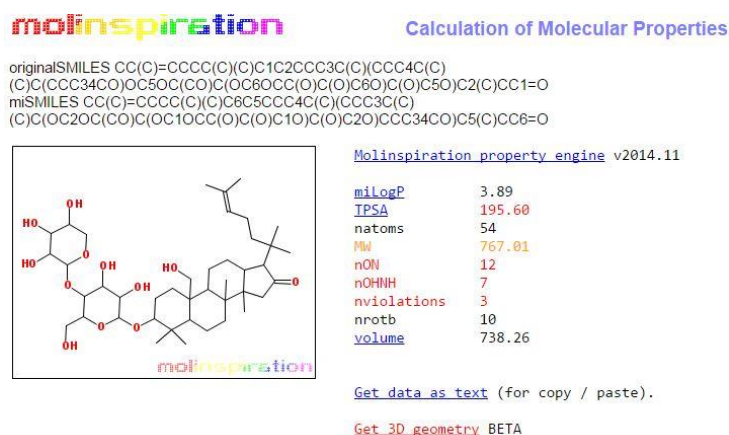

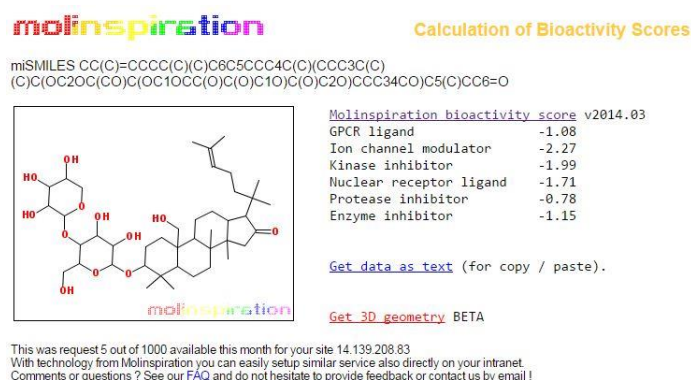**Fig 5.3 Molecular properties of Bacoside A**

Fig 5.4 Bioactivity Scores of Bacoside A

The bioactivity scores of bacoside A were considerable and it was showing a good drug-likeness properties. These results are showing that most of the molecular properties of Bcoside A are satisfying the Lipinski's rule of five. The molecular weight of the compound is only likely to be violating the rule.

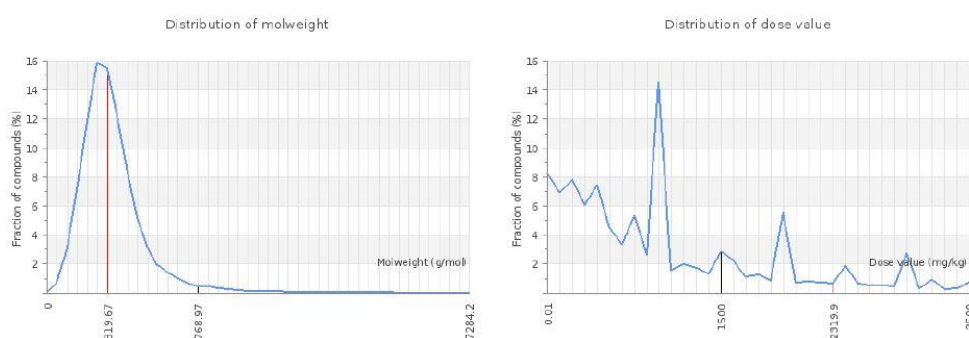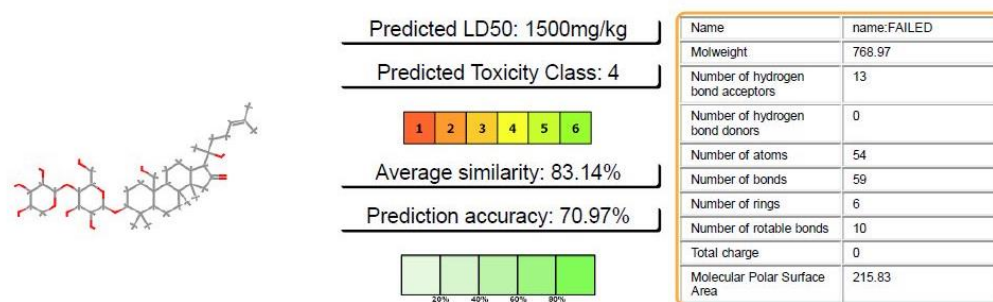PART I: Oral toxicity prediction results for input compound

Predicted LD50: 1500mg/kg	
Predicted Toxicity Class: 4	

| 1 | 2 | 3 | 4 | 5 | 6 |

Average similarity: 83.14%

Prediction accuracy: 70.97%

| 20% | 40% | 60% | 80% |

Name	name:FAILED
Molweight	768.97
Number of hydrogen bond acceptors	13
Number of hydrogen bond donors	0
Number of atoms	54
Number of bonds	59
Number of rings	6
Number of rotable bonds	10
Total charge	0
Molecular Polar Surface Area	215.83

Fig 5.5 Toxicity prediction results of Bacoside A

Toxicity of Bacoside A was predicted by the toxicity predictor tool "protox". The results showed that class 4 toxicity was observed. Class 4 toxicity was not harmful. *In silico* analyses are showing positive results for Bacoside A to be Drug molecule. Numerous in vitro studies proved its anti alzheimeric potential. So such important plant metabolite should be produced more for its increasing requirements.

5.3 SELECTION OF PLANT

From the results of Molecular docking and the literature proofs Bacopa monnieri was selected for the project and Bacoside A was the target molecule in this report work to be produced in the callus cultures.

5.4 CALLUS INDUCTION

5.4.1 CALLUS INDUCTION FROM FIELD GROWN EXPLANT

Leaves were used as explants. Different concentrations of Auxins were used alone and in combination with Cytokinin in MS agar Media. The Hormones used, their concentration and the observations were mentioned in the table 5.3.

Tables 5.3 Standardisation of Callus induction

HORMONE mg/L			Callus induction	Observation
IAA	NAA	BAP		
0.25	-	-	-	Root induction
0.50	-	-	-	Root induction with multiple shoot induction
1.00	-	-	-	Root induction with multiple shoot induction
0.25	-	0.10	+	Root induction with multiple shoot induction Callus initiation also observed
-	0.25	-	++	Callus induction with root formation
-	0.50	-	++	Callus induction with root formation
-	1.00	-	+	Callus initiation
-	0.25	0.10	++	Callus induction with less root formation
- : no induction + : Poor callus initiation ++ :Good callus initiation				

Fine calluses were induced by the use of auxin NAA at the concentration 0.25 mg/L (Fig 6.6) and 0.50 mg/L (Fig 5.7). Some root formation also observed with the callus. To inhibit the root induction the cytokinin BAP at the concentration 0.10 mg/L was added with auxins.
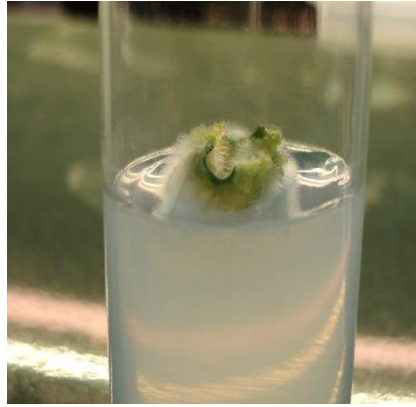
Fig 5.6 Induced callus at 0.25mg/L of NAA

Fig 5.7 :Induced callus at 0.50mg/L of NAA

Usage of auxin IAA has no significant effect on callus induction. It induced root induction with multiple shoot from leaf explant (Fig 5.8). If the inoculated culture maintained at dark condition there was no formation of multiple shoots (Fig5.9).. But IAA was not much effective in callus induction of Bacopa monnieri

Fig 5.8 Multiple shoot and root formation

Fig 5.9 Effect of IAA in dark condition

5.4.2 PREPARATION OF IN VITRO PLANT

To avoid the threats of contamination and to increase the explant purity in vitro grown plant is the good option. The combination of Auxin and cytokinin at the concentration of 1.0 mg/L IAA and 0.1 mg/L BAP was used in MS media for in vitro plant preparation. Root induction (Fig 5.10) and the Elongation (Fig 5.11) of shoots were observed with rapid growth.
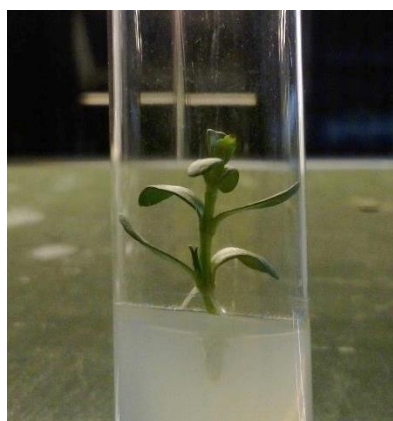

Fig 5.10 Root induction   **Fig 5.11 In vitro plant**

5.4.3 CALLUS INDUCTION FROM IN VITRO GROWN EXPLANT

Callus induction from the field grown leaf explants experiment results shows that 0.50 mg/L concentration of NAA is able to induce the fine callus. To avoid the root induction low concentration of cytokinin should be added.

Leaf and shoots from the in vitro grown plants were used as explants. The combination of 0.5 mg/L of NAA and 0.10 mg/L of BAP is supplemented with MS agar media at pH 5.8. The explants were sliced from in vitro grown plant and they were inoculated aseptically in media. Fine callus was induced from leaf explants. Callus

initiation was observed at 15th day from inoculation. Images of callus induced from at different time interval were shown in Figure 5.12,5.13 and 5.14. callus from the shoot explant was shown in figure 5.15. shoot explant was not much efficient in callus induction
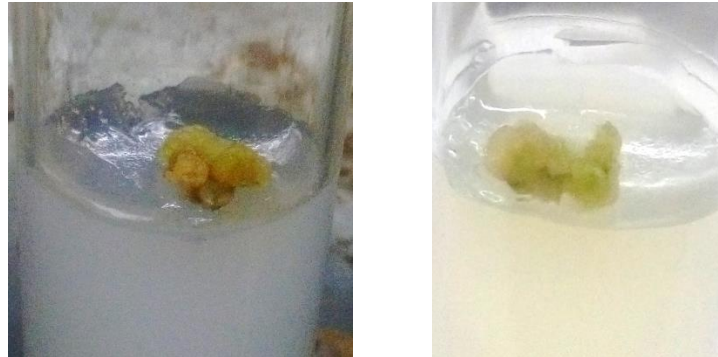

Figure 5.12 callus induced from in vitro grown Leaf explant (14 days old)

Figure 5.13 14 Days old Callus induced from in vitro grown Leaf explant at varying hormonal concentration [A) 0.25 mg/l NAA B) 0.50 mg/l NAA C) 1.0 mg/l NAA D)0.5mg/l NAA+0.1 mg/l BAP]

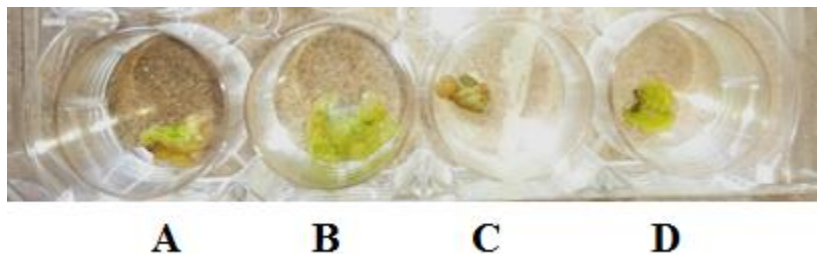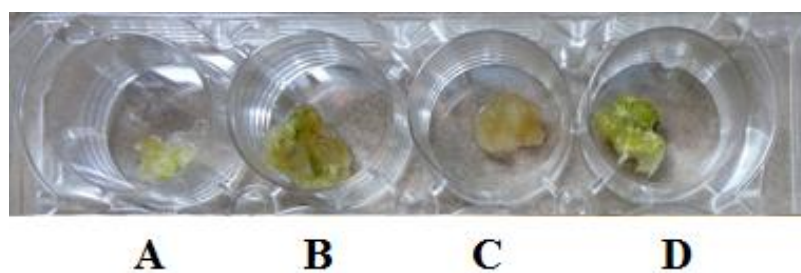**Figure 5.14 21 Days old Callus induced from in vitro grown Leaf explant at varying hormonal concentration [A) 0.25 mg/l NAA B) 0.50 mg/l NAA C) 1.0 mg/l NAA D)0.5mg/l NAA+0.1 mg/l BAP]**

Figure 5.15 Callus induced from in vitro grown shoot explant (23 days old)

Table 5.4 Callus growth by the influence of NAA and BAP

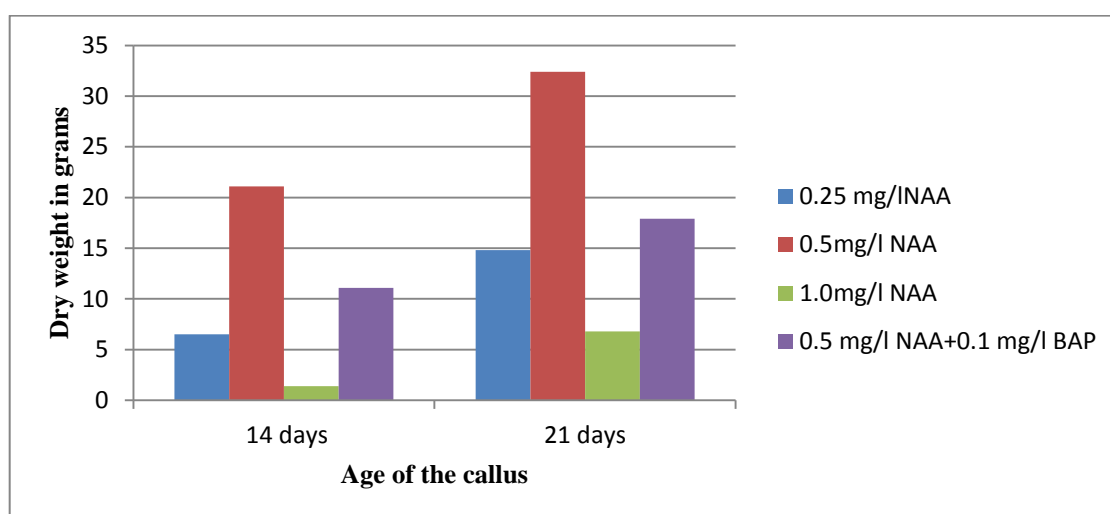| Concentration of hormones (mg/l)		Dry weight of the callus in milligrams		Growth Rate of callus Day^{-1}
NAA	BAP	14 days old	21 Days	
0.25	-	6.5±0.6	14.8±0.8	0.182418
0.50	-	21.1±2.3	32.4±1.1	0.076506
1.00	-	1.4±0.2	6.8±0.6	0.55102
0.50	0.10	11.1±0.4	17.9±0.4	0.087516

Fig 5.16 Comparison of callus growth at 14 and 21 days

The dry weights of callus induced from varying concentrations of hormones at different time interval were mentioned in table 5.4 and the comparative bar graph was shown in figure 5.16.

5.5 SUSPENSION CULTURES

5.5.1 PLACKETT BURMAN DESIGN

The results of placket Burman design showed the most influencing parameter of suspension culture for the growth of callus. The results were shown in the table 5.5.

Table 5.5 Plackett Burman Experiment results

Experiment number	Factor A NO3/NH4 RATIO	Factor B Sucrose g/l	Factor C Phosphate mM	Factor D Inoculum g/l	Response Dry cell weight(g/l)
1	05:01	30	1.2	1	0.242
2	02:01	60	2	0.2	0.14
3	02:01	60	1.2	1	0.208
4	05:01	60	1.2	0.2	0.028
5	05:01	60	2	1	0.2
6	02:01	30	1.2	0.2	0.064
7	02:01	30	2	1	0.186
8	05:01	30	2	0.2	0.018

Table 5.6 The value of regression coefficient (t-value) from Plackett Burman experiments

S.No	Name	Code	t-value	Priority of factor for affecting biomass
1.	NO_3/NH_4 ratio	A	-0.42	-
2.	Sucrose	B	0.25	2
3.	Phosphate	C	7.46×10^{-3}	3
4.	Inoculum	D	4.87	1

Plackett Burman experiment result showed that inoculum size and the sucrose concentrations were the major factors affecting the biomass production. It has indicated by the t- value.(Table5.6). the Least affecting parameters for biomass production was nitrate concentration since the t-value was negative. These effects can be understood by half normal plot in figure 5.16. Choi *et al*.,1994 reported that Saponin production increased with sucrose levels of upto 60g/l. they also reported inhibition of cell growth at 70 g/l concentration of sucrose.
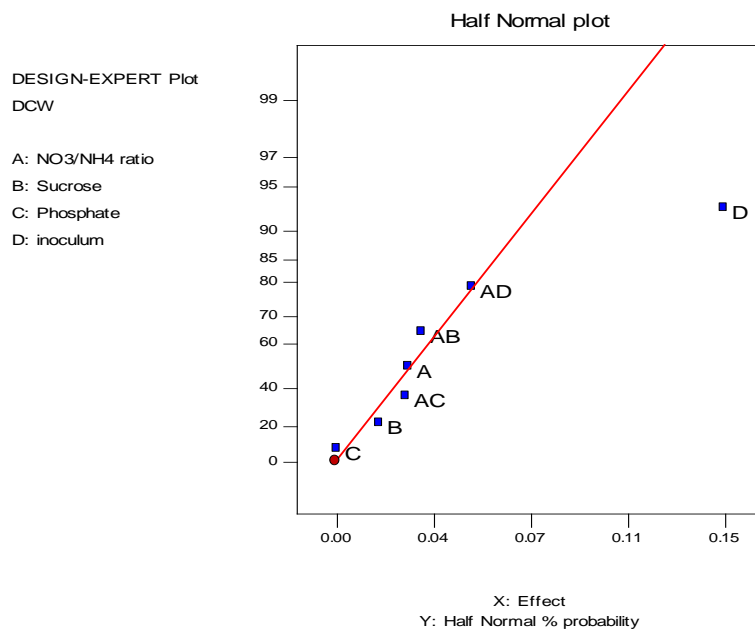
Half Normal plot

DESIGN-EXPERT Plot
DCW

A: NO3/NH4 ratio
B: Sucrose
C: Phosphate
D: inoculum

X: Effect
Y: Half Normal % probability

Fig 5.17 Half Normal Plot

5.6 ELICITATION AND ESTIMATION OF BACOSIDE A

Suspension culture was treated with three elicitors namely Copper sulphate, Chitosan and Casein Hydrolysate at varying concentrations of 50μg/l, 100μg/l and 200μg/l. There was no extractable amount of yield was obtained. So the amount of Bacoside produced could not be estimated by HPLC. The standard HPLC graph of Bacoside A at concentration of 100μg/ml was shown in figure 5.17.
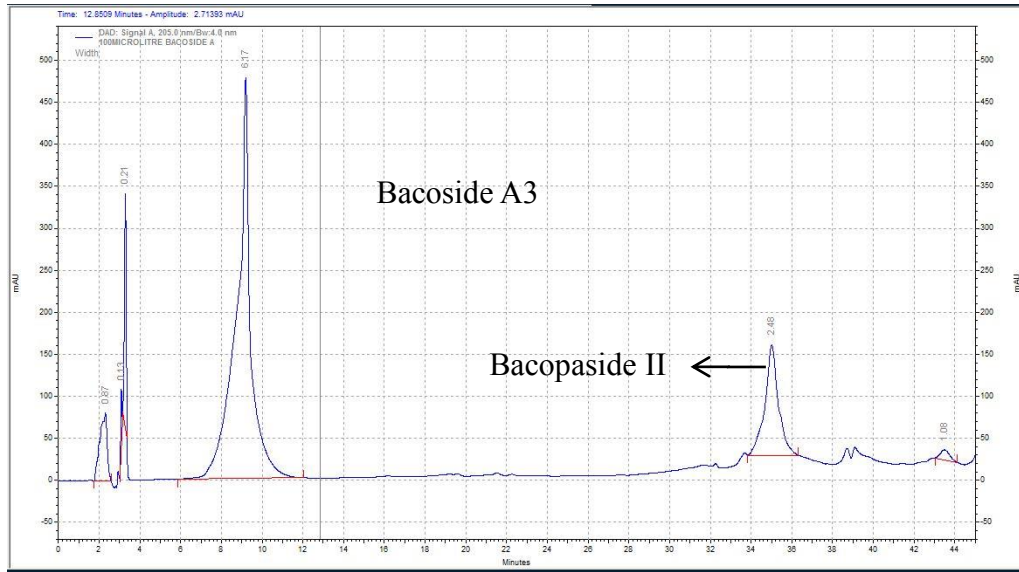
Fig 5.18 Standard HPLC graph of Bacoside A

CHAPTER 6

CONCLUSION

The *In Silico* studies are showing the potential of Bacoside A to be a drug for Alzheimer. The docking potential of Bacoside A on AChE and MAGL is comparatively higher than any other reported phytochemicals. Bacoside A is having binding energy of -20.1 and -12.7 on the targets AChE and MAGL respectively. It has been calculated by Molecular Docking using AUTODOC vina software tool.

From the results of Toxicity predictor, we can come to a positive conclusion that Bacoside A not much toxic. It is coming under Class 4 toxicity which is practically non-toxic. The druglikeness of Bacoside A was measured by bioactivity score using molinspiration tool.

The hormone concentrations were standardised for better callus induction.The 0.50 mg/l concentration ofNAA resulting in better callus induction than other concentrations. IAA was not showing significant callus inductions.To inhibit the root inductions small amount of cytokinins need to be added.

The most influencing parameters for the production of biomass in suspension cultures were analysed by Plackett Burman design. Inoculum size and the sucrose concentrations were the most influencing factors.

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