ISOLATION,CHARACTERIZATION AND EVALUATION OF ANTI-ALZHEIMER ACTIVITY ON THE AERIAL PARTS OF *OXYSTELMA ESCULENTUM* R.BR.

A Dissertation submitted to
THE TAMIL NADU Dr.M.G.R. MEDICAL UNIVERSITY
CHENNAI-600 032

In partial fulfilment of the requirements for the award of the Degree of

MASTER OF PHARMACY
IN
PHARMACEUTICAL CHEMISTRY

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COLLEGE OF PHARMACY
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CERTIFICATE

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EXAMINERS

1.

2.

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LIST OF ABBREVATIONS

AD	ALZHEIMER DISEASE
O.ESCULENTUM	OXYSELMA ESCULENTUM
MRI	MAGNETIC RESONANCE IMAGING
CT	COMPUTED TOMOGRAPHY
OECD	ORGANIZATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
UV	ULTRA-VIOLET SPECTROSCOPY
FT-IR	FOURIER TRANSFORM INFRA-RED SPECTROSCOPY
1 H-NMR	PROTON NUCLEARNMAGNETIC RESONANCE SPECTROSCOPY
LC-HRMS	LIQUID CHROMATOGRAPHY-HIGH RESOLUTION MASS SPECTROSCOPY
TLC	THIN LAYER CHROMATOGRAPHY
CPCSEA	THE COMMITTEE FOR THE PURPOSE OF CONTROL AND SUPERVISION OF EXPERIMENTS ON ANIMALS
MDA	MALONDIALDEHYDE
PDB	PROTEIN DATA BANK
ANOVA	ANALYSIS OF VARIANCE
SEM	STANDARD ERROR OF MEAN
RPM	REVOLUTIONS PER MINUTE
Rf	RETENTION TIME
LD_{50}	MEAN LETHAL DOSE
IC_{50}	INHIBITION CONCENTRATION AT 50%
AChE	ACETYLCHOLINESTERASE
GSH	REDUCED GLUTATHINE
DTNB	DINITROBENZOIC ACID

CHAPTER – 1
INTRODUCTION

ALZHEIMER DISEASE

Alzheimer disease (AD) is a progressive neurodegenerative disease results in death of brain cells.It causes memory loss and cognitive decline.The symptoms are mild at first and becomes more severe overtime.The disease was named after the German Physician Aloes Alzheimer in 1906. According to National Institute on Aging Alzheimer disease is the sixth major cause of death in U.S.Old aged people are more prone to AD.[1]

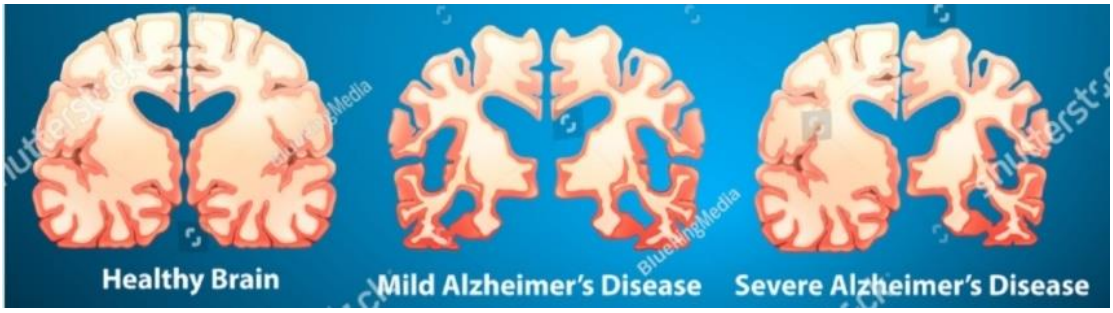


Fig:1 Progression of Alzheimer Disease. *[5]*

The main features of AD is the

- Presence of Plaques and Tangles in brain
- Loss of connections between the nerve cells in brain.

DEMENTIA

Dementia is a general term for the symptoms affecting memory,communication and thinking. Alzheimer disease is the most common form of dementia.Types of dementia are

- Alzheimer disease
- Vascular dementia
- Dementia with lewy bodies
- Temporal lobar dementia.[2]

CAUSES OF ALZHEIMER DISEASE

The brain has 100 million nerve cells.Each nerve cell connects with one another to form communication networks.Each group of nerve cells have specific function.some neurons are involved in thinking,learning and memory.Some neurons are involved in hearing and smelling.They process and store the information and communicate with other cells.In diseased condition brain cells fails to function normally,which disrupts the neurons and triggers a series of toxic events.Neurons get damaged and loses connection to each other and eventually die.[3]

AD are focussed on two types of proteins **PLAQUES AND TANGLES.**

They leads to destruction of nerve cells in the cerebral cortex of brain.

PLAQUES

Beta amyloid is a fragment of larger protein.When these fragments closer together they appear to have toxic effects on neurons and disrupts the cell to cell communication.These clusters form larger protein called amyloid plaques.These biochemical events leads to neuronal death with subsequent production of free radicals.[4] These beta amyloid protein is formed by the cleavage of the amyloid precursor protein (APP).These event is mediated by the enzyme β-secretase.

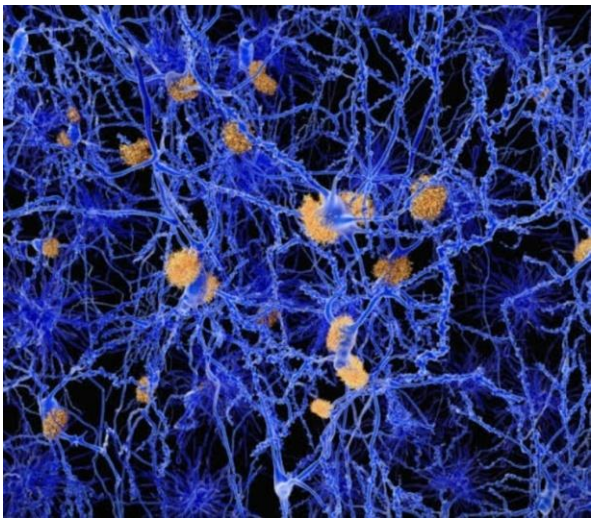


Fig:2 Accumulation of plaques in neurons[6]

TANGLES

Tau proteins play a part in neuronal internal support and transport system to carry nutrients and other essential materials. AD tau proteins change their shape and organize themselves to a new structure called neurofibrillary tangles.These tangles disrupt transport system and are toxic to nerve cells.[4]

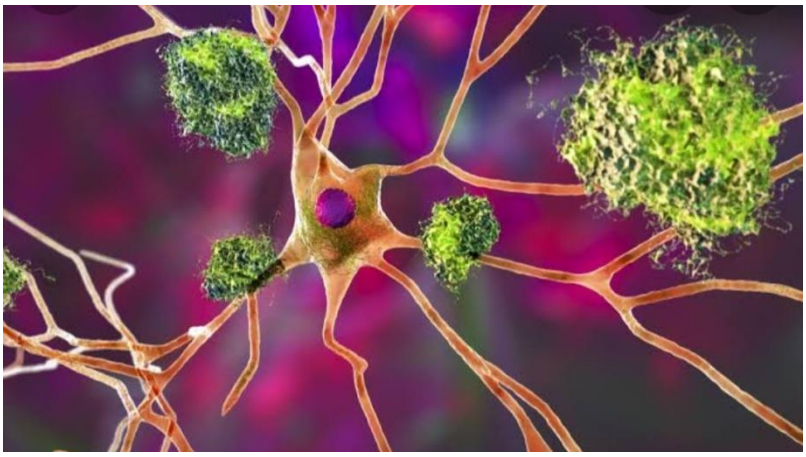


Fig:3 Accumulation of tangles in neurons[7]

NEUROTRANSMITTER IN ALZHEIMER DISEASE

Both Cholinergic and Glutamatergic transmission involved in the etiology of Alzheimer disease.

The loss of memory is considered to be the result of shortage of neurotransmitter Acetylcholine. It is possible to increase the level of this transmitter by inhibiting the activity of enzyme acetylcholinesterase which splits or breakdown the transmitter substance.

Glutamatergic hypothesis links the cognitive decline in patients with Alzheimer to neuronal damage resulting from overactivation of N-methyl-D-aspartate (NMDA receptors) by glutamate. Low level activation of NMDA receptors which are pivotal in learning and memory which may result from deficiencies in glutamate reuptake.[8]

TYPES OF ALZHEIMER DISEASE[9]

- **Early onset AD (**Occurs between 40-60 years of age)
- **Late onset of AD (**Occurs above the age of 65)
- **Familial AD (**Rare AD caused by mutations in genes)

ALZHEIMER DISEASE – A GLOBAL BURDEN

The old aged people are more prone to AD.Alzheimer affects about 36.5 millions of the world population.An estimated 0.7%of the gobal population has dementia cases.The total number of persons affecting from AD has doubled from 1990 to 2019.Japan has the highest prevalence (3,097 cases per 100,000) f ollowed by Italy,Slovenia,Monaco,Greece and Germany.The prevalence is higher in high income countries like Western Europe compared to Asia and Africa.Women are more likely to be affected than men.More than 6 million Americans are living with AD.[10,11,16]

In United States,Alzheimer and dementia deaths have increased 16% during COVID -19 pandemic.

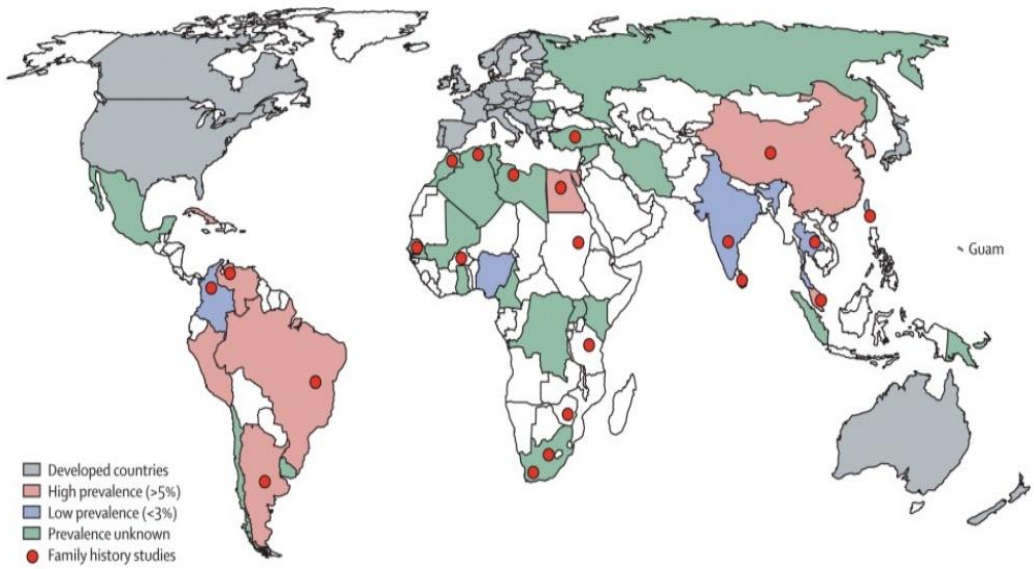


Fig:4 Worldwide prevalence of Alzheimer disease[12].

FACTORS RESPONSIBLE FOR ALZHEIMER DISEASE

Alzheimer disease caused by a combination of genetic,lifestyle and environmental factors that affect the brain overtime.The factors responsible for AD are as follows[14]

- Aging
- Family history
- Genetics
- Cardiovascular problems
- Type 2 Diabetes
- Poor diet and excercising habit
- Head injury
- Lack of mental ability
- Smoking
- Drinking

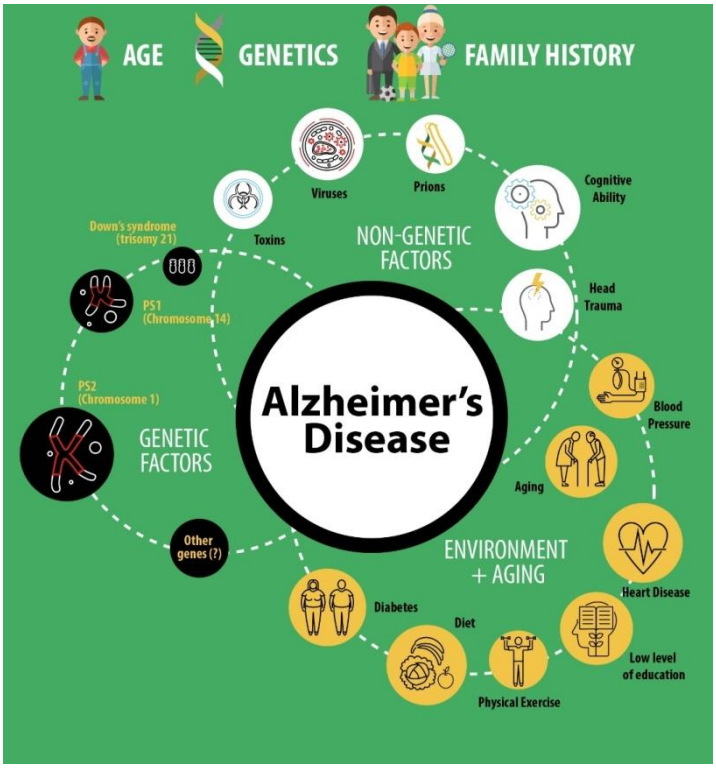


Fig:5 Factors the cause of AD.[13]

SYMPTOMS OF ALZHEIMER DISEASE

The symptoms progresses slowly over several years.The symptoms differ from individual to individual. The other conditions like stroke, delirium are also responsible for the symptoms to worsen.The mild and moderate symptoms are[17]

Early symptoms are

- ✓ Trouble in thinking
- ✓ Forget recent conversation,name of places
- ✓ Hard to make decision

Middle stage symptoms are

- ✓ Confusion and disorientation
- ✓ Impulsive behaviour
- ✓ Mood swings

Later stage symptoms are

- ✓ Hallucinations and delusions
- ✓ Weight loss
- ✓ Unintentional passing of urine
- ✓ Gradual loss of speech
- ✓ Significant problem with short and long term memory.

DIAGNOSIS OF ALZHEIMER DISEASE

Alzheimer disease is usually diagnosed bsed on the persons medical history,history from relatives and behavioural observations.The presence of characteristic neurological and neurophsychological features and absence of alternative conditions supports the diagnosis[18]

Recent advances to diagnose the Alzheimer disease are

- Blood studies
- Brain MRI /CT scan
- SPECT (Single **Positron** Emission Computed Tomography)
- PET (Positron Emission Tomography)
- Lumbar puncture
- Genotyping
- Electroencephalography

MANAGEMENT OF ALZHEIMER DISEASE

- To maintain patients brain function as far as possible
- To treat psychiatric and behavioural sequale
- To focus on emotional and supportive care of concerned patient
- To reduce morbidity and mortality as far as possible
- To improve quality of life

ORAL ANTI-ALZHEIMER DRUGS

The deficiency in cholinergic neurotransmission of AD leads to cholinesterase inhibitor as first line symotoms of disease.[19] The clinical benefits of these agents include improvements,stabilised or less than expected decline in function,cognition and behaviour.Several new Alzheimer drugs have been approved in recent years but the exact cause of AD is unknown

It is important to understand the safety and efficacy of these medication for proper use therapies for alzheimer disease.

There are different class of anti alzheimer drugs are available[20]

S. No	Drug class	Examples of drug	Adverse effect
1	Cholinesterase Inhibitor	Donepezil, Rivastigmine Galantamine, Tacrine	Muscle cramps, bradycardia, Hepatotoxicity
2	NMDA antagonist	Memantine	Cataract, Agitation
3	PPAR Gamma antagonist	Pioglitazone	Nausea, vomiting
4	Antioxidants	Gingo biloba, Vit E, Melatonin	Haemorrhage
5	Gamma secretase inhibitor	Semagacest	GIT irritation
6	Statins	Simvastatin, pravastatin	Liver damage
7	Others	Heavy metals, Estrogen, Anti-inflammatory drugs	Stomach ulcers, Dizziness, Headache

MEDICINAL HERBS TO TREAT ALZHEIMER

Herbal medicines have their origins in ancient cultures, including those of the Egyptian, Indians and Chinese. It involves the use of medicinal plants to treat AD and enhances general health and well beings. In fact, many pharmaceutical drugs are based on the synthesized adaptations of naturally occurring compounds found in plants.[21]

In recent years, interest in herbal medicine has increased, leading to a greater scientific interest in the medicinal use of plants in treating disease and improving health, often without any significant side effects. Herbal medicines and natural products are the oldest remedies known to mankind. Medicinal plants have been used by all cultures throughout the history. In present scenario, the demand for herbal products is growing, exponentially throughout the world.

In human body, the nervous system coordinates and regulates the various voluntary and involuntary activities of the body. the central nervous

system and the autonomic nervous system are interlinked and some drugs affect the CNS producing reactions associated with the autonomic system .

Drugs involved with the CNS may have general stimulatory or depressant action with anticonvulsant and psychopharmacological activities. Memory deficit is a major global health problem. Current therapies are inadequate and have numerous adverse effects. There is an urgent need for possible alternative treatments for AD and memory deficit. Various medicinal plants are prescribed to enhance the memory. We have reviewed the literature on medicinal plants used in the treatment of AD and memory deficit.

Several medicinal plants have been used for decades in different cultures to improve memory such as *Valeriana officinalis*,*Punica granatum* l., *Salvia officinalis*, *Myristica fragrans Bacopa monnieri* linn, *Centella asiatica* linn and *Evolvulus alsinoides* linn. Elufioye et al. (2012) reported some plants used as anti-aging and memory enhancing activities in Sagamu, Nigeria that are *Bacopa floribunda*, *Angraecum eichlerianum*, *Parquet inanigrescens*, *Cleome gynandra*, *Dalbergia lacteal*, *Capsicum fructescens*, *Aframomum melegueta*, *Digitaria debilis*, *Musa sapientum*, *Bryophyllum pinnatum*, *Abrus precatorius*, *Ficus exasperate*, *Dioscorea mangenotiana*, *Jatropha curcas*, *Spondia smombin*, *Capsicum frutescens*, *Cola acuminate*, *Mirabilis jalapa*, *Elaies guineensis*, *canna indica*, *Ipomoea mauritania*, *Bambusa vulgaris*, *Cordia millenii*, *Piper guineense*, *Dioclea sarmentosa*, *Cucumeropsis mannii*, *Eleusine indica*, *Ocimum basilicum*, *Khaya ivorensis*, *Carpolobia alba*, *Carapa procera*, *Entandrophragma utile*, *Xylopiaae thiopica*, *Garcinia kola*, *Theobroma cacao*, *Milicia excels*, *Blighia sapida*, *Baphia nitida*, *Peperomia pellucid*, *Vernonia amygdalina*.

These herbal plants shows various potential activities like free radical scavenging activities, Anti-amyloidogenic,Anticholinesterse, Hypolipidemic, Antioxidant and Anti inflammatory activities.

The major steps in the discovery of bioactive compounds from plants are solvent extraction, bioassays, isolation, characterization, toxicological

evaluation, preclinical and clinical investigation of isolated bioactive compounds.[22]

TERPENOIDS AS ANTI-ALZHEIMER AGENTS

Ginsenosides from *Panax ginseng*

Ginsenosides are a series of derivatives of the dammarane-type triterpenes with some sugar moieties attached, which are active components in ginseng.Among the diverse ginsenoside Rg1, Rg2,Rg3 reduces the β-amyloid protein levels by promoting Aβ degradation and by enhancing reprilysis gene expression.[44]

Ginkgolides and *Bilobalide from Ginkgo biloba*.L

Ginkgolides are cyclic diterpenes of labolane type commonly isolated from G.biloba.Extract of G.biloba leaves which contains 24% flavanoid glycosides, 6% terpenes, 5-10% organic acids has been extensively evaluated for its neuroprotective effects.Ginkgolides revrse the Aβ induced reduction of acetylcholinesterase release from hippocampal brain.

Cannabinoids from *Cannabis sativa*`L

Cannabinoids are the aromatic compounds containing a monoterpene isolated from *C.sativa*. This plant contains about 60 cannabinoids,, and one of potential anti-alzheimer agent is Tetrahydro cannabinol (THC).THC competitively inhibits AchE and increases the availability of Ach helps in memory.

Other terpenoids having potential anti-alzheimer effects

Cornel iridoid glycosides loganin isolated in the fruits of *Cornus officialis* improves memory deficits and tenuates hippocampal neuronal loss by improving the brain improvement for repair and promoting neuronal survival.

Oleanolic acid, tenuifolin is triterpene has reported to be beneficial for AD.

A screening effort to identify potent AchE inhibitors from medicinal herbs leads to isolation of ursolic acid.Ursolic acid competitively inhibits AchE in dose dependent manner.

FLAVANOIDS AS ANTI-ALZHEIMER AGENTS

Flavanoids are a class of secondary metabolites that always attract the attention of pharmaceutical industry due to their versatility in therapeutic properties.More than 9000 flavanoids have been identified.[43]

Fiavanols (rutin,quercetin,catechin,epicatechin), Isoflavanes(glycetin), Flavanones, Flavanes, Anthocyanins etc.are used to treat AD.

Flavanoids highlight the potential effects against AD by protect neurons against oxidative stress,subsequently reduce the reactive oxygen species production suppresses the neuro-inflammation.

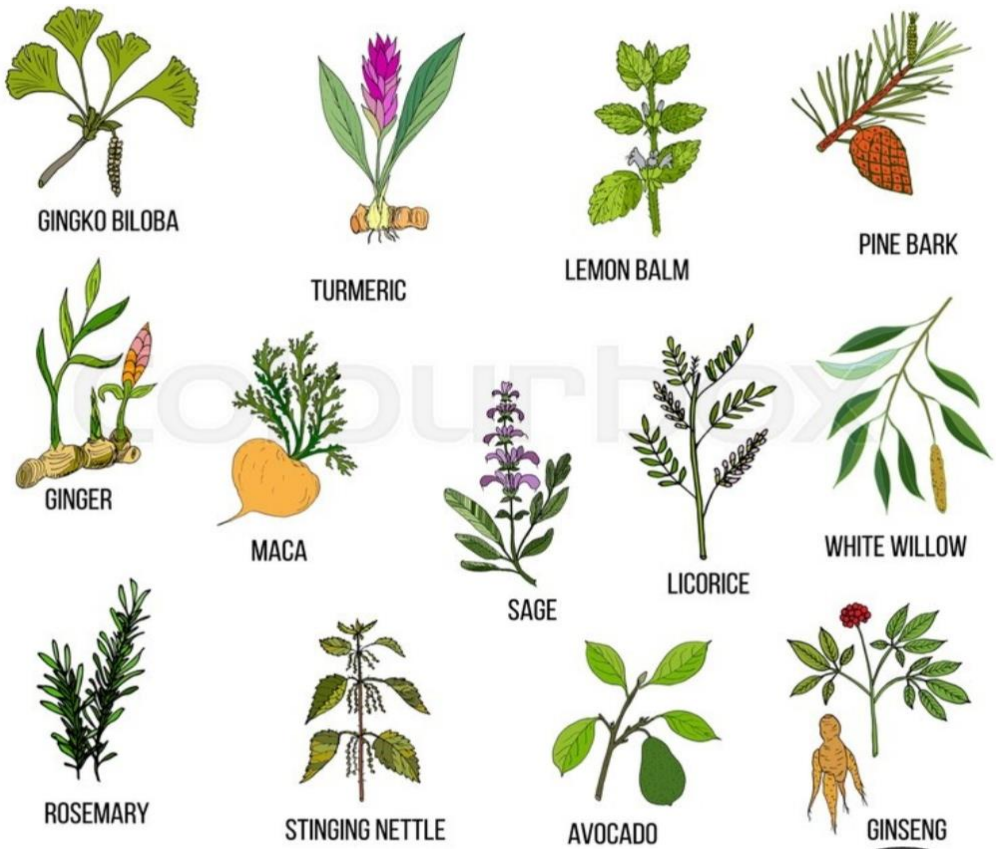


Fig:6 Herbs used for Alzheimer disease.[23]

IN VITRO ANTI-ALZHEIMER MODEL

The bioactive compounds needed to be validated by *in vitro* and *in vivo* model.The anti-alzheimer compounds are assayed by *in vitro* acetylcholinesterase inhibition assay.The acetylcholinesterase enzymes are involved in the breakdown of neurotransmitter substance Acetylcholine.Therefore inhibition of acetylcholinesterase enzyme leads to availability of neurotnsmitter acetylcholine which delays the development of disease.[24]

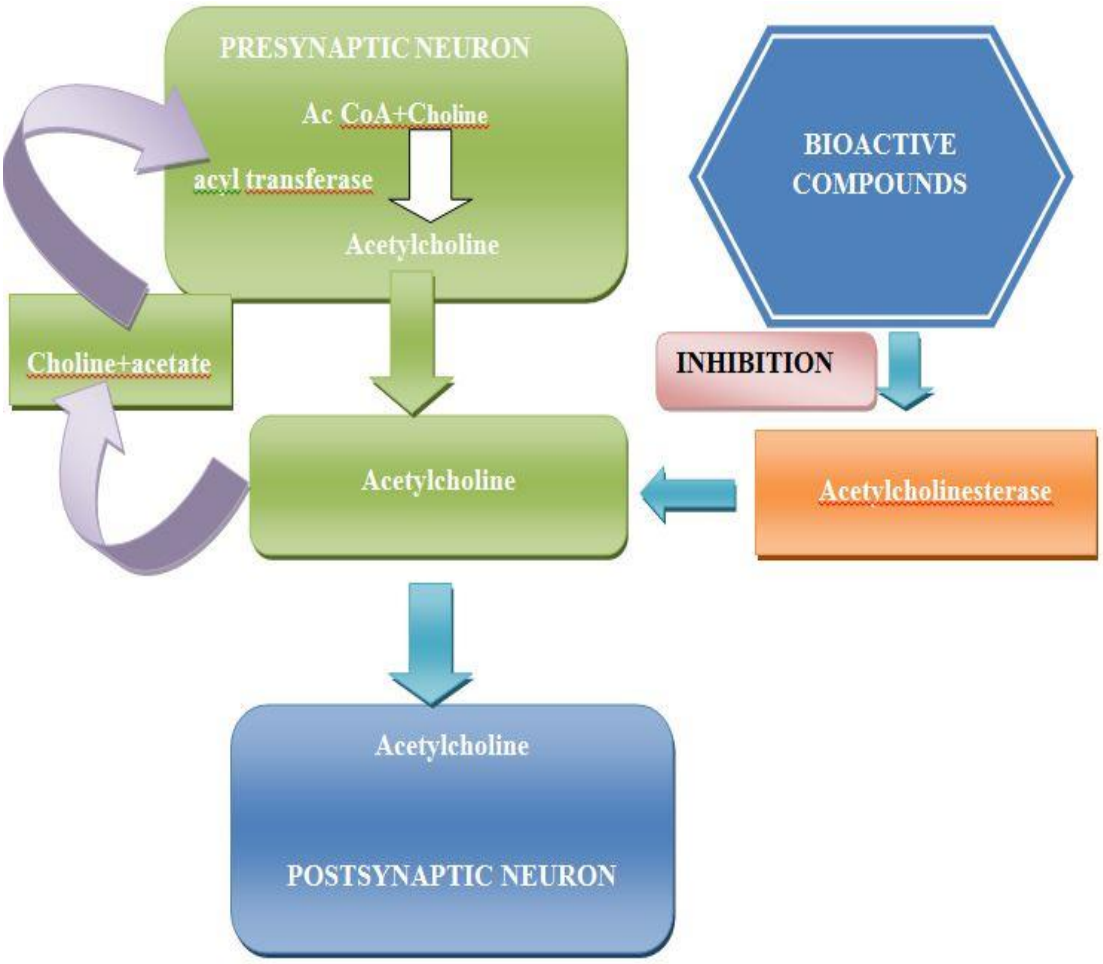


Fig:7 Inhibition of acetylcholinesterase enzyme[26,27]

BIOACTIVE COMPOUNDS

↓

Inhibits acetylcholinesterase enzyme

↓

Prevents breakdown of acetylcholine into choline and acetate in synaptic cleft

↓

Increase the availability of neurotransmitter acetylcholine in post synaptic membrane

↓

Anti-alzheimer activity[24]

IN VIVO ANTI-ALZHEIMER MODEL

Scopolamine induced Alzheimer disease

Scopolamine is muscarnic receptor antagonist which blocks cholinergic neurotransmission leading to memory impairement.Scopolamine is used to treat motion sickness and antispasmodic.Administration of Scopolamine leads to increasing beta-amyloid deposition and increase in reactive oxygen species results in deficits in memory.[28]

MOLECULAR DOCKING APPROACH

The molecular docking study was performed to findout the potential molecular target for natural anti-alzheimer compounds.The docking study was carried out by using software such as as AutoDock®, Argus lab®, Glide® etc.The docking score (binding energy)reveals that the compounds contained in the extracts of plants have capacity to be used as inhibitor for Alzheimer target.

LIST OF SOME PATENTS FOR HERBAL TREATMENTS OF ALZHEIMER

PATENT APPLICATION NUMBER	TITLE	INVENTION
US20020146467A1	Herbal composition for the treatment and prevention of dementia	The present invention relates to herbal composition for the prevention of dementia comprising Polygonium multiflori(*Polygonium multiflorum),*Polygalae radix,Caryophyllus flos,Xingiberis Rhizoma crodus in conventional herb composition to maximize anti-dementia effect with minimal amount.[29]
US7429397B2	Herbal formulation as memory enhancer in Alzheimer condition	This invention provides a novel herbal formulation used as brain tonic,improves memory nd helps in recalling of thoughts.[30]

In this present research,the plant Oxystelma esculentum R.BR has been selected. The literatures pertaining to phytochemical evaluation are very scarceand anti-alzheimer activity has not been investigated.Hence an attempt has been made to evaluate the invitro and invivi anti-alzheimer activity of aerial parts of Oxystelma esculentum R.BR. and to isolate the active constituent from active extract responsible for anti-alzheimer activity.The isolated pure compound has been attempted for molecular docking analysis.

CHAPTER – 2
REVIEW OF LITERATURE

PHYTOCHEMICAL REVIEW OF *OXYSTELMA ESCULENTUM* R.BR

1. **Savitha S and Balamurugan S; (2016)[45]** evaluated the phytochemical constituents in the extracts of *Oxystelma esculentum* R.BR with pet ether, chloroform, ethyl acetate, methanol as solvents. The phytochemical analysis of plant revaled the presence of alkaloids, tannins, steroids, saponins, terpenoids, flavanoids, glycosides and phenolic compounds. All these extracts show various level of antimicrobial activity but methanol extract shows potent antimicrobial activity.

2. **Pandya DJ and Anand IS; (2012)[46]** isolated epicatechin from methanlic extract and Kaempferol from pet ether extract *Oxystelma esculentum* R.BR.and their structure were elucidated.

3. **Poornima.N and Umarajan KM;(2009)[47]** determined the anatomical features of *Oxystelma esculentum* R.BR. root, rhizome, stem, leaf, petiole for description of its purity and identity and also investigated the phytochemical analysis revealed the presence of tannins, flavanoids, terpenoids, cardiac glycosides and alkaloids.

4. **Durairaj Ashok Kumar et al; (2008)[40]**evaluated the antioxidant and free radical scavenging properties of *Oxystelma esculentum* R.BR. The total antioxidant activity of methanol extract of *O.esculentum* increases with increasing concentration. The methanol extract scavenges the hydrogen peroxide in dose dependent manner. Finally the extract of *O.esculentum* proved its strong anti oxidant activity and used as a natural antioxidant.

ANTI-ALZHEIMER ACTIVITY OF OTHER MEDICINAL PLANTS

5. **Vaishali J Mahadik and Monika N; (2020)[48]** performed the cognition enhancing effect of various extracts of fruits of *Sesbania glandiflora*, pet ether extract, benzene extract, chloroform extract, acetone extract and ethanolic extract in scopolamine induced amnesia mice. Morris water method and Elevated plus maze method was done to evaluate the cognitive performance. Scopolamine was used to induce amnesia and Piracetum was used as standard. Various biochemical parameters such as acetylcholinesterase, glutathione, malondialdehyde from brain homogenate were evaluated. Pretreatment with various extracts possess cognition enhancing property in scopolamine induced amnesia mice

6. **Prachi Saini et al; (2019)[49]** investigated *in vivo* anti-alzheimer activity in amnesia induced albino mice from isolated compounds Karanjin and Embelin. Karanjin was isolated *from Pongomia pinnata* and Embelin from *Embelia aribes*. The anti-alzheimer activity of isolated compounds is evaluated through elevated plus maze model and Morris water maze model on swiss albino mice. Diazepam was used for induction of Alzheimer effects and Piracetum was used as standard treatment. The increase and decrease in transfer latency was recorded in elevated plus maze model. The isolated compounds and standard significantly reversed amnesia induced by diazepam and improved learning and memory of mice in dose dependent manner.

7. **Anil Yilmaz and Mehmat Boga; (2016)[50]** isolated two terpenoids from diclromethane extract and two novel terpenoids from ethanol extract of *Nepta obtusicrena*. These isolated novel terpenes shows anti-alzheimer activity by lipid peroxidation inhibition and potent acetylcholinesterase inhibition.

REVIEW ON ALZHEIMER TARGET PROTEIN

8. **Hemalatha S, Jason Tom Abraham; (2021)[58]** docked the potential compounds of *Brassus flabellifer* against the Alzheimer target proteins acetylcholinesterase and β-secretase enzyme. Five compounds showed best interaction with acetylcholinesterase and three compounds showed best interaction with β-secretase

9. **Zhen Zhong et al; (2018)[57]** evaluated potential ability of *Gingko biloba* to bind with acetylcholinesterase enzyme by molecular docking. Docking results indicated the ligand bind tightly with the active site of human acetylcholinesterase, with hydrogen bond interactions. Among the different isolated compounds of *Ginkgo biloba* Diosmetin compound was found to be the best predicted docking score.

10. **Rithvik Ganesh et al; (2017)[56]** performed molecular docking studies of plant alkaloid derivatives as inhibitors of various drug targets of Alzheimer disease. 150 derivatives of *Curcumin, Bacopaside, Ginkolide B* are docked with multiple target proteins β-secretase, cholinesterase, tau proteins.Results are found to be 23 ligands are good fit with the drug target. *Ginkgolide B* shows excellent binding studies with the multiple drug targets.The proposed drug act as cholinesterase inhibitor, thus used as symptomatic drug to relieve Alzheimer disease.

CHAPTER – 3
AIM AND OBJECTIVES

AIM

To isolate, characterize and evaluate an Anti-alzheimer activity from the aerial parts of *Oxystelma esculentum* R.BR.

OBJECTIVES

- To carryout successive extraction on dried powdered leaves of *Oxystelma esculentum* R.Br. by **hot continuous percolation** in soxhlet apparatus using solvent such as Hexane, chloroform, ethyl acetate and ethanol.
- To carry out preliminary **phytochemical screening** for the extract for the detection of active constituens.
- To perform bioactive guided isolation.
  - ✓ The extracts were planned to undergo *in vitro* **anti Alzheimer activity** by acetylcholinesterase inhibition assay.
  - ✓ Based on IC_{50} values of all the extract against acetylcholinesterase an active extract will be selected for isolation by column chromatographic method.
- Acute toxicity study will be carried out for an active extract by **OECD423** guidelines.
- The active extract of safer dose $1/5^{th}$ and $1/10^{th}$ will be subjected for *in-vivo* Anti-alzhiemer study in Scopolamine induced amnesia mice.
- To carryout isolation of selected active extract by **column chromatography** using gradient elution technique.

- The active extract will be subjected for analysis by Quadrapole Time of Flight (QTOF) for library database.
- To evaluate *in vitro* anti-alzheimer activity of an isolated compound.
- The isolated compound will be characterized by **UV, IR, ^{1}H-NMR.**
- To evaluate *in-silico* molecular properties of an isolated compound by **Molinspiration Cheminformatics®** software tool.
- To perform molecular docking studies of the isolated compound on Alzheimer target protein using **Autodock® software Version1.5.6.**

CHAPTER – 4
SCHEME OF WORK

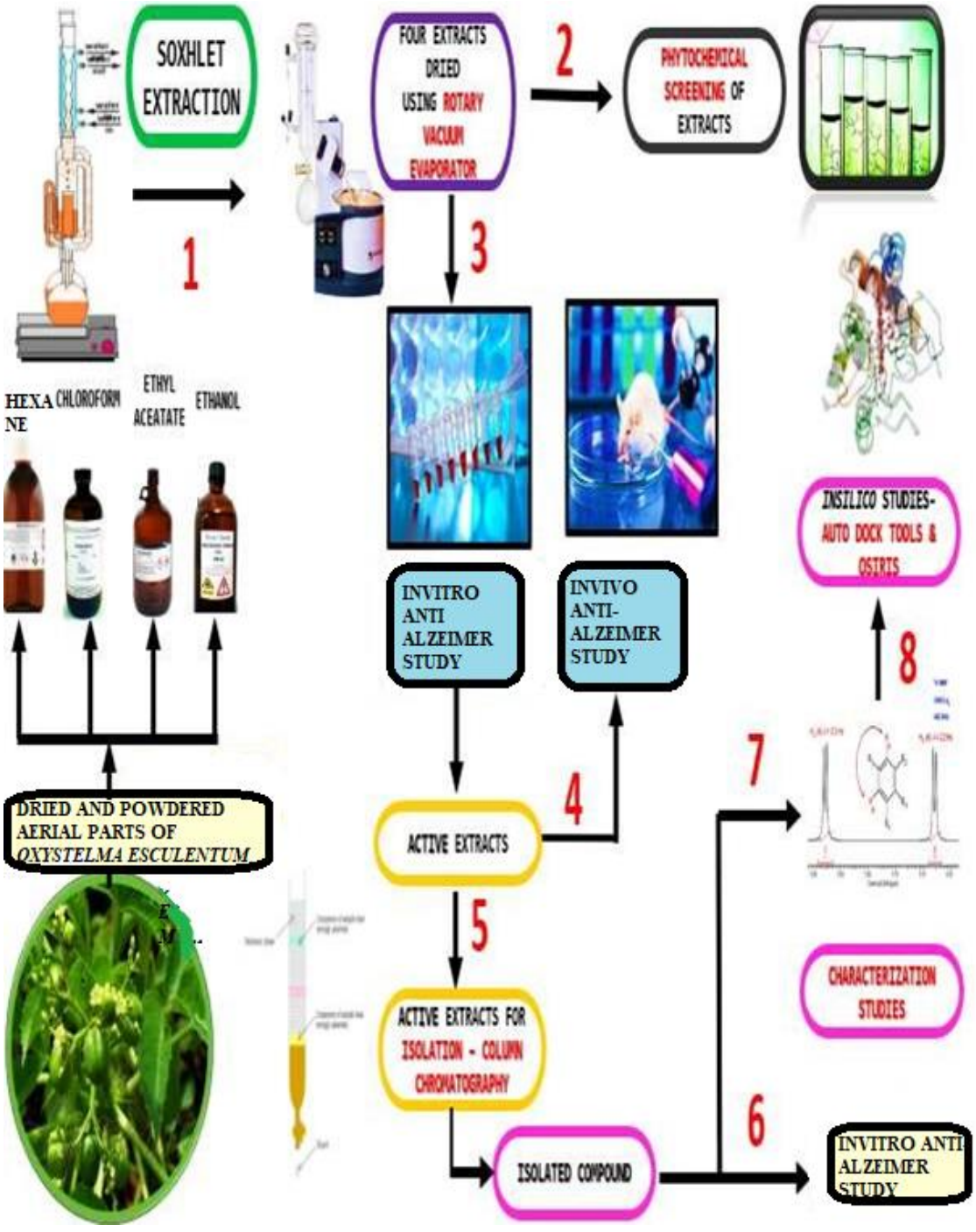


Fig:8. Outline of research

CHAPTER – 5
PLANT PROFILE

Fig-9: Plant of Oxystelma Esculentum R.BR[37]

OXYSTELMA ESCULENTUM R.BR.

Synonym	:	Oxystelma Alpine Decne
Family	:	asclepiadaceae

Vernacular names[38]

English	:	Rosy Milkweed Vine
Tamil	:	Usippaalai
Telugu	:	Dudhipala
Malayalam	:	Kulapala
Kannada	:	Dugdhika
Marathi	:	Dudhani
Gujarathi	:	Jaladhudhi
Hindi	:	Dudhialata

Habit	:	Twinging Subshrubs
Habitat	:	Moist Ground Canal Banks.
Colour of the flower	:	Whitish Purple
Distribution	:	Uttarpradesh, Maharastra, Andhrapradesh, Bihar, Tamilnadu (Pudukottai, Kanyakumari, Coimbatore)

Description

Rosy milkweed vine is a perennial creeper, which becomes 1-2m long.it has fibrous roots emerging from lower nodes of stem.[41] numerous slender,twinning stems are branched. Oppositely arranged linear-lance like leaves with round bases are 4-6 cm long. White purple handsome flowers occurs as raceme like cymes 10-15 cm long.flowers can be sometimes almost white. The drooping,5 petalled,sauce shaped flowers are pink purple veined. It has corona of 5 erect lobes in the centre lance like follicles are 3-6 cm long and are edible. The plants are good source of famine food.[37]

Flowering of this plant is from July to February

Constituents[42]

- Pregnane glycosides
    1. Oxysine
    2. Oxyline
    3. Esculentum
- Cardenolides
    1. Oxystelmoside
    2. Oxystelmine

- Flavanoids
- Terpenoids
- Cardiac glycosides

Medicinal uses[41]

The plant has many therapeutic uses which are vital importance in curing the modern world like

- ✓ Cancer,
- ✓ Hepatitis,
- ✓ Kidney disorders,
- ✓ Stress related disorders and
- ✓ Microbial infections.

CHAPTER-6
MATERIALS AND METHODS

6.1 COLLECTION OF PLANT

The leaves of ***Oxystelma esculentum*** **R.BR**. were collected from Tamil Nadu(forest of kalakatu) Tirunelveli District,India. Taxonomic identification was made from botanical survey of medicinal plants, Siddha Unit, Government of India, Palayamkottai authenticated by V.Chelladurai Botanists. Authenticated register No:XCH 40379. Fresh aerial parts of the plant were shade dried at room temperature, ground into coarse powder and stored in airtight containers.

6.2 PREPARATION OF PLANT EXTRACTS

The dried powdered aerial parts of *Oxystelma esculentum* R.BR. was extracted sequentially by hot continuous percolation method by **Soxhlet apparatus** using **hexane, chloroform, ethyl acetate and ethanol** as solvent.The collected extracts were concentrated by using rotatory vacuum evaporator and kept in desiccator.

Fig:10. Soxhlet extraction process of Oxystelma esculentum. R.BR.

6.3 PRELIMINARY PHYTOCHEMICAL ANALYSIS OF EXTRACTS

All the extracts were subjected to preliminary phytochemical screening for the detection of various plant constituents present.The nature of the constituents present in the plant helps to determine the biological and pharmacological activity. The hexane, chloroform,ethyl acetate and ethanolic extracts of *Oxystelma esculentum* R.BR.was subjected to the following chemical tests to identify the various active constituents present.[31,32,33]

TESTS FOR ALKALOIDS

DRAGENDROFF'S TEST

The extract was treated with Dragendroff's reagent and observed for the formation of yellow colored precipitate indicates the presence of alkaloids.

WAGNER'S TEST

The extract was treated with Wagner's reagent and observed for the formation of a reddish-brown precipitate indicates the presence of the alkaloids**.**

MAYER'S TEST

The extract was treated with Mayer's reagent formation of white precipitate or creamy coloured precipitate indicates the presence of alkaloids.

HAGER'S TEST

The extract was treated with Hager's reagent and observed for the formation of yellow precipitate indicates the presence of alkaloids.

TEST FOR CARBOHYDRATES

MOLISCH'S TEST

To 2ml of the extract 1ml of alpha-naphthol solution was added and concentrated sulphuric acid was added through the sides of test tube. Purple or reddish violet color at the junction of the two liquids revealed the presence of carbohydrates.

FEHLING'S TEST

To 1ml of the extract equal quantities of Fehling's solution A and B were added while heating formation of a brick red precipitate that indicates the presence of carbohydrates.

BENEDICT'S TEST

To 5ml of Benedict's reagent 1ml of the extract solution was added and boiled for 2minutes and cooled.Formation of red precipitate shows the presence of carbohydrates.

TESTS FOR GLYCOSIDES

LEGAL'S TEST

The extract was dissolved in pyridine and sodium nitroprusside solution was added to make it an alkaline.The formation of pink red to red color shows the presence of glycosides.

BALJET TEST

To 1 ml of the ethanolic extract was added with 1 ml sodium picrate solution and the yellow to orange color reveals the presence of glycosides.

BONTRAGER'S TEST

A few ml of dilute Hydrochloric acid was added to1ml of the extract solution .It was then boiled,filtered and the filtrate was extracted with chloroform.The chloroform layer was then treated with 1ml of ammonia. The formation of red color shows the presence of anthraquinone glycosides.

KELLER KILLIANI TEST

The extract was dissolved in acetic acid containing traces of ferric chloride and it was then transferred to a test tube containing sulphuric acid. At the junction,formation of a reddish brown color, which gradually became blue confirms the presence of glycosides.

TESTS FOR PHYTOSTEROL

LIBERMANN BURCHARD TEST

3ml of extract was mixed with 3ml of acetic anhydride. It was heated and then cooled. Few drops of concentrated sulphuric acid were added. Appearance of blue color shows the presence of phytosterol.

SALKOWSKI'S TEST

Dissolve the extract in chloroform and equal volume of concentrated sulphuric acid was added. Formation of bluish red to cherry red color in chloroform layer and green fluorescence in the acid layer represents the steroid components present in the extract.

TEST FOR FLAVONOIDS

SHINODA TEST

The extract was treated with 5ml of 95%ethanol.Add few drops of concentrated hydrochloric acid and 0.5g of magnesium turnings. Pink color was observed which shows the presence of flavonoids.

TEST FOR TANNINS AND PHENOLIC COMPOUNDS

FERRIC CHLORIDE TEST

1ml of the extract was added with ferric chloride and observed for the formation of a dark blue or greenish black color indicates the presence of Tannins and Phenolic compounds.

GELATIN TEST

The extract was treated with 1%gelatin containing 10% NaCl and observed for the precipitation which indicates the presence of Tannins and Phenolic compounds.

TESTS FOR PROTEINS AND AMINOACIDS

BIURET TEST

1ml of the extract was treated with 1ml of 40% sodium hydroxide solution followed by 2drops of 1% copper sulphate solution. Formation of a violet color shows the presence of proteins.

XANTHOPROTEIC TEST

1ml of the extract was treated with 1ml of concentrated nitric acid. A white precipitate was formed, it was boiled and cooled. Then 20% of sodium hydroxide or ammonia was added orange color was formed indicates the presence of aromatic amino acids.

LEAD ACETATE TEST

The extract was treated with 1ml of lead acetate. Formation of a white precipitate indicates the presence of proteins.

TEST FOR SAPONINS

About 1 ml of ethanol extract was diluted separately with distilled water to 20 ml and shaken in a graduated cylinder for15minute s.A1%1cm layer of foam indicates the presence of saponins.

TEST FOR FIXED OILS

SPOT TEST

A small quantity of the extract was pressed between two filter papers. Oil stains on the filter paper indicates the presence of fixed oils.

6.4 IN VITRO ANT-IALZHEIMER ACTIVITY ON EXTRACTS OF OXYSTELMA ESCULENTUM R.BR. BY ACETYLCHOLINESTERASE INHIBITION ASSAY

All the four extracts were subjected to in-vitro anti-Alzheimer's activity by acetylcholinesterase enzyme inhibition assay.

Procedure

Acetylcholinesterase Inhibition Assay

The acetylcholinesterase inhibitor activity of the test samples (hexane,chloroform, ethyl acetate and ethanol) where assayed by the following adaptation of spectrophotometric method reported by elman et al.[34]

The cuvette used as a blank to control for the nonenzymatic hydrolysis of acetylcholine contained a mixture of 500 µL of 3mM DTNB solution (in 0.1M potassium phosphate pH 8), 100 µL of 15mM AChI (in water), 275 µL of 0.1Mpotassium phosphate pH 8, and 100 µL of each cork and corkback ethanol-water extract solutions at the different concentrations (25, 50,100,200,400 and 800 µg/ml). In the reaction cuvette, 25 µL of buffer was replaced by AChE solution 0.16 U/mL. The resulting solutions were placed in a spectrophotometer. The thiocholine formed during the hydrolysis of acetylcholine reacts rapidly with DTNB and a yellow compound is formed. The reaction was monitored for 5 min at 405nm and the absorbance registered every minute. Velocities of reaction were calculated Enzyme activity was calculated as a percentage of the velocities compared to that of the assay using buffer solution instead of inhibitor (cork or corkback ethanol-water extracts). Positive assays were performed in triplicate.

$$\% \text{ of inhibition} = [(\text{OD of control} - \text{OD of test})/(\text{OD of control})] \times 100$$

The extract Exhibiting minimum IC50 value was selected as an active extract far isolation of anti-Alzheimer's compound using column chromatography.

6.5. ACUTE TOXICITY STUDY OF ACTIVE EXTRACT OF OXYSTELMA ESCULENTUM R.BR.IN MICE

Determination of acute oral toxicity is usually the initial screening step in the assessment and evaluation of the toxic characteristics of all compounds. The types of toxicity tests which are routinely performed by pharmaceutical manufacturers in the investigation of new drug involved in acute, sub-acute and chronic toxicity. Acute toxicity is involved in estimation of LD50(the dose which has proved to be lethal(causing death) to 50% of the tested group of animals).

The acute toxicity study of active ethanolic extract of *Oxystelma esculentum* R.BR. will be carried out as per (OECD) draft guidelines 423. The acute toxic class method is based on biometric evaluation with fixed doses, adequately separated to enable a substance to be ranked for classification purpose and hazard assessment.[35]

ACCLIMATIZATION OF ANIMALS:

OECD 423 guidelines will be followed. Swiss albino mice (25-30g) were obtained from the Centre for experimental animals in Madras Medical College (CPCSEA Registration no: 1917/GO/ReBi/2016/CPCSEA valid till 19-09-2026). After seven days of acclimatization,the three mice were randomly assigned for the acute toxicity groups. Animals will be allowed free access to standard pellet diet and water *ad libitum*. They will be maintained in controlled laboratory conditions of 12 hours dark/light cycle 22±2ºC temperature and 45-60% humidity.

ADMINISTRATION OF ACTIVE EXTRACT

The three mice were made to fasting prior to dosing (food was withdrawn overnight and water was withdrawn 3hrs before drug administration) following the period of fasting. The animals are weighed and the ethanol extract was administered in a single dose,as 1% suspension in carboxy methyl cellulose by oral intubation. Food was withheld for further one hour after the

administration of drug. The starting dose level was selected for the study with a dose of 2000mg/kg body weight.

OBSERVATIONS

Animals were observed individually after dosing periodically during the first 30 minutes to first 24 hours ,with special attention given during the first 4 hours and daily thereafter, for a total of 14 days. The time at which signs of toxicity appear and disappear was observed systematically and recorded for each animal.

Additional signs of toxicity such as changes in bodyweight, skin and fur, eyes and mucus membranes, respiratory system, circulatory system, autonomous system and central nervous system, somato motor activity and behaviour pattern were also recorded. Attention was given to observe the tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.

The mice will be observed regularly for 14 days to note the compound related mortality/morbidity or other toxic symptoms. Any mortality during the experiment for 14 days was observed and recorded. If no deaths were reported,the study was repeated with same dose to confirm the results.$1/5^{th}$ and $1/10^{th}$ of the safer dose will be selected for *in vivo* study.

6.6 In vivo anti-Alzheimer activity (Scopolamine induced amnesic mice)

Grouping and induction of amnesia

A total of 30 Swiss male mice were selected in this study which weighed approximately 20–30 g each, aged 6–7 weeks old.

The animals were divided randomly into five groups, each consisting of six animals. The animals had water ad libitum and food pellets. They were allowed to acclimatize to the laboratory environment for a week before the experiments. Group I, Control, was administered with normal saline orally and Group II, Negative Control, was administered with scopolamine 1mg/kg i.p.[54,36]

Groups III and IV were treated orally with a low dose of an extract of Oxystelma esculentum R.BR. and a high dose of an extract of Oxystelma esculentum R.BR. respectively, and induced with amnesia. Group V, standard, Donepezil will be administered orally. The experimental study and the drug treatment (extract & standard) duration was 15 days; scopolamine 1 mg/kg body weight (Deng et al., 2019) was administered i.p from the 8th day to the 14th day in Groups II, III, and IV & V. On the day of the behavioural test, the extract & standard (Donepezil) was administered 1 hour prior to the test.[51]

Table-1: Grouping of animals and their doses.

Group	Drug	Treatment	No. of. Animals
1.	Control	Normal saline	06
2.	Disease control	Scopolamine(1mg/kg)	06
3.	Test dose 1	Scopolamine+ low dose of active extract	06
4.	Test dose 2	Scopolamine+ high dose of active extract	06
5.	Standard	Scopolamine +donepezil(5mg/kg)	06

Behavioural study Y-maze test

Y-maze with the specifications of 40 x 8 x 15 cm (L x W x H) will be used. The arms were constructed in such a way that they are 120°C symmetrically disposed to each other. The floor was made up of dark opaque polyvinyl plastic. On the 15th day of the treatment, 1 hour before the test, the extract and donepezil were treated in the treatment group and all the mice were subject to the test. All the three arms of the maze were labelled as A, B, and C, respectively. The mice were placed at the end of the arm labelled “A” and one food pellet was placed at all the three arms in order to motivate the movement of the mice.

Each arm of the maze is labelled as either arm A, B, or C. In each session, the animal is placed in arm B and allowed to explore the three arms for

5 minutes. Number of arm entries and number of alternations are scored live in order to calculate the percent alternation. The entry is considered when all four limbs are within the arm. The alternation percentage is calculated by dividing the number of alternations by number of possible triads x 100. The maze is cleaned with Virkon solution or 70% alcohol between animals to eliminate odour traces. [53]

Open-Field Test

The open-field test is used to provide a qualitative and quantitative measurement of exploratory and locomotor activity in rodents. The apparatus consists of an arena surrounded by high walls, to prevent escape, and the floor of the open field is divided into squares. The Open Field Test measures hyperactivity through locomotion, exploration and anxious behaviour. The open field box consisted of a square box (60cm x 60cm x 25cm); made out of plywood with outlined a series of squares.

The open field represents a new stressful environment for the animal and allows for evaluation of locomotor activity, level of exploration, and emotional response in animals, in a single day. Animals that received Scopolamine and were analysed for memory in the Y-maze were immediately placed in the open field. The following parameters were noted for a period of 5 minutes for each mouse: the number of "crossing" (number of crossed lines or crossed tiles), the number of "rearing" (when the animal is placed on its hind legs by resting on the wall of the device with its front legs), and the time spent in the centre.[53]

TRACTION TEST

The effect of scopolamine, as well as the extract *of O.esculentum*, on motor co ordination of the mice was evaluated using the traction test.The experimental set up was done using a horizontal bar of 12 mm in size, one 12 inch length fixed in two poles at a height of 40 cm. The height of set up allows the animl sufficient time and space to land by losing muscle grip on its feet due to righting reflex.The setup was arranged on the tabletop and the animals were allowed to get a grip with their hind limbs on the horizontal bars.The total motivation time in seconds to hang on to the bar from the aversion to falling is

considered as grip index.The motivation time is considered to be proportional to muscle grip strength and balance. Once done with setup, the mice were suspended on the static bar and time taken for the mice to re establish themselves and retain on the bar was recorded.[53]

Biochemical studies

Tissue Preparation.

Immediately after the behavioural tests, the mice were sacrificed by cervical decapitation, and then, the brains were quickly removed and placed in boxes containing frozen saline for its solidification for 10min. The brain was introduced into a graduated cylinder followed by the addition of PBS (pH 7.4) to obtain the 10% homogenate. Each tube was centrifuged at 10000 rpm/min at 4° C for 15minutes, and the supernatant was collected for biochemical analysis.[54]

Estimation of Malondialdehyde.

A MDA assay was performed according to the protocol described by Wilbur et al. [21], with some modifications. For this assay, 500 ml of homogenate was introduced in the test tubes and 500 µl of Tris-HCl buffer (50 mM, pH 7,4) in the control tube. In each tube, 250 ml of trichloroacetic acid (TCA) 20% and 500 µl of TBA 0.67% were added. The tubes were closed with glass beads and then incubated in water bath for 10 min at 90° C. They were then left in room temperature for cooling before being centrifuged at 3000 rpm for 15 minutes. The supernatant was piped, and the absorbance was read with a spectrophotometer at 530 nm against the control. The concentration of MDA in mol/g was determined using the formula of Beer Lambert using the molar extinction coefficient 1:56 × 105 mmol-1 cm-1

Estimation of Catalase activity.

The activity of catalase was measured by the method of Aebi. In 0.1 ml of supernatant, 1.9 ml of 50 mM phosphate buffer (pH 7.0) was added. The reaction was initiated by the addition of 1.0 ml of newly prepared 30 mM

H2O2. The rate of decomposition of H2O2 was measured by spectrophotometry from absorbance changes at 240 nm. Catalase activity was expressed in units/mg protein. 2.8.3.

Estimation of Reduced Glutathione (GSH).

The reduced glutathione (GSH) was assessed in the brain supernatant using Ellman's reagent as described by Ellman [24]. Twenty (20)µl of brain homogenates was mixed with 3 ml of Ellman's reagent at room temperature. After one hour, the absorbance of the yellow compound was read at 412 nm using a spectrophotometer. The amount of glutathione was calculated with the formula of Beer-Lambert using the extinction coefficient value of 13.600 mol-1 cm-1.

Statistical analysis

Data is reported as Mean +_ SEM. Statical comparisons were determined by analysis of variance (ANOVA) and means were separated using Duncan's multiple range test ($P<0.05$).

6.7 Isolation of active extract by column chromatography

The ethanolic extract was active and shows better *in vitro* anti-Alzheimer activity compared to other extracts.Hence the bioactive guided isolation was performed and the ethanol extract will be subjected for column chromatographic isolation by gradient elution technique.

Column Packing

- Dry packing technique was employed for the isolation by column chromatography.
- Initially cotton plug is kept at the bottom of the column and preheated silicagel(100-200mesh size) was added to3/4th of the column length.
- Then, 20 gm of ethanol extract was dissolved in 20 ml of ethanol to dissolve the extract completely, if any debris found,it was filtered.

- The clear liquid was then adsorbed by silica gel (60-120 mesh size) with constant stirring. Then it is kept in incubator at 40˚c for 3 hours to obtain free flowing powder. This flowing powder was packed above the silicagel bed.

- A few grams of silica gel are added on the top of the sample and cotton was kept overit.

- The mobile phase (100 % Hexane) is continuously run over the column for about 5-6 times for tight packing of the column.

Elution Process – Gradient Elution Technique

The ethanol extract was eluted by gradient elution technique using various solvent systems. The collected elutes was marked as fractions and collected in a beaker. Further, TLC was performed for all fractions to identify the fraction which exhibits similar Rf value .The fractions sshowing similar Rf value was mixed together and evaporated.The fractions which shows single spot in UV (254nm and 366nm) and in iodine chamber is considered as pure and can be designated as an isolated compound.[57]

Table-2: Solvent composition for Isolation

S. no	Solvent system	Solvent ratio
1.	Hexane	100%
2.	Hexane: Ethyl Acetate	95:5,90:10,85:15,80:20,75:25,70:30, 65:35,60:40, 55:45,50:50,45:55,40:60, 35:65,30:70,25:75,20:80, 15;85,10:90,5:95
3.	Chloroform	100%
4.	Chloroform: Ethyl acetate	97:3,94:6,91:9,88:12,85:15,82:18,79:21, 76:24,73:27,70;30, 67:33,64:36,61:39,58:42, 55:45,52:48,49:51,46:54,43:57, 40:60,37:63, 34:66,31:69,28:72,25:75,22:78,19:81,15:85, 10:90
5.	Ethyl acetate	100%
6.	Chloroform: Ethanol	98:2,96:4,94:6,92:8,90:10,88:12,86:14,84:16, 82:18,80:20, 78:22,76:24,74:26,72:28,70:30, 68:32,66:34,64:36, 62:38, 60:40,58:42, 56:44,54:46,52:48,50:50,45:55,40:60, 35:65, 30:70, 25:75,20:80,15:85,10:90

6.8. In vitro anti-alzheimer activity of isolated compound by acetylcholinesterase inhibition assay

Acetylcholinesterase inhibition assay

The effect of isolated compound on acetylcholinesterase inhibitory activity will be determined using acetylcholinesterase enzyme.where assayed by the following adaptation of spectrophotometric method reported by Elman et al.[34]

The cuvette used as a blank to control for the non enzymatic hydrolysis of acetylcholine contained a mixture of 500 µL of 3mM DTNB solution (in 0.1M potassium phosphate pH 8), 100 µL of 15mM AChI (in water), 275 µL of 0.1Mpotassium phosphate pH 8, and 100 µL of each cork and cork back ethanol-water extract solutions at the different concentrations (25, 50,100,200,400 and 800 µg/ml). In the reaction cuvette, 25 µL of buffer was replaced by AChE solution 0.16 U/mL. The resulting solutions were placed in a spectrophotometer. The thiocholine formed during the hydrolysis of acetylcholine reacts rapidly with DTNB and a yellow compound is formed. The reaction was monitored for 5 min at 405nm and the absorbance registered every minute. Velocities of reaction were calculated Enzyme activity was calculated as a percentage of the velocities compared to that of the assay using buffer solution instead of inhibitor (cork or cork back ethanol-water extracts). Positive assays were performed in triplicate.

Acetylcholinesterase inhibition = [(OD of control - OD of test)/(OD of control)]×100

6.9 Characterisation of isolated compound

The isolated compound will be characterized by following analytical techniques

-UV spectroscopy, IR ,^{1}H-NMR and Mass spectroscopy.[59]

6.10 I*n-silico* molecular property prediction of isolated compound

The isolated compound was evaluated for molecular property by Molinspiration Cheminformatics software tool.This tool will evaluate the logP

prediction,Aqueous solubility, Molecular weight,Fragment based drug likeness,Overall drug score etc., directly from the chemical structure.

The Violations of Lipinskis rule of 5 for this compound is predicted by using this tool.

6.11 Molecular docking study of isolated compound against Alzheimer target protein

Molecular docking is generally used to detect the protein-ligand orientation and interaction. Autodock Tools package version 1.5.6 was utilized to create the docking input files.The grid region was surrounded by the active site for binding. So, grid region was selected on the basis of amino acid residues representing the binding site of Rivastigmine as the standard drug obtained from PDB ID- 1 GQR(Human Acetylcholinesterase)[56], 1 SGZ (β-SECRETASE)[57,58] and considered as the best active region for the favorable interaction.The gridbox was set at 126×126×126A˚forx,y and z axis and covered the active site of the target protein. The Lamarckian Genetic Algorithm (LGA), a local search algorithm was utilized for ligands conformers searching. During the docking process, a maximum of 10 conformers were considered for the compound. After completion of the conformer the lowest binding energy was chosen. The conformational similarity by visualizing the binding site and its energy (Kcal/mol) and the docked amino acid residues forming hydrogen bonds and other parameters like intermolecular energy(Kcal/mol) and inhibition constant(μM) were analyzed by Autodock tool. Ten best poses were generated for the ligand and score during Auto Dock-

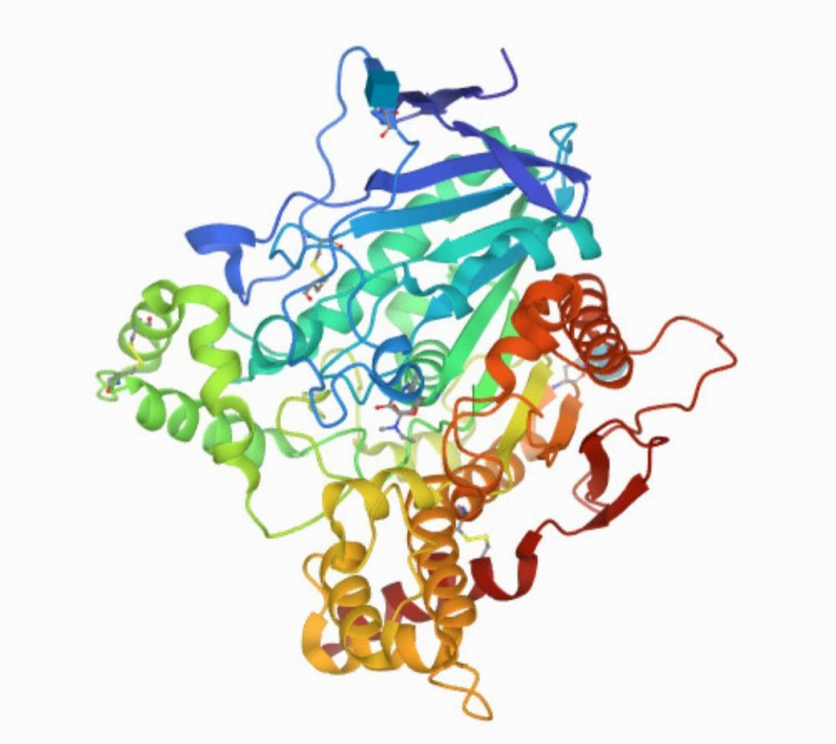


Fig 11: 3D structure of Human acetylcholinesterase

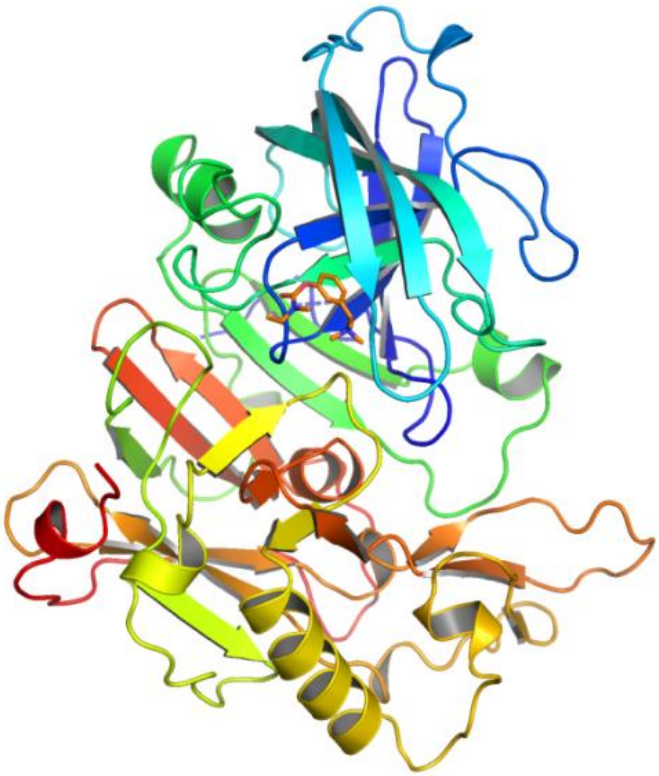


Figure 12. Structure of β-secretase

AUTODOCK and ADT

Short Tutorial:

Overview:

Working with Auto Dock4 includes 3 steps:

1. Preparation of receptor & ligand files.
2. Calculation of affinity maps by using a 3D grid around the receptor & ligand.
3. Defining the docking parameters and running the docking simulation.

The preparation step starts with pdb files of receptor (protein.pdb) and ligand(ligand.pdb), which are added hydrogens and then saved as protein.pdb & ligand.pdb.The calculation of affinity maps in the "Grid" section requires the above pdb files to be assigned charges & atom types, and also that the nonpolar hydrogens are merged. This is done automatically by ADT,and the resulting files need to be saved as protein.pdbqt & ligand.pdbqt, which is the only format AutoGrid& Autodock can work with .Calculation of affinity maps is done by AutoGrid and then docking can be done by Autodock. The newest docking algorithm is LGA (Lamarckian Genetic Algorithm).

For running ADT, you should be following next steps:

1. Install MGL tools.
2. Download autogrid4.exe,autodock4.exeandAD4.1_bound.datfiles.
3. Install Molegro Molecular Viewer.
4. Install ChemDraw including 3DPro.

After having finished installation of above tools, you can run ADT. Here, I recommend you to follow my current protocol for running it:

1. Create a work folder for your job and tag it based on your favorite

name.(e.g.project)

2. Running ADT in start menu in windows: after running you should set its directory:following these steps:

   a) Open ADT> File> Preferences>set>Startup Directory (in this section you should put on work folder path and set it). for example: “C:\Users\project

3. prepare ligand and protein in ".pdb" format. Notice, you must have labeled them by"Ligand" and "Protein" keywords. Protein can be preprocessed byMolegro Molecular Viewer& Ligand can be initially prepared using in Chem Bio Draw and can be saved as pdb.

After having finished these steps you can open ADT: Well! you should do the below ways:

1-openADT:

File>Read Molecule>Select Protein File(“.pdb”file)Then,

Edit>Hydrogens>Add>Polar Only >OK .

Edit >Hydrogens> Merge Non-Polar >Continue (in this step if you see any warning please click on "continue") then:

Edit >Charges>Compute Gasteiger>Ok

Edit>Misc>Repair Missing Atoms>Ok/Select all> Dismiss

File > Save > Write PDB>>Sort Nodes (Check) > OK > (Overwrite) YES

Well! in the next step you should try prepare the ligand:Ligand>Input>Open>SelectLigand File (“.pdb” file) > OK

Now repeat above steps and then: (in this section you must add charge to your ligand.

So, click on Edit menu and:

Edit> Charges> Add Kollman charges

Ligand >Output>Save as PDBQT

****

Now you should be executing AutoGrid4:

For Auto Grid following the below steps:(preparing gpf file)

1. Grid >Macromolecule> Choose> Click (Protein) > Select Molecule > OK > save as PDBQT
2. Grid> Set Map types >Choose Ligand> Click (Ligand) > Select Ligand
3. Grid>GridBox> Set the BOX> File>Close Saving Current
4. Grid> Output > Save GPF> grid.gpf> save

****

After having finished above steps following the below ways:

For Autodock following the below steps:(preparing dpf file)

1. Docking>Macromolecule>Set Rigid filename>Select Protein.pdbqt‟ >Open
2. Docking>Ligand>Choose>Click Ligand‟ >Select Ligand>Accept
3. Docking>SearchParameters>GeneticAlgorithm>Accept
4. Docking>Output>Lamarckian GA> Save file as dock.dpf‟

****

Now copy the autogrid4.exe, autodock4.exeandAD4.1_bound.datfile sinto the destination folder (working folder) and check whether 2 pdbqt files and grid.gpf and dock. dpf files are there.

After having finished above steps following the below ways:

1. Open cmd (command prompt). Windows +R and type cmd enter
2. In command prompt go the destination folder by cd commands.
3. Run the following commands
4. Autogrid4.exe -p grid.gpf -l grid.glg (wait for the response)
5. Autodock4.exe-pdock.dpf-ldock.dlg(wait for the response)

Now the dock.dlg file is your docking result.

---

1. Analyze >Docking>Open> Select „dock.dlg"> Open >Assign Ligand New Name> OK
2. Analyze > Macromolecule > Choose> Click Protein > Select Macromolecule
3. Analyze> Conformations> Play, Ranked By Energy or Play > Click on the „&"Button
4. Set Play Options >Check „Build H-Bonds"> View the hydrogen bonds formed> Check „Show Info"> View the Interaction Energy > Build Current Write Complex>Save as Result.pdb save …

For better ligand interactions you can open the result.pdb in Molegro Molecular Viewer and View it and save the ligand interaction image.

CHAPTER – 7
RESULTS AND DISCUSSION

7.1. PERCENTAGE YIELD EXTRACTS OF *OXYSTELMA ESCULENTUM* R.BR.

Table-3: Percentage yield and appearance of extract of *Oxystelma esculentum* R.BR.

EXTRACTS	APPEARANCE	PERCENTAGE YEILD (W/W)
HEXANE	**GREEN**	11.2%
CHLOROFORM	**DARK GREEN**	9.2%
ETHYL ACETATE	**YELLOWISH GREEN**	7.3%
ETHANOL	**REDDISH BROWN**	12.5%

The appearance of concentrated and dried extracts of hexane, chloroform, ethyl acetate and ethanol were observed and percentage yield was calculated for the extracts described in the table 3.

7.2 PHYTOCHEMICAL SCREENING OF EXTRACTS

From the preliminary phytochemical screening, it was found that all the extracts on the aerial parts of *Oxystelma esculentum* R.BR.showed the presence of different primary and secondary metabolites. The observation was listed below.

Table-4: Phytochemical screening on aerial parts of *oxystelma esculentum* R.BR.

COMPOUNDS	HEXANE EXTRACT	CHLOROFORM EXTRACT	ETHYL ACETATE EXTRACT	ETHANOL EXTRACT
Alkaloids	-VE	+VE	+VE	+VE
Reducign sugar	-VE	+VE	+VE	+VE
Saponins	+VE	+VE	-VE	-VE
Phytosterols	-VE	+VE	+VE	+VE
Tannins	-VE	+VE	-VE	+VE
Flavonoids	-VE	+VE	+VE	+VE
Protein and Aminoacid	-VE	-VE	-VE	+VE
Terponoids	+VE	+VE	+VE	+VE
Glycosides	-VE	+VE	+VE	+VE
Fixed oils and fats	+VE	+VE	-VE	-VE

NOTE: +VE INDICAT E THE PRESENCE OF PHYTOCONSTITUENT

-VE INDICATE THE ABSENCE OF PHYTOCONSTITUENT

RESULT

As illustrated in table 4 hexane extract shows the presence of terpenoids, saponins and oils and fats. Chloroform extract shows the presence of alkaloids, carbohydrate, phytosterols, tannins, glycosides and oil and fats. Ethyl acetate extract shows the presence of alkaloids, carbohydrates, saponins, phytosterols, phenols, terpenoids, glycosides and flavanoids. Ethanolic extract shows the presence of glycosides, flavonoids, phytosterol, alkaloids, terpenoids and phenolic compounds.

DISCUSSION

The above phytochemical investigation has revealed that the **ethanolic extract** on the aerial parts of *Oxystelma esculentum* R.BR.have various phytochemical constituents compared to other extracts like glycosides,

flavanoids, phytosterol, alkaloids, terpenoids and phenolic compounds. These active constituents possesses numerous pharmacological activities like anti microbial,anti inflammatory,stress related disorders and as diuretics.

7.3 INVITRO ANTI-ALZHEIMER ACTIVITY OF EXTRACTS

Table 5. Effect of extracts of *Oxystelma esculentum* R.BR on acetylcholinesterase inhibition

Conc. (µg)	Standard (Galantamine)	Hexane (HEX)	Chloroform (CH)	Eethyl Acetate (EA)	Ethanol (E)
25	0.844868	0.158457	0.374019	0.261059	0.261059
50	1.395604	0.333459	0.261059	0.374019	0.414937
100	0.311203	0.274455	0.374019	0.374019	0.364303
200	0.158456	0.215940	0.374019	0.364303	0.364303
400	0.392732	0.274455	0.261059	0.059940	0.316913
800	0.364303	0.215940	0.261059	0.844868	0.571324
IC50 Value	14.22	726.07	171.41	72.01	38.54

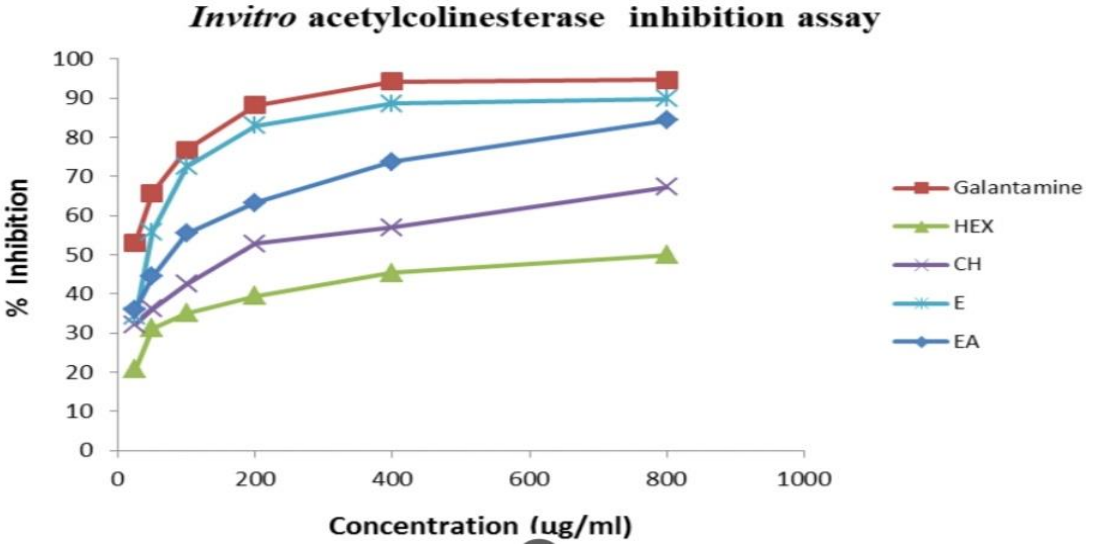
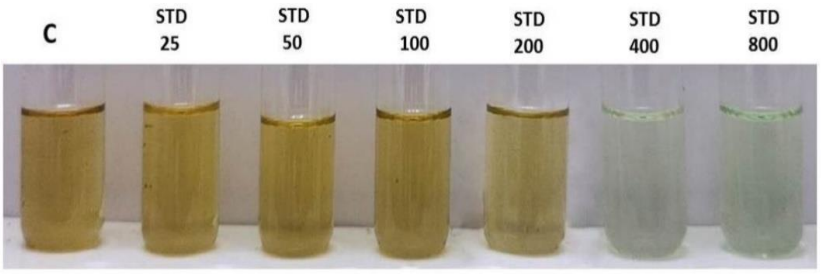
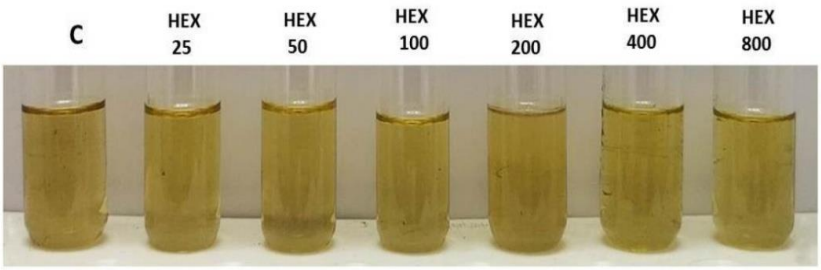


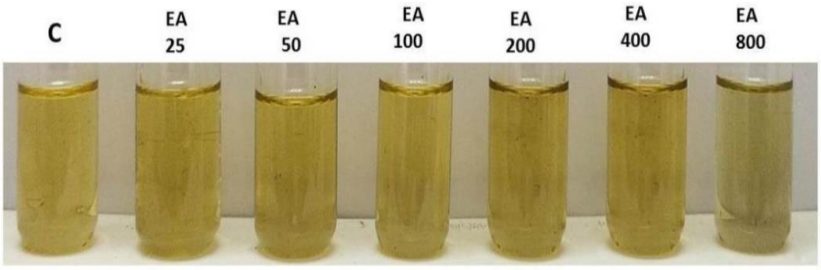
Fig-13: Graph representing acetylcholinesterase inhibition assay of Oxystelma esculentum R.BR.



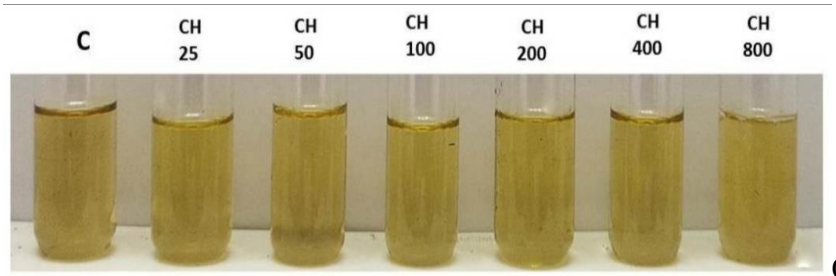
STD



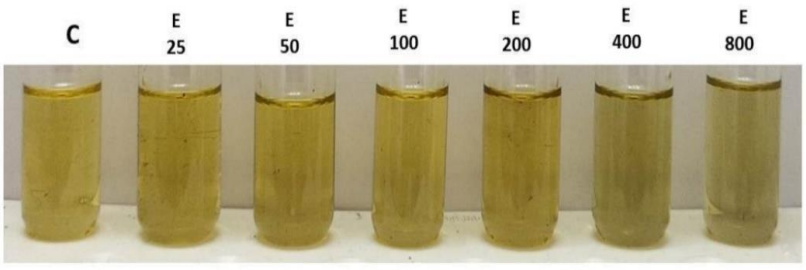
HEXANE



ETHYL ACETATE



CHLOROFORM



ETHANOL

Fig:14 In vitro acetylcholinesterase assay showing different concentration of dilution of Oxystelma esculentum R.BR.

RESULTS

As illustrated in the above table 5, all the extracts were subjected to acetylcholinesterase inhibition assay. It was observed that ethanol extract has good acetylcholinesterase inhibition compared with other extracts with an IC_{50} value of 38.54. The Galantamine was used as a standard, and its IC_{50} values was found to be 14.22. The percentage inhibition of acetylcholinesterase for the extracts was described in Figure 13.

DISCUSSION

From the above results, it was determined that,ethanol extract of *Oxystelma esculentum* R.BR has good *in vitro* anti-alzheimer activity by inhibiting acetylcholinesterase enzyme with an IC_{50} value 38.54 respectively.

The acetylcholinesterase enzyme begins the breakdown of the neurotransmitter acetylcholine into choline and acetate which causes deficiency of acetylcholine in postsynaptic neuron of brain leads to memory deficits. The acetylcholinesterase enzyme (AChE) is an attractive target for the discovery of mechanism-based inhibitors because of its role in hydrolysis of the neurotransmitter acetylcholine. AChE inhibitors as rivastigmine, donepezil, or galantamine are currently the most effective agents to treat cognitive symptoms of AD and have other possible therapeutic applications in the treatment of other neurodegenerative disorders.[62,63]

Recently, research on prevention and treatment of Alzheimer has focused on naturally occurring acetylcholinesterase (AChE) inhibitors from plants, namely, polyphenolic compounds such as flavonoids with inhibitory capacity similar to that of the currently prescribed AChE inhibitor drugs.

Hence based on the above evidence, we have carried out *in vitro* screening of extracts of *O esculentum* against acetylcholinesterase target enzyme. It was observed that ethanol extract shows promising inhibition against the enzyme compared with other extracts. Furthermore ethanol extract was considered as an active extract and subjected for *in vivo* anti Alzheimer activity and for the isolation of compounds by column chromatography

7.4 ACUTE TOXICITY STUDY OF ETHANOL EXTRACT OF *OXYSTELMA ESCULENTUM* R.BR IN MICE.

Table 6. Results of acute toxicity in mice on administration of ethanol extract of *Oxystelma esculentum* R.BR at the dose of 2000mg/kg

OBSERVATION	30 MINS	4 HOURS	24 HOURS	14th DAY
BODY WEIGHT	-	-	-	-
PRETERMNAL DEATH	-	-	-	-
CONVULSIONS	-	-	-	-
RIGHTING REFLEX	+	+	+	+
LACRIMATION	-	-	-	-
SALIVATION	-	-	-	-
RESPIRATION	+	+	+	+
DIARRHOEA	-	-	-	-
SEDATION	-	-	-	-
EXCITATION	+	+	-	+
AGGRESSION	+	+	+	+

NOTE + INDICATES PRESENCE AND - INDICATES ABSENCE.

RESULT

As described in table 6, the ethanol extract was found to be non-toxic and safe upto the dose of 2000mg/kg body weight. The behavioural changes and mortality were not observed for tested mice. Hence, LD_{50} value is expected to increase the dose level above 2000mg/kg body weight. From this 2000mg/kg,1/10th (200mg/kg) and 1/5th (400mg/kg) were selected as Low dose and High dose treatment for *in vivo* studies.

7.5. *IN VIVO* ANTI-ALZHEIMER ACTIVITY OF ETHANOL EXTRACT ON SCOPOLAMINE INDUCED AMNESIA IN MICE

Y MAZE METHOD

Fig:15. Y Maze method

Table 7. Percentange alterations of different group of drug treated mice by Y Maze method

GROUP	PERCENTAGE ALTERATIONS
CONTROL	60.33± 2.50
SCOPOLAMINE	16.54± 1.50***
SCOPOLAMINE+200mg/kg *of O.esculentum*	65.45 ±6.55***
SCOPOLAMINE+400mg/kg of *O.esculentum*	76.50 ±5.45***
STD – DONEPEZIL	72.34± 4.56***

Values are expressed as Mean± SEM (n=6). The percentage alteration significantly decreased for the scopolamine group (***p< 0.001 vs control). The administration of *O.esculentum* at 200mg/kg and 400 mg/kg increases the

percentage alterations (***p <0.001) vs scopolamine . One way Anova followed by Dunnets t test.

RESULT

The results obtained for the effect of *O.esculentum* on percentage of spontaneous alterations in Y maze are depicted in table 7. The control group was compared with scopolamine induced group and observed that the reduction was significant.After the treatment, both low dose 200mg/kg and high dose 400mg/kg and standard Donepezil treated group shows a significant improvement (p <0.001) in memory and learning ability.

OPEN FEILD METHOD

Fig 16. Open feild test in mice.

Table 8. Number of crossing, rearing and time spent in centre

Parameter	Control	Scopolamine Disease	Low dose of ethanol extract	High dose of ethanol extract	Standard Donepezil
Number of crossing	75±2.8	45±3.1***	60±3.4***	71±4.7***	75±1.8***
Number of rearing	9±1.9	5±0.8***	6±1.5	8±1.1	10±1.1**
Time spent in minutes	4±1.1	2.5±0..8***	3±1.0	4.5±0.8**	5±2.0***

Each value represents the Mean±SEM (n=6).The number of crossing, rearing and time spent in the open feild decreases for the scopolamine group

(***p<0.001 vs control) and the administration of *O.esculentum* extract at 200mg/kg and 400mg/kg body weight increases this number of crossing and rearing (**p<0.01 and ***p<0.001 vs scopolamine). One way ANOVA followed by Dunnets t-test.

RESULT

The treatment of ethanolic extract of *O.esculentum* (200mg/kg and 400mg/kg) exhibited a significant (**p<0.01; ***p<0.001) effect respectively on the number of crossings and number of rearings of open feild acquisition of memory compared to the negative control group(scopolamine group).

The results obtained indicated that the high dose of 400mg/kg of *O.esculentum* exhibited better exploratory behaviour in number of crossing table 8.The effect of ethanolic extract of *O.esculentum* on line crossing indicated that the administration of 200 mg/kg and 400mg/kg exhibited significant difference of p<0.01 and p`,0.001.In rearing the result obtained indicated that the animal treated with 400mg/kg exhibit a significant p<0.001 compared to disease induced group.

TRACTION TEST

Table 9. Retention time of mice by Traction test

GROUP	RETENTION TIME IN SECONDS
CONTROL	14.00± 1.10
SCOPOLAMINE	4.50± 1.50**
SCOPOLAMINE+200mg/kg of O.esculentum	9.5 ±2.55*
SCOPOLAMINE+400mg/kg of O.esculentum	28.57 ±2.00***
STD – DONEPEZIL	26.34± 4.56***

**p<0.01 Negative control- Scoolamine induced at 400mg/kg

***p<0.001.Oneway anova followed by dunnets t test

RESULT

In the traction test, the Scopolamine induced group exhibited loss of muscle contraction, which is considered as a sign of neurotoxcity in general. The animals in the negative control ($p<0.01$) showed a significant reduction in retention time and downfall in ashort duration of time when compared to normal control group. The administration of *O.esculentum* has shown a significant effect on enhancing motor co ordination and balancing ability in mice.The animals after treatment with *O.esculentum* with 400mg/kg exhibited significant ($p<0.001$) spending more time balancing compared to scopolamine induced group.

DISCUSSION

The present study was undertaken to investigate whether *Oxystelma esculentum* could improve memory impairment in mice. Medicinal plants are playing a significant role in the management of memory deficit and Alzheimer's disease. Scopolamine promoted amnesia in the animals through impaired memory by blocking the muscarinic cholinergic receptors in the brain as earlier reported.

In neuroprotective evaluation, behavioral studies with Y-maze and open-field habituation memory exhibited promising results on cognitive improvement after scopolamine-induced amnesia. In the Y-maze test, the number of entries in each arm was observed and recorded. There was a significant improvement, which indicates that there is a significant effect produced by the ethanol extract of *O. esculentum* on treating memory and learning impairment.

The open-field test was conducted followed by the Y-maze test. The purpose of this test was to measure the exploratory behavior of the mice in a new environment, anxiety level, and the level of cognition. In the open-field test, the study showed that the administration of scopolamine exhibited a significant effect on declining the memory and learning level in the mice and it was clearly observable in the line crossing test, rearing, and number of head dipping test.[61] Open-field exploration and habituation memory are considered

to be disturbed in age-related disease in animal models and studies suggest that phytoconstituents rich in polyphenols and flavonoids and terpenoids tend to improve AD

In our study, it is evident from the open-field test that the treatment with ethanolic extract of *O.esculentum* enhanced cognitive performance. In the traction test, the time taken for the mice to retain on the retort bar was recorded and this test was carried out to examine the changes in sensorimotor performance during aging. In this test, it was indicated that only the high dose group (ethanolic extract of *O.esculentum* 400 mg/kg) produced a significant effect toward amnesic mice, whereas the low dose group had no significant activity toward the amnesia induced by scopolamine, but an increase in the mean was observed in this group compared to the negative control group. This shows that ethanolic extract of *O.esculentum* is involved in neuroprotection and improves the sensorimotor performance in aging.

Administration of scopolamine for a prolonged period of time causes amnesia and this induction model is associated with Alzheimer's model. Scopolamine is a nonselective muscarinic acetylcholine receptor which has the tendency to reduce the induction of long-term potentiation in the brain which relates to the decrease in memory and learning ability

BIOCHEMICAL ESTIMATION

Table-10. The biochemical estimation of Malondialdehyde, Glutathione, Catalase in homogenised mice brain

Group	Group Name	MDA (mmol/g)	Glutathione (mmol/g)	Catalase (mmol/g)
A	**Control**	59.30±3.54	5.56±0.36	287.56±10.34
B	**Disease Control (Scopolamie)**	97.32±3.14**	1.57±0.10**	107.32±4.65***
C	**Ethanol Extract (200mg/kg) + Scopolamine**	70.87±3.87*	3.72±0.16*	250.44±8.78**

Group	Group Name	MDA (mmol/g)	Glutathione (mmol/g)	Catalase (mmol/g)
D	**Ethanol Extract (400mg/kg) + Scopolamine**	64.8±4.47***	4.36±0.09**	315.72±18.89***
E	**Standard Donepezil**	59.3±6.89***	4.98±0.12**	387.60±10.14***

Each value represents the mean SEM (n=6). The level of MDA increases and the antioxidant level decreases for the scopolamine group (**p<0.01, ***p<0.001) vs control and the administration of *O.esculentum* extract decrease the level of MDA by increasing the level of antioxidant enzymes (*p<0.05, **p<0.01, ***p<0.001) vs scopolamine.Oneway ANOVA followed by Dunnets t test.

RESULT

The administration of scopolamine induces oxidative damage in animals.The MDA levels increases significantly p<0.001 in the mice of group receiving only scopolamine of 97.32 3.14 compared to the control which has a level of 59.30 3.54.The level of MDA significantly decreases in the group of animals that received the treatments with the dose of *O.esculentum* ethanol extract.

Scopolamine significantly decrease the glutathione level to 1.57 1.0 p<0.01.Treatment with 400mg/kg of ethanol extract of *O.esculentum* significantly increases the glutathione level of 4.98 0.12 p<0.01

Scopolamine also significantly decrease the level of catalase to 107.32 4.65 p<0.001 compared to control. Administration of 400mg/kg of ethanol extract of *O.esculentum* significantly increase the level of 315.72 18.89

Catalase and Glutathione increased significantly with administration of Donepezil p<0.001.The results are shown in the table 10.

DISCUSSION

Scopolamine induced memory disorders are associated with increased oxidative stress in the brain characterized by an increased Malondialdehyde levels which is harmful effect of reactive oxygen species. The results of these present study showed a collapse in brain antioxidant defence mechanism characterized by a higher MDA level and lower catalase, Glutathione levels, while the level of MDA decreases significantiy.Finally the ethanol extracts of *O.esculentum* possesse anti-oxidant potentials by increasing the catalase, Glutathione, and decreasing MDA levels significantly in memory impaired mice. Thus inhibition of reactive oxygen takes place by ethanol extract of plant.

7.6 ISOLATION OF ETHANOL EXTRACT BY COLUMN CHROMATOGRAPHY

Table 11. Total number of fractions collected and their solvent systems

S.No	Fractions	Solvent system & ratios
1	F-1	Hexane (100%)
2	F-2 to F-38	Hexane:Ethyl acetate (95:5,90:10,85:15,80:20,75:25,70:30,65:35, 60:40,55:45,50:50,45:55,40:60,35:65,30:70,25:75, 20:80,15;85,10:90,5:95
3	F-39	Ethyl acetate (100%)
4	F-40 to F-78	Ethyl acetate:Chloroform(97:3,94:6,91:9,88:12, 85:15,82:18,79:21,76:24,73:27,70;30,67:33, 64:36,61:39,58:42,55:45,52:48,49:51,46:54, 43:57,40:60,37:63,34:66,31:69,28:72,25:75, 22:78,19:81,15:85,10:90
5	F-79	Chloroform(100%)
6	F-80 to F-113	Chloroform:Methanol(98:2,96:4,94:6,92:8, 90:10,88:12,86:14,84:16,82:18,80:20,78:22, 76:24,74:26,72:28,70:30,68:32,66:34,64:36, 62:38,60:40,58:42,56:44,54:46,52:48,50:50, 45:55,40:60,35:65,30:70,25:75,20:80,15:85,10:90

Table 12. R_f value for isolated compound

Isolated Compound	TLC Solvent System	No. of SPOT	R_f Value
V 1	Chloroform:methanol (8:2)	1	0.84
	Chloroform:methanol (8:2)	1	0.85
	Chloroform:methanol (7:3)	1	0.90

Fig17. Separation of compounds by column

RESULT

The ethanol extract was eluted with various solvents by gradient elution method. Each fraction was collected as 100 ml. The ftraction were analysed by Thin Layer Chromatograohy for determination of Rf value. The yellow colour band was eluted and collected from the fraction number 85 – 88 in solvent composition Chloroform:Methanol (94:6, 91:9, 88:12, 85:15). The collected yellow fractions showed single spot in TLC, UV (254nm,366nm), and in Iodine vapour with the same Rf value in various solvent composition of chloroform and methanol. Hence the fractions were mixed together and evaporated to get a

crystals. The isolated compound designated as V1. The obtained yellow crystals were then subjected for characterization in UV, IR, and 1H-NMR and LC-MS

7.7 CHARACTERIZATION OF ISOLATED COMPOUNDS

RESULTS AND DISCUSSION

The compound V1 is a yellow crystal compound.

The melting point of this compound was found to be 67^{o}C

The UV was performed in (ethyl acetate) λ max was found to be 230nm

FT-IR in (cm^{-1})

3334 cm^{-1} Aromatic OH groups bonded to cyclopentane pyran system.

3030 cm^{-1} Aromatic C-H stretch

1218 cm^{-1} pure stretching vibration of cyclopentane pyran system

Mass m/z: 625.48 $[M+1]^{+}$;

^{1}H-NMR ($CDCl_3$): 5.32 (1H, s, =CH), 3.89 – 3.95 (10H, m, -CH); 3.82 – 3.85 (4H, m, -CH); 3.63 – 3.65 (4H, d, -CH); 3.42 – 3.44 (4H, m, -CH); 2.11 – 2.14 (4H, m, -CH);1.78 (1H, m , -CH); 1.28 – 1.33 (6H, m, -CH2); 1.22 – 1.25 (4H, m, -CH2); 1.21 (6H, d, -CH_3).

From the characterization reports of above compound and from the library database which was obtained from QTOF HRLCMS. The isolated compound was found to be KANOKOSIDE D (an iridoid glycoside) – Terpene Glycoside.

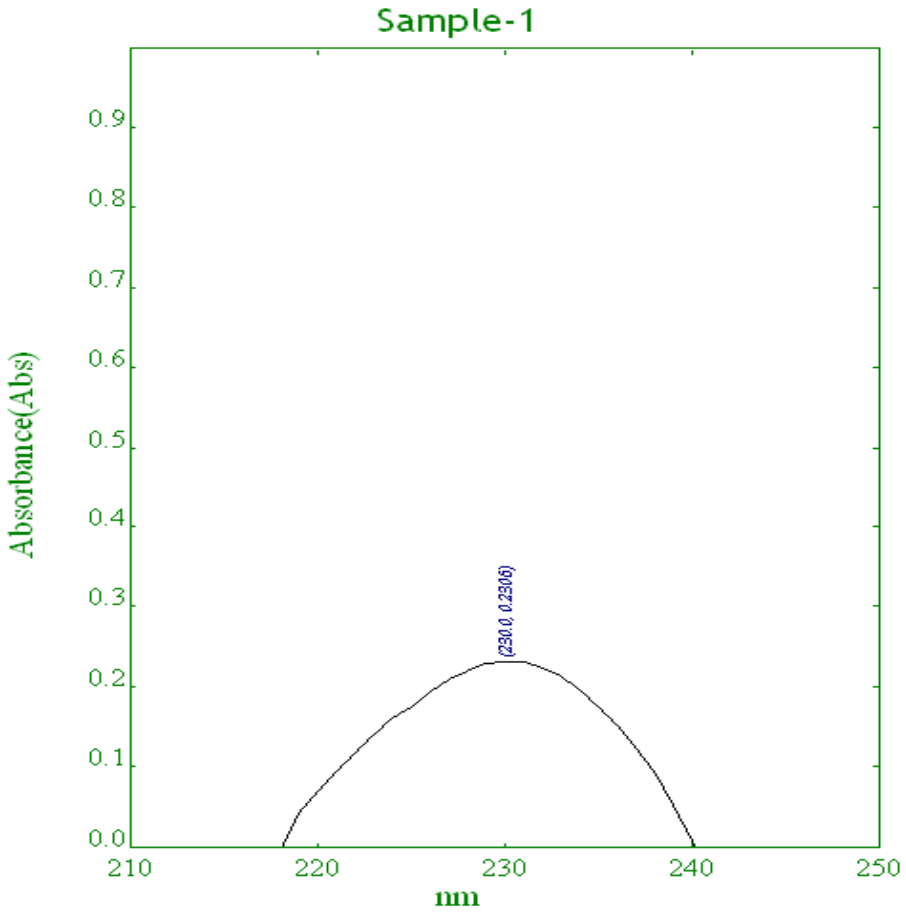


Fig 18. UV SPECTRUM OF ISOLATED COMPOUND V1

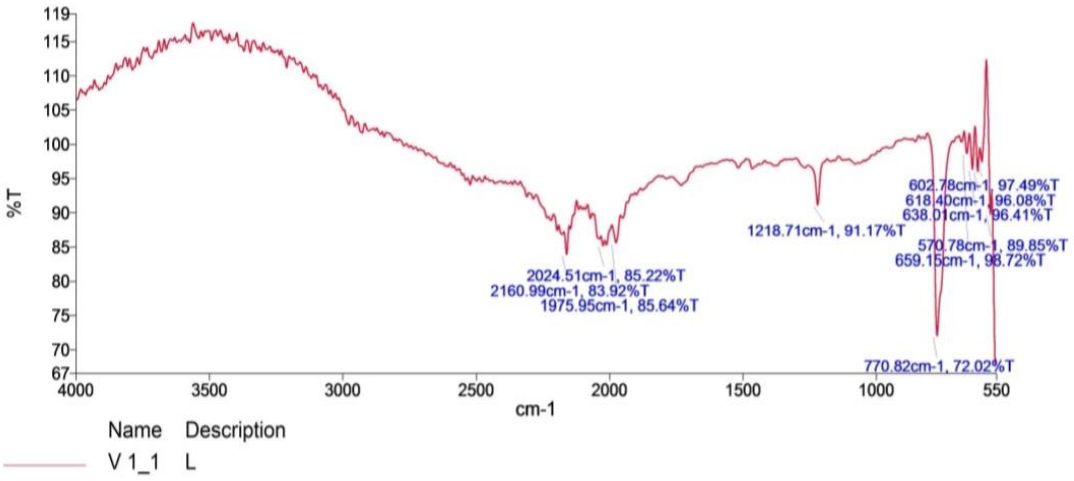


Fig 19. FT-IR SPECTRUM OF ISOLATED COMPOUND V1

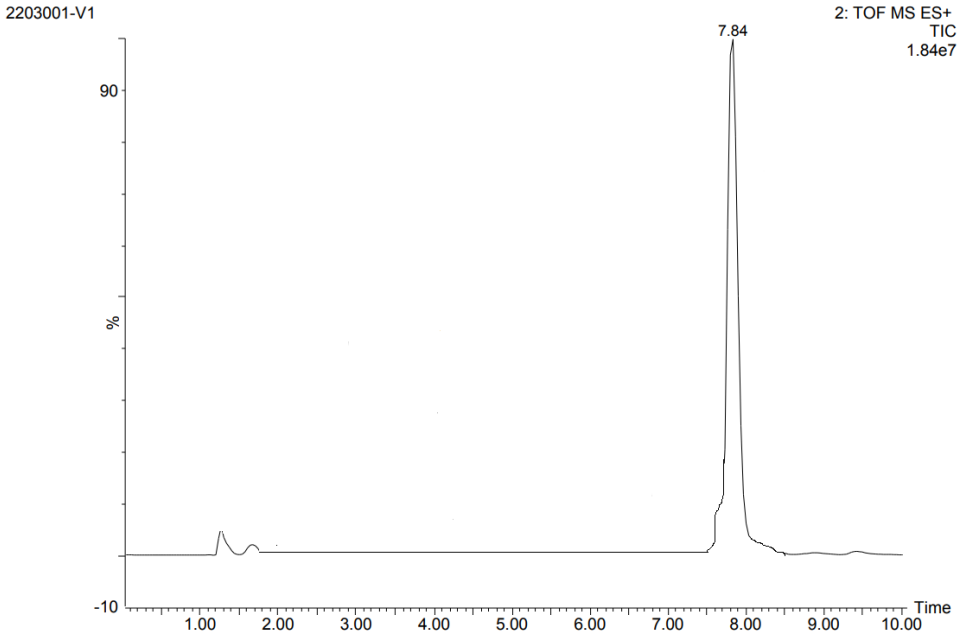


Fig 20. HR LCMS OF ISOLATED COMPOUND V1

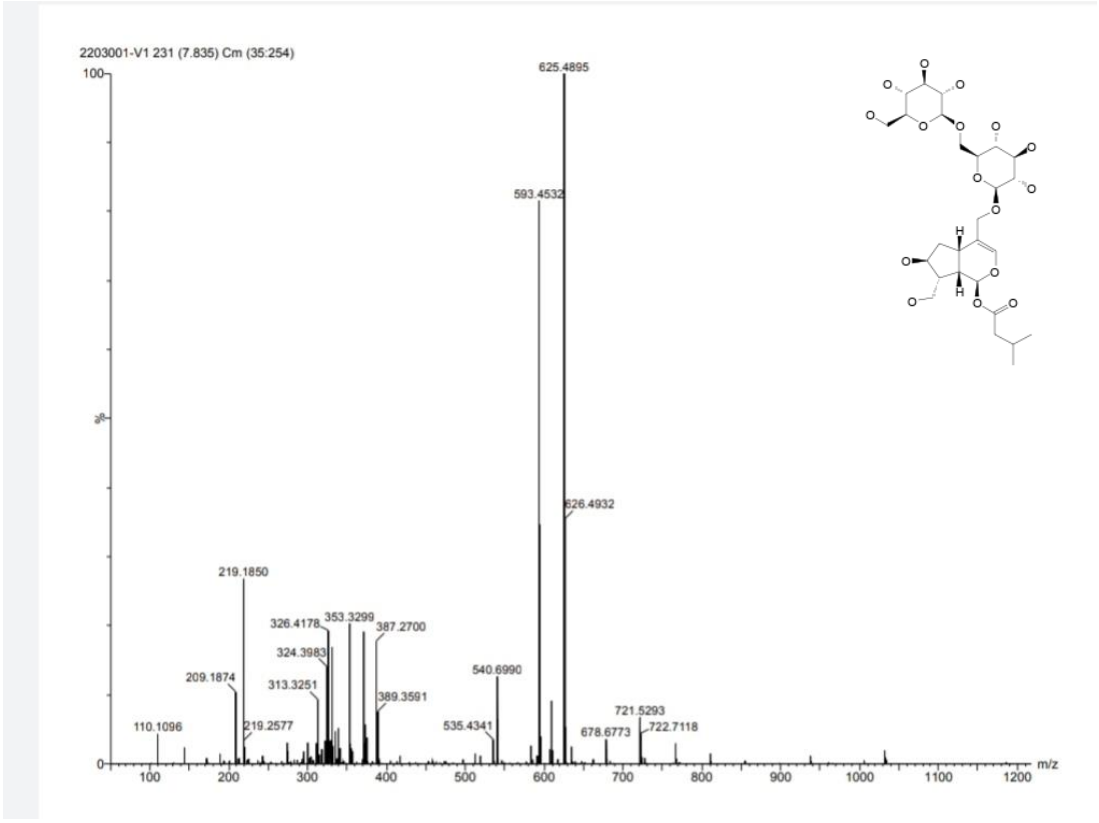


Fig 21 MASS SPECTRUM OF ISOLATED COMPOUND V1

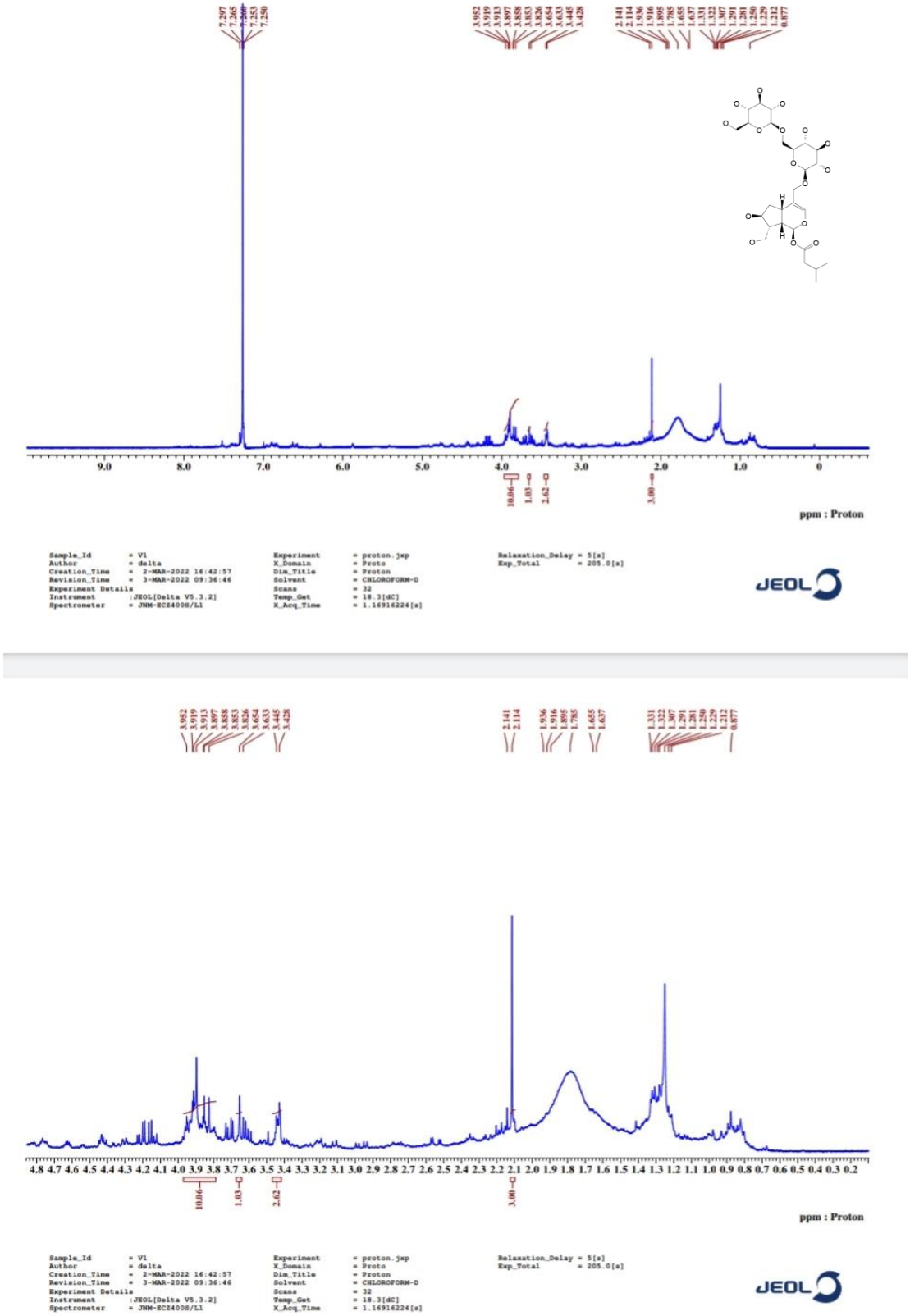


Fig 22. PROTON NMR OF ISOLATED COMPOUND V1

7.8 *IN VITRO* ANTI-ALZHEIMER ACTIVITY OF KANOKOSIDE-D BY ACETYLCHOLINESTERASE INHIBITION ASSAY

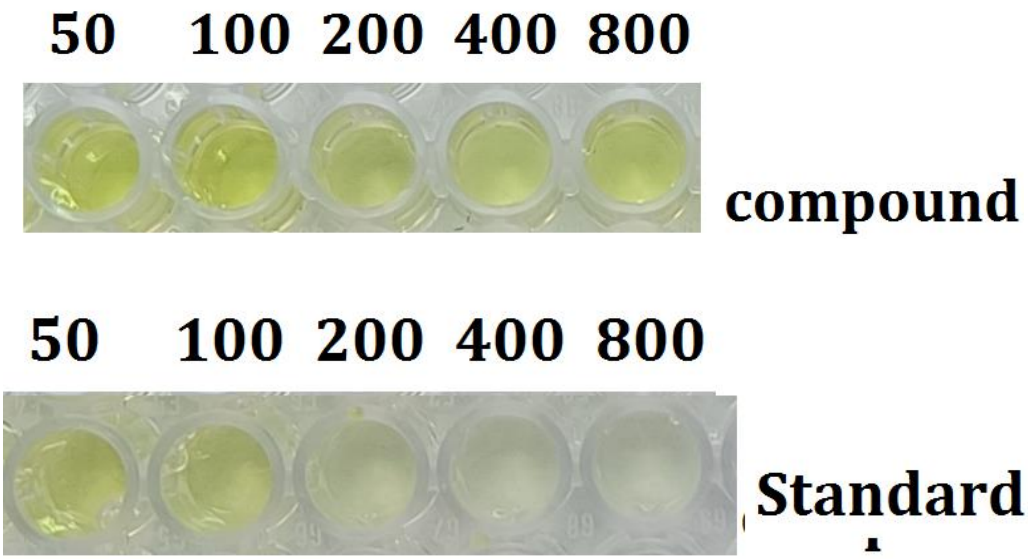


Fig23. Different concentrations of dilution of Kanokoside D by acetylcholinesterase inhibition assay

Table 13. Percentage inhibition of Kanokoside D on acetylcholinesterase inhibition assay

Concentration (in µg level)	STD (DONEPEZIL)	KANOKOSIDE D
50	36.96	30.56
100	53.97	43.02
200	68.13	49.72
400	83.79	61.53
800	90.09	77.63
IC_{50}	85.89	135.54

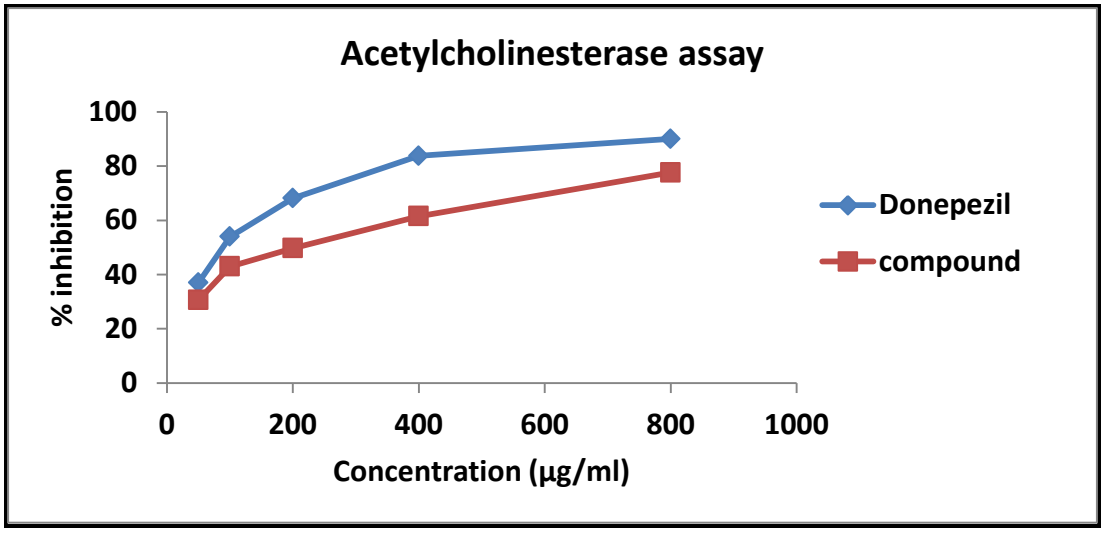


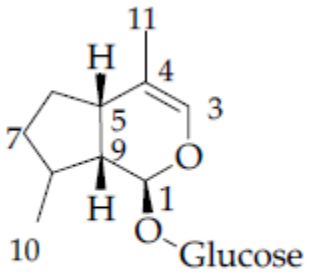
Fig 24. Graph representing acetylcholinesterase inhibition assay of Kanokoside D

RESULT

As depicted in the table 12. the isolated Kanokoside a terpene glycoside (Iridoid) was investigated for acetylcholinesterase inhibitor. It was observed that Kanokoside D had good acetylcholinesterase inhibitor with an IC_{50} value of 135.54 compared with the standard Donepezil has greater inhibitor of 85.89.

DISCUSSION

Kanokoside D is an Iridoid glycoside. Iridoids are a class of monoterpenoid compounds constructed from 10-carbon skeleton of isoprene building units. These compounds in their aglycones and glycosylated forms exist in nature to contribute to mechanisms related to plant defenses and diverse plant-animal interactions.[60]



Iridoid glycoside

Therapeutic Potential for Alzheimer's Disease

The vast arrays of neuroprotective effects of iridoids and some monoterpenes have been investigated. The β-amyloid peptide (Aβ) formation, aggregation and function have been the major target areas of AD for *in vitro* experiments. In a study by the β-secretase (recombinant human BACE1) inhibitory activities of some monoterpenes have been evaluated.

Insights into the Mechanism of Action of Iridoids and Other Monoterpenes in AD

The role of Aβ in the pathology and as therapeutic target for AD has been reviewed in the various literatures Recent review articles from our laboratories have also shown that many polyphenolic compounds such the flavonoids, diterpenoids and cinamate derivatives display therapeutic potential for AD through multiple mechanisms involving Aβ. Hence, the formation, aggregation and toxicity of Aβ can all serve as targets for therapeutic agents.

The predominant forms of the pathological Aβ in the brain are Aβ1–40 and to a lesser extent Aβ1–42 which are formed through the amyloidgenic β-secretase-dependent pathway. The selective inhibition of this enzyme by monoterpenes without much effect on the non-amyloidogenic marker enzyme (α-secretase) is an interesting finding. A large body of evidence also suggests that monoterpenes ameliorate the Aβ-induced cytotoxicity both in cultured neuronal cells and various animal models of AD.

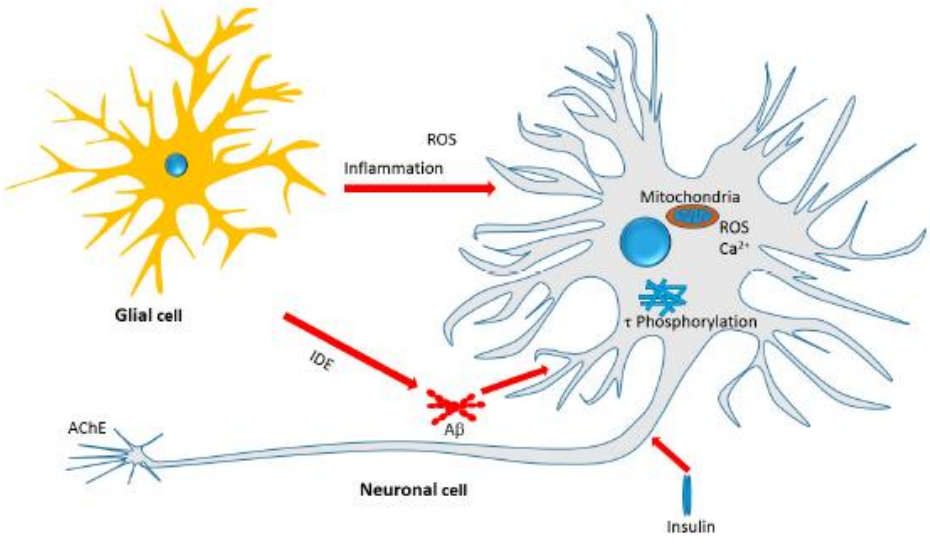


Fig 25: Targets of iridoid glycosides

Therapeutic targets of iridoids and other monoterpenes discussed in this review. Antiinflammatory effect, amelioration of oxidative stress, mechanisms related to Aβ formation, aggregation and clearance, τ-protein phosphorylation and aggregation, and neurotoxicity associated with mitochondrial dependent and independent mechanisms are among the therapeutic targets.

7.9 IN SILICO MOLECULAR PROPERTY PREDICTION OF KANOKOSIDE D

The molecular properties of Kanokoside D were calculated by using Molinspiration and Chemoinformatics and data's given below. From the data, it rvealed that the Kanokoside D obeys the Lipinski's rule of 5 (with some violations).Hence the compound has drug-likeness property and it can be a potential drug candidate.

Lipinski's rule of five

Molecular weight <500 Da

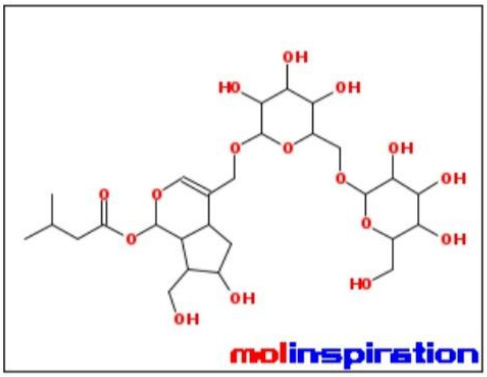
ClogP < 5

Number of hydrogen bond donors < 5

Number of hydrogen bond acceptors < 10

Number of rotatable bonds < 7

Polar surface area < 140 A^2



Molinspiration property engine v2021.1

miLogP	-2.62
TPSA	254.53
natoms	43
MW	624.63
nON	16
nOHNH	9
nviolations	3
nrotb	12
volume	544.91

Get data as text (for copy / paste).

Get 3D geometry BETA

Fig 26. Insilico predicted molecular properties of Kanokoside D

RESULTS AND DISCUSSION

The physicochemical properties of the Kanokoside D possessing molecular weight 624.63 dalton, total polar surface area 254.53 and hydrogen bond donor 9 which slightly violating the Lipinski`s rule of five.

7.10 MOLECULAR DOCKING STUDY OF KANOKOSIDE D AGAINST ALZHEIMER TARGET PROTEIN

- **Acetylcholinesterase**
- **β-Secretase**

DOCKING OF STANDARD RIVASTIGMINE WITH HUMAN ACETYLCHOLINESTERASE ENZYME

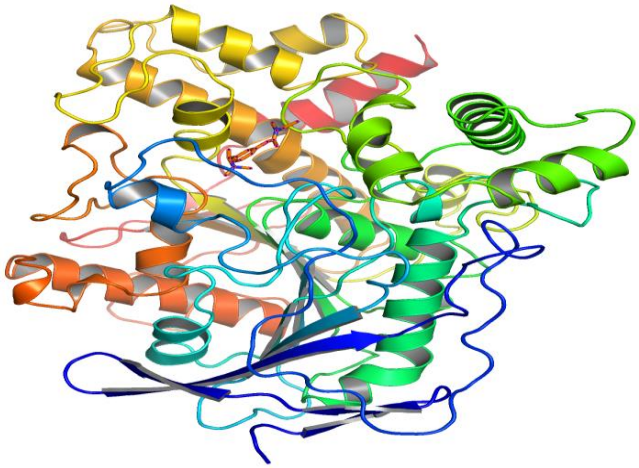


Fig 27. Docking of rivastigmine in target protein of acetylcholinesterase.

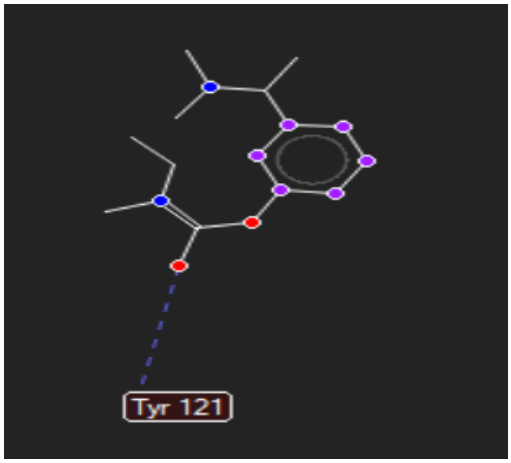


Fig 28. Molecular interaction between Rivastigmine with acetylcholinesterase

DOCKING OF KANOKOSIDE D WITH HUMAN ACETYLCHOLINESTERASE

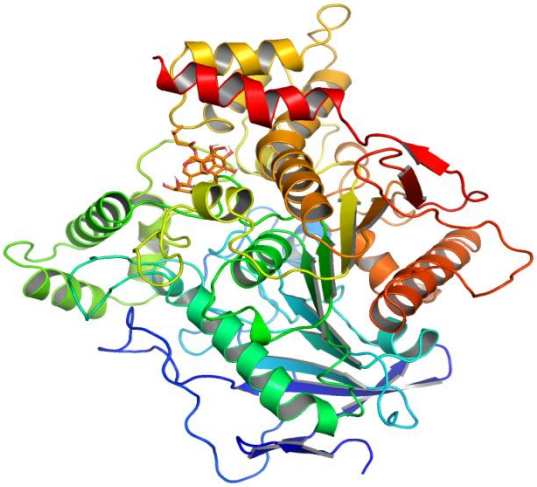


Fig29. Docking of Kanokoside D with Hman acetylcholinesterase

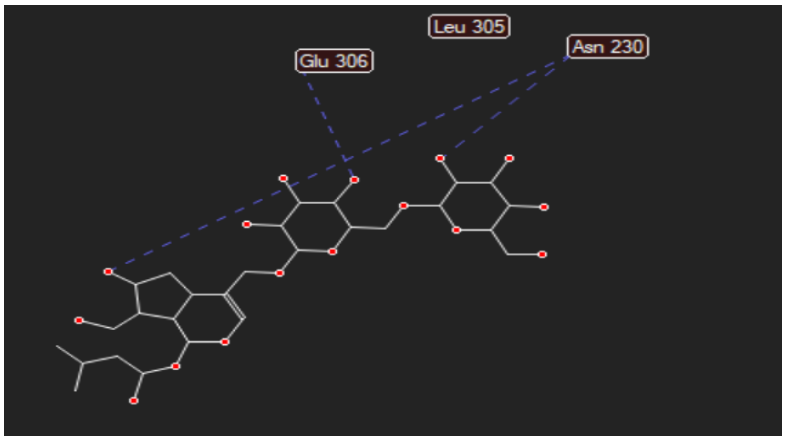


Fig 30. Molecular interaction between Knokoside D and acetylcholinesterase

Table 14. Docking scores of Rivastigmine and Kannokoside D

Protein	Ligand	Predicted Binding Energy (kcal/mol)
Human Acetylcholinesterase PDB ID:1GQR	Rivastigmine	-5.82
	Kanokoside D	-4.38

RESULT AND DISCUSSION

The molecular docking study of Kanokoside D against Alzheimer target protein investigated in fig 29. The ligand (Kanokoside D) form hydrogen bond

interactions with amino acid Asparagine 230, luecine 305 and Glutamic acid 306 was observed in the fig 30. The predicted binding energy of Kanokoside D against acetylcholinesterase was found to be -4.38 kcal/mol compared with the standard binding energy of -5.82 kcal/mol.

DOCKING OF STANDARD RIVASTIGMINE WITH B-SERETASE ENZYME

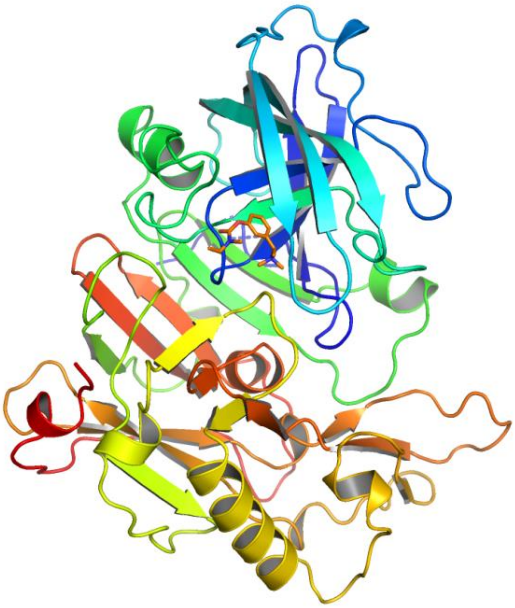


Fig 31. Docking of Rivastigmine with β-Secretase enzyme

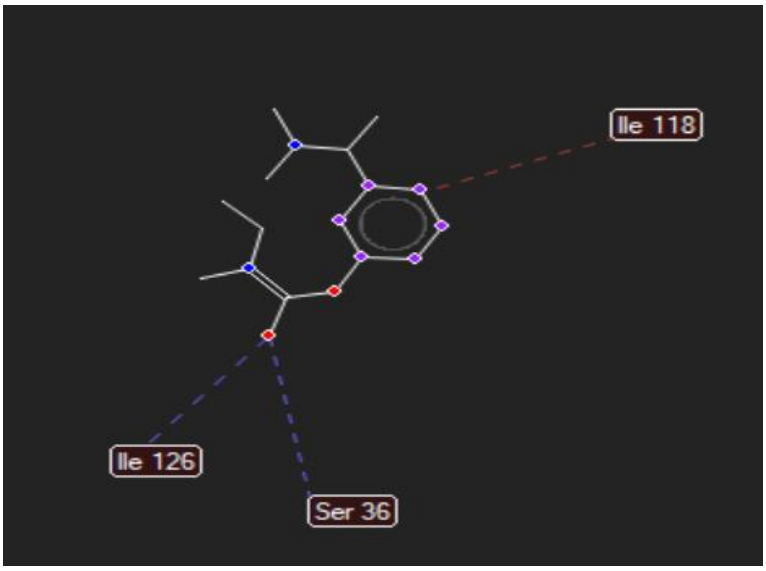


Fig 32. Molecular interaction between rivastigmine and β-secretase

DOCKING OF KANOKOSIDE WITH B-SECRETASE ENZYME

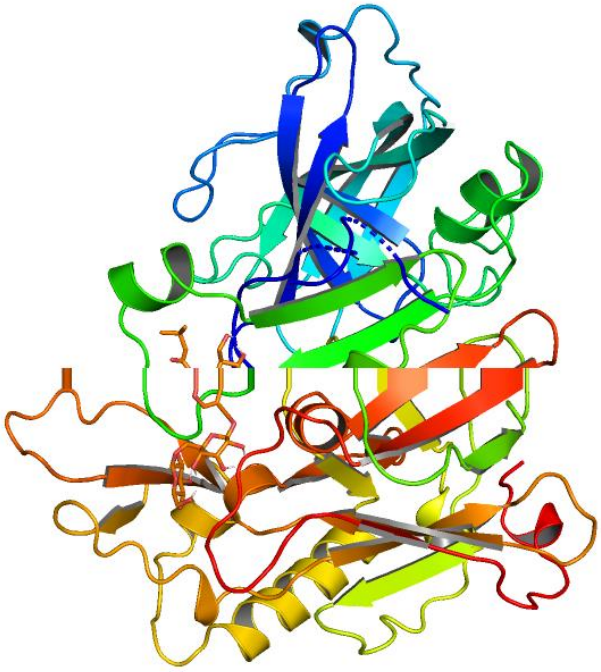


Fig 33. Docking of Kanokoside D with β-secretase

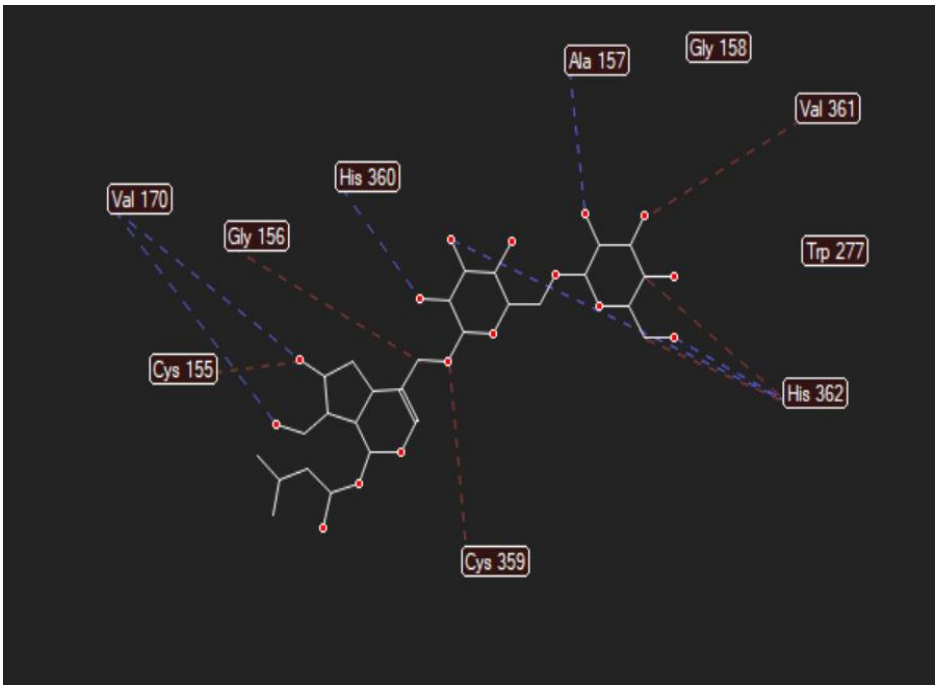


Fig34 Molecular interaction between Kanokoside D and β-secretase

Table 15. Docking score of Rivastigmine and Kannocoside

Protein	Ligand	Predicted Binding Energy (k cal/mol)
β –Secretase PDB ID:1 SGZ	Rivastigmine	-4.67
	Kanokoside D	-6.82

RESULT

The molecular docking study of Kanokoside D against β-secretase.The ligand form hydrogen bond interactions with amino acid Alanine 157, valine 361, histidine 360, cysteine 359 and valine 170 was observed in the docking study Fig 32 .The predicted binding energy was found to be -6.82k cal/mol compared with the standard rivastigmine -4.67 k cal/mol

It is clearly indicates **that Kanokoside D show potential role in AD and active against β-secretase enzyme. The selective inhibition of this enzyme play an important role in AD**. It was also proved in our present research findigs of *in vitro* acetylcholinesterase inhibition assay of Kanokoside D with IC_{50} value of 135 compared with Donepezil 85.

DISCUSSION

In this docking study we present an approach to control alzheimer disease by targeting the enzymes Human acetylcholinesterase and β-secretase enzyme with standard drug Rivastigmine and Kanokoside D an isolated compound from *Oxystelma esculentum* R.BR. Acetylcholinesterase responsible for breakdown of transmitter substance acetlcholine, β-secretase enzyme responsible for the formation of beta-amyloid plaques in brain leads to the disease. Hence for inhibition we are targeting this enzymes.

The ligand (Kanokoside D) was found to have a better binding energy of -4.38, -6.82 kcal/mol as compared to standard drug Rivastigmine -5.82, -4.67 kcal/mol. Hence an isolated compound has better binding energy into acetlcholinesterase and β-secretase enzyme.

CHAPTER-8
SUMMARY AND CONCLUSION

Oxystelma esculentum R.BR.aerial parts was selected for the present research work to investigate the anti-alzheimer activity. First the aerial parts of *O.esculentum* was sequentially extracted by hot continous percolation method by using solvents such as Hexane, Chloroform, Ethyl acetate and Ethanol. All the extracts were screened for preliminary phytochemical screening to determine the secondary metabolites present in the extract. It was observed that the ethanol extract possess maximam phytoconstituents compared to other extracts, containing glycosides, flavonoids, terpenoids, alkaloids, phytosterol and phenolic compounds. These active constituents possess numerous pharmacological activities.

Invitro acetylcholinesterase inhibition assay

All the extracts were investigated for *in vitro* acetylcholinesterase inhibition assay. It was observed that ethanol extract exhibits good acetylcholinesterase inhibition compared with other extracts with an IC_{50} value of 38.54. The polyphenolic compounds such as flavonoids, terpenoids, glycosides have inhibitory capacity against acetylcholinesterase similar to that of currently prescribed synthetic drugs. Hence ethanol extract was considered as an active extract and subjected for *in vivo* anti-alzheimer activity and for isolation of compounds by column chromatography.

Acute toxicity study

The ethanol extract was found to be non toxic and safe upto the dose of 2000mg/kg body weight. The LD_{50} value is expected to increase the dose level above 2000mg/kg. From this 1/10 th(200mg/kg) and 1/5th(400mg/kg) was selected for *in vivo* studies

In vivo anti Alzheimer activity of ethanol extract of *O.esculentum*

In vivo anti Alzheimer activity was investigated by evaluating the ethanol extract against Y Maze, Open feild and Traction test method.The treatment of 200mg/kg and 400mg/kg of ethanol extract of *O.esculentum* showed a significant increase in ($p<0.001$) spantaneous alterations and improve the memory and learning ability in mice.

In open feild method, ethanol extract of *O.esculentum* at 200mg/kg and 400mg/kg exhibited a significant increasing effect on number of crossings and number of rearings compared to scopolamine group. The high dose of 400mg/kg of extract exhibited a better exploratory behaviour in mice in number of crossings.

In traction, the administration of *O.esculentum* has shown a significant effect on enhancing the motor co-ordination and balancing ability in mice. At 400mg/kg of ethanol extract of *O.esculentum* exhibits significant ($p<0.001$) spending more time balancing ability compared to scopolamine induced group.

Biochemical estimation

The administration of scopolamine induces oxidative damage in animals MDA level increases significantly in the mice of group rearing scopolamine.

The level of MDA significantly decrease in the group of animal that received the treatments with the dose of ethanol extract of *O.esculentum*.

Treatment of 400mg/kg of ethanol extract of *O.esculentum* significantly increase the glutathione level $p<0.001$

Administration of 400mg/kg of ethanol extract significantly increase the level of catalase.

Isolation of compound from active extract by Column chromatography

The ethanol extract shows promising effect in both *in vitro* and *in vivo* anti Alzheimer activity. Hence ethanol extract was subjected to column chromatography isolation.The ethanol extract was eluted with various solvents by gradient elution technique. The yellow colour band was eluted and collected

from the fraction 85-88 in the solvent composition chloroform:methanol. The collected yellow fractions showed a single spot in TLC, UV (254nm,366nm), and in iodine vapour. Hence the fractions were mixed together and designated as V1.

The isolated compounds was characterized by UV, FT-IR, ^{1}H-NMR and LC-MS. From the characterization report the isolated compound was determined as Kanokoside D, which is a Iridoid glycoside.

Acetylcholinesterase inhibition of Kanokoside D

The isolated Kanokoside D was investigated for acetlcholinesterase inhibition assay. It was observed that Kanokoside D has good AchE inhibition with an IC_{50} value of 135.54 compared with the standard Donepezil has a greater inhibition of 85.89.

In silico molecular property and docking study of Kanokoside D

The molecular properties of Kanokoside D were calculated by using In silico Molinspiration Cheminformatics tool showed that the Kanokoside D compound slightly violating the Lipinski's rule of 5 (3 violations). The compound Kanokoside D was docked with two targets Human acetylcholinesterase and β-secretase. The ligand (Kanokoside D) showed a better binding energy -4.38 with that of standard Rivastigmine having -5.82 with the target acetylcholinesterase. The ligand form hydrogen bond interaction with Asparagine 230, leucine 305 and glutamic acid 306.

The molecular docking study of Kanokoside D against β-secretase shows a better binding energy of 6.82 k cal than the standard Rivastigmine having -4.67 k cal. The ligand showing hydrogen bond interactions with Alanine 157, Valine 361, histidine 360, cysteine 359 and valine 170.

It clearly indicates that Kanokoside D shows potential role in AD.

CONCLUSION

Ethanolic extract of *O.esculentum* shows promising role in anti-alzheimer activity by evaluating against scopolamine induced amnesia. The phytochemical constituents in the ethanol extract may be responsible for the decrease in neurotoxcity in mice.The Kanokoside D an iridoid glycoside, first isolated from the plant and have a role in inhibition of neurotoxcity and improvement of memory in mice.

CHAPTER-9
FUTURE PROSPECTUS

- In future study Kanokoside D will be evaluated for in vivo anti-alzheimer study in mice.
- Iridoid glycosides (Kanokoside D) a potential inhibitory effect in numerous cancer. So in future the in vitro cell line in cancer also studied.
- The Kanokoside D will be evaluated in future for effective drug delivery system.

CHAPTER-10
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Palayamkottai – 627002, Tamil Nadu, India
Website: www.stxavierstn.edu.in Email: xrfsxc@gmail.com

Date: 18-7-2021

Authentication Certificate

Based on the Taxonomic features and personal observations the plant material/s submitted by S. Vanmugilan, Madras Medical College, Chennai is botanically identified and conformed as here under with the Register No: XCH 40379.

Botanical Name: Oxystelma esculentum (L.f) R.Br. ex Schultes

Syn. Name: -

Family: Asclepiadaceae

Part: Aerial parts

Reference: Flora of Tamilnadu Vol. II : 88, 1987
Botanical Survey of India, Coimbatore

Place: palayamkottai

Signature
Dr. S. MUTHEESWARAN, M.Sc., M.Phil., Ph.D.
Scientist
Xavier Research Foundation
St. Xavier's College
Palayamkottai - 627 002.
Tamil Nadu, India

MADRAS MEDICAL COLLEGE, CHENNAI – 600003

INSTITUTIONAL ANIMAL ETHICS COMMITTEE

PROCEEDINGS

PRESENT: Dr. A. JERAD SURESH, MPharm., PhD., MBA

Roc. No: 5/AEL/IAEC/MMC/2022 Dated: 01-11-2021

Sub: IAEC, MMC, Ch-3 – Approval of Laboratory Animals – Regarding

Ref: IAEC Meeting held on 21-10-2021

This order is issued based on the approval by the Institutional Animal Ethics Committee Meeting held on 21-10-2021, Thursday.

Project Proposal ID Number	18/2021-2022
CPCSEA Registration Number	1917/GO/ReBi/2016/CPCSEA Valid till 19-9-2026
Name of the Researcher with ID Number	S. VANMUGILAN 261915709
Name of the Guide	Dr. M. Sathish, MPharm., PhD
Project Title	Isolation Characterization and Anti-Alzheimer activity on aerial parts of Oxystelma esculentum R. BR.,.
Date of submission of proposal to IAEC	07-10-2021
Date of IAEC meeting	21-10-2021
Date of submission of modified proposal to IAEC	22-10-2021
Date of Approval	21-10-2021
Validity of the Approved Proposal	One Year
Number & Species of Laboratory Animals Approved	**36 Swiss Albino Mice Approved.**

5/2/22

Chairperson
Institutional Animal Ethics Committee
Madras Medical College
Chennai-600003

COLLEGE OF PHARMACY
MADRAS MEDICAL COLLEGE
CHENNAI-600 003

To
Dr. M. Sathish, MPharm, PhD.,
Assistant Professor, Dept. of Pharmaceutical Chemistry,
College of Pharmacy,
MMC, Ch-3.

Copy to:
Special Veterinary Officer, Animal Experimental Laboratory,
Madras Medical College, Ch-3.

Received copy
M Sathish

71st Indian Pharmaceutical Congress

Theme: Healthcare System - Role of Regulators

Chennai, TamilNadu.

Certificate of Participation

This is to certify that Mr. / Ms. Vanmugilan S.

of _____ *has participated / qualified to ~~Semifinals~~ / qualified to ~~Finals~~ in the Quiz Competition of 71st Indian Pharmaceutical Congress 2019 held at Sri Ramachandra Institute of Higher Education and Research (DU) during 20th to 22nd December 2019, Chennai, TamilNadu.*

Shri.Ravi Uday Bhaskar	*Dr.P.V.Vijayaraghavan*	*Dr.M.Dhilip Kumar*	*Dr.A.Jerad Suresh*
President - 71st Ipc 2019	*Chairman - Loc*	*Secretary - Loc*	*Chairman - Quiz*

Organised by
Indian Pharmaceutical Congress Association

Hosted by
All India Drugs Control Officers' Confederation

CBCMT

VIT
Vellore Institute of Technology
(Deemed to be University under section 3 of UGC Act, 1956)

1920-2020 Swansea University Prifysgol Abertawe

Indo-UK Virtual Conference

'Current Innovations and the Future of Therapeutic Developments'

CIFTD-2020

Certificate of Participation

This is to certify that VANMUGILAN.S of COLLEGE OF PHARMACY, MADRAS MEDICAL COLLEGE. has participated in the Indo-UK Virtual Conference 'Current Innovations and the Future of Therapeutic Developments' CIFTD-2020, organized by Centre for Biomaterials Cellular & Molecular Theranostics (CBCMT) at Vellore Institute of Technology (VIT), Vellore, India and Swansea University, United Kingdom during 1st-3rd June, 2020.

Prof. Geetha Manivasagam
Chair, CIFTD-2020
CBCMT, VIT

Prof. Venkat Kanamarlapudi
Chair, CIFTD-2020
Swansea University, UK

Dr. Sudhagar Pitchaimuthu
Convener, CIFTD-2020
Swansea University, UK

Dr. Loganathan Rangasamy
Convener, CIFTD-2020
CBCMT, VIT

K.K. COLLEGE OF PHARMACY

1/161 Sankaralinganar Road, Gerugambakkam (Near Porur),Chennai 600128

AFFILIATED TO THE TAMILNADU DR.M.G.R MEDICAL UNIVERSITY, GUINDY, CHENNAI
APPROVED BY AICTE & PCI, NEW DELHI & GOVT. OF TAMILNADU

Certificate of Participation

This Certificate is awarded to

Vanmugilan.S

for attending Webinar Series -XII on the topic

"PHARMACY CAREER: THE NEED OF DIGITAL TOOLS"

Mr. MANOJ KUMAR YADAVA

on 8th July, 2020.

His/Her Participation is highly appreciated.

PROF. KR. ARUMUGAM	Dr. A.MEENA	Dr. V. VAIDHYALINGAM	Dr. A. SHANTHI	Dr. S. RAMALAKSHMI
Chairman	Principal	Director	Vice-Principal	Organizing Secretary

One day workshop on Artificial Intelligence in Drug Discovery

Organized by
CSIR-North East Institute of Science & Technology

Sl. No. AIDD1987

Certificate of Participation

This is to certify that

Vanmugilan.S

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has participated in the
one day workshop on "Artificial Intelligence in Drug Discovery" organized by
CSIR-North East Institute of Science and Technology, Jorhat on 01-09-2020.

Debabrata Das

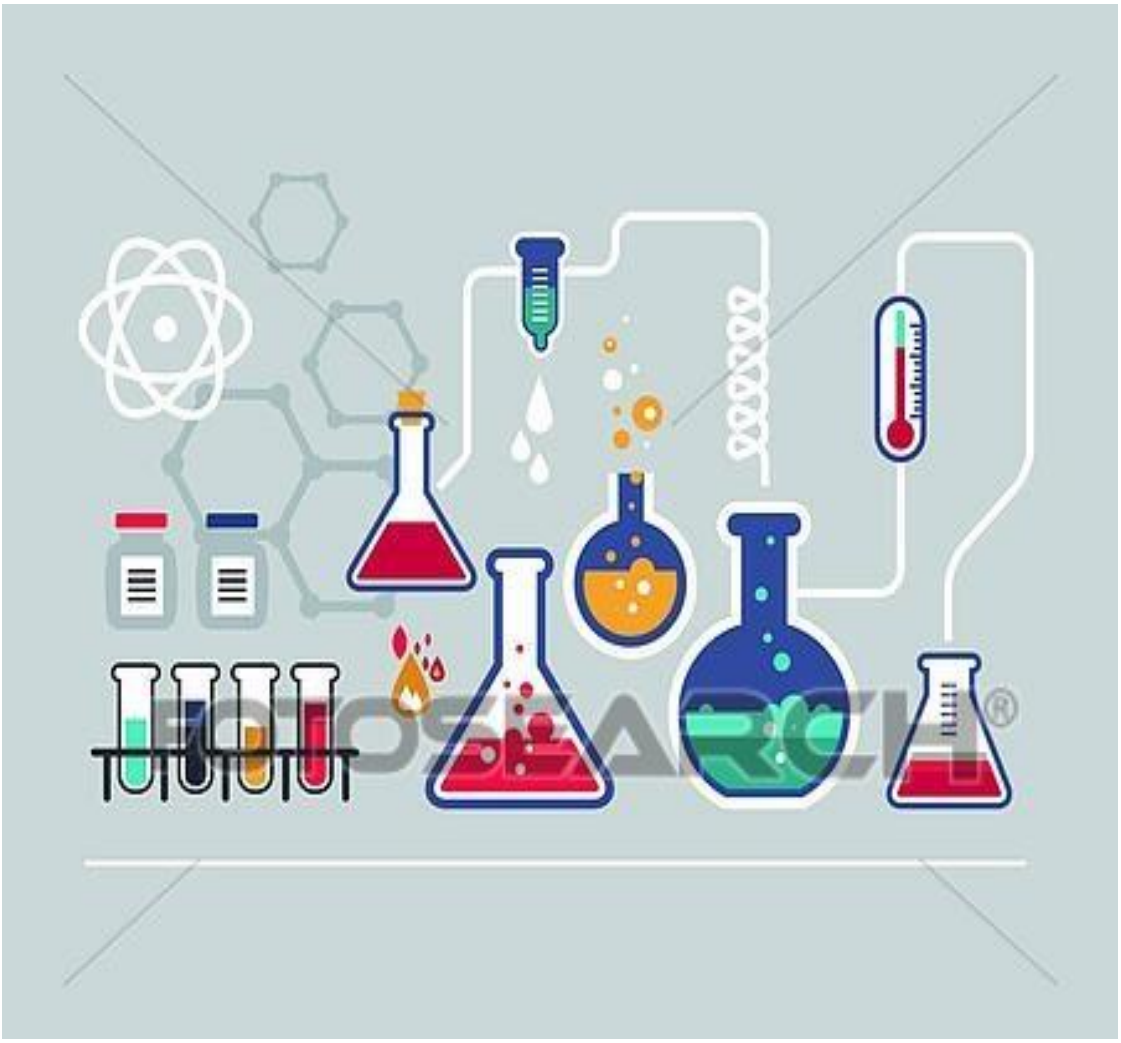
Mr. Debabrata Das
Coordinator
CSIR-NEIST, Jorhat

Dr. G. Narahari Sastry
Director, CSIR-NEIST
Jorhat, Assam


Introduction

Review of literature

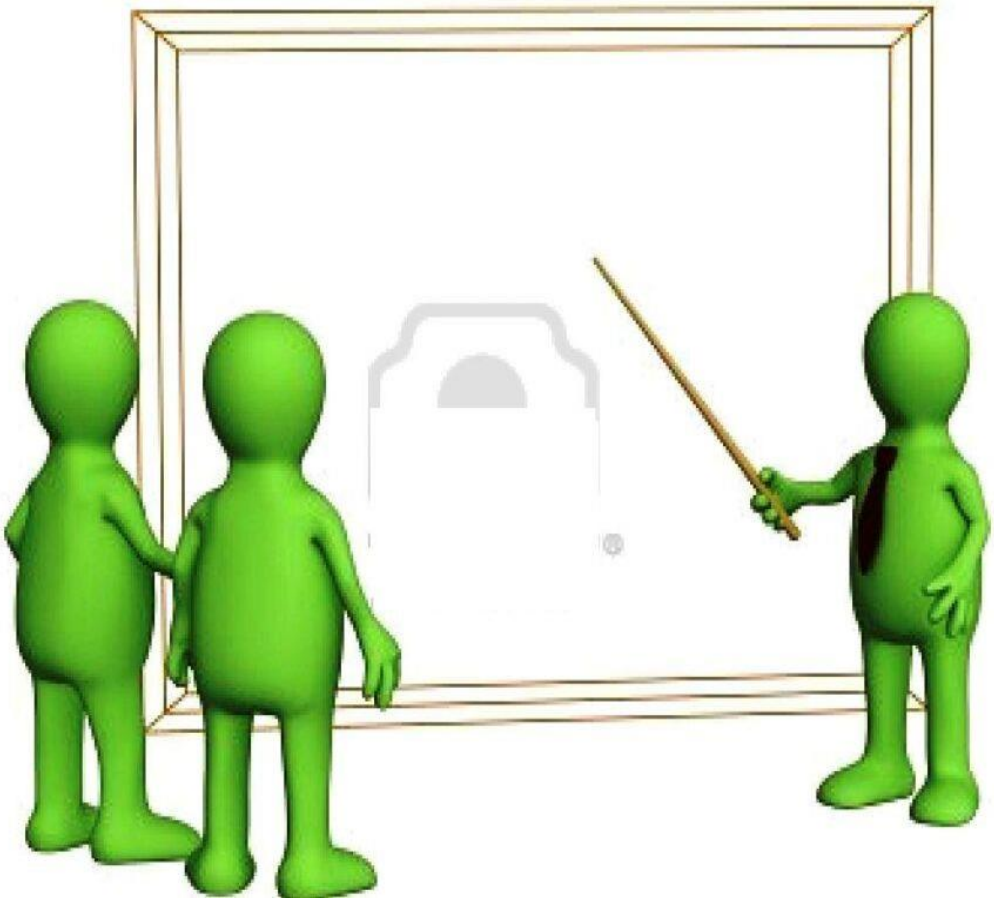
Aim and objectives



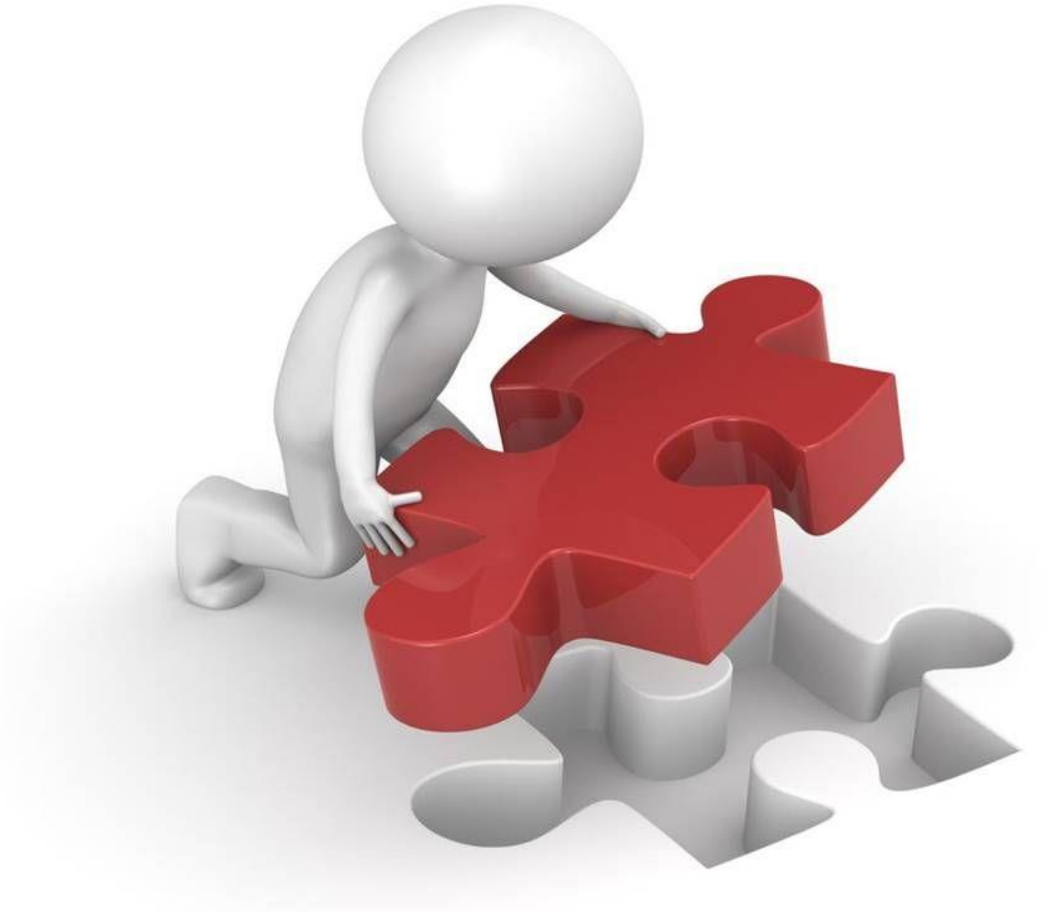
Scheme of work

Plant profile

Materials and methods

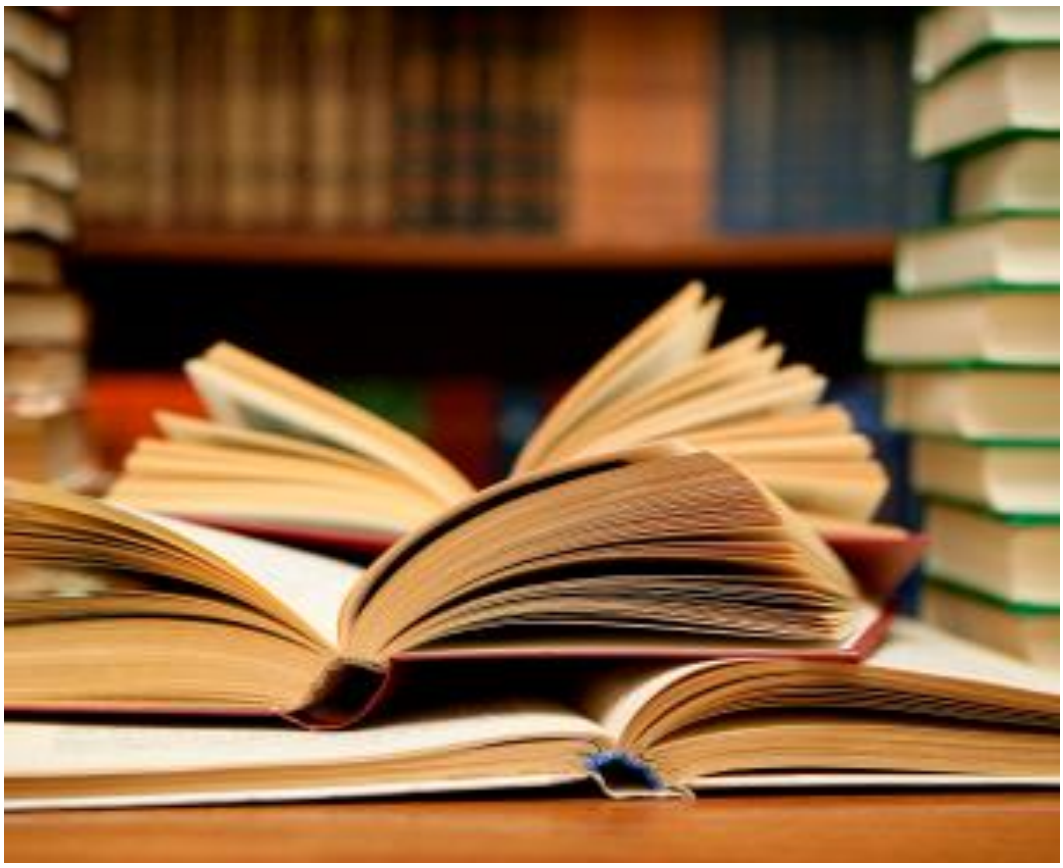


Results and discussion



Summary and Conclusion

Future prospectus



References

annexure