In-Vitro Screening for Cytotoxicity and Acetylcholinesterase inhibitory activity of Pakistani Medicinal Plants

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Thesis Acceptance Certificate

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Declaration

I hereby declare that this research study has been done for partial fulfilment of requirements for the degree of Master of Science in Biomedical Sciences. The intellectual content of this thesis is a product of my own work and no portion of the work referred to in this thesis has been submitted in any other degree or other institute of learning. I also certify that the thesis has been written by me. The help I received during my research work and preparation of the thesis, itself has been acknowledged. Moreover, I certify that all sources and literature used have been cited in the thesis.

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Copyright Statement

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List of Abbreviations

Abbreviation	Full form
ACh	Acetylcholine
AChE	Acetylcholinesterase
BuChE	Butyrylcholinesterase
AChEI's	Acetylcholinesterase inhibitors
AD	Alzheimer's disease
APP	Amyloid precursor protein
Aβ	Amyloid beta
CNS	Central nervous system
Conc.	Concentration
ACTI	Acetylthiocholine Iodide
DPPH	1, 1-diphenyl-2-picrylhydrazyl
DTNB	5,5'Disulfanediylbis (2- Nitrobenzoic acid)
IC_{50}	Conc. required for 50% Inhibition
LC_{50}	Conc. required for 50% Lethality
CC_{50}	Conc. required for 50% Cytotoxicity
NFTs	Neurofibrillary tangles
ROS	Reactive Oxygen Specie
SD	Standard deviation
TLC	Thin Layer Chromatography

ml	Milli liter
µg	Micro gram
$Na_2HPO4.2H_2O$	Di-sodium hydrogen phosphate dehydrate
$NaH_2PO4.2H_2O$	Sodium dihydrogen phosphate dihydrate
FBS	Fetal bovine serum
MeOH	Methanol
BuOH	Butanol
EtOAc	Ethyl acetate
Hex.	Hexane
NaOH	Sodium Hydroxide
H_3PO4	Phosphoric acid
ppm	Parts per million
AR	Argyrolobium roseum
ZF	Zygophyllum Fabago
CC	Citrullus colocynthis
BSLT	Brine Shrimp Lethality Test
MTT	Methyl thiazole tetrazolium
HUH	Liver Cancer Cell Line
MCF-7	Breast Cancer Cell Line

ABSTRACT

Alzheimer's disease (AD) is an advancing age-related neurodegenerative disease that is depicted by the decline of acetylcholine neurotransmitter leading to cognitive decline. Inhibition of acetylcholinesterase (AChE) is deemed as a promising tactic in the administration of AD. The shortcoming of presently approved drugs in use for AD is inherent toxicity and side effects compromising the drug efficacy studies. The present study was designed to investigate the cytotoxic and anticholinesterase potential of 24 extracts of three plant species, namely *Argyrolobium roseum, Zygophyllum fabago* and *Citrullus colocynthis*. In vitro bioactivity screening was performed for various plant extracts obtained through detailed fractionation of crude plant extracts. Anticholinesterase potential was evaluated by Ellman's method and cytotoxic activity was assessed by Brine shrimp lethality assay and MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) cell proliferation assay using 2 human cancer cell lines (HUH-liver & MCF-7-breast). Eight extracted fractions of plant species, revealed promising inhibition as above 50% on AChE at concentration of 100µg/ml. While three butanol fractions of C.colocynthis and Z.fabago plant displayed significant IC_{50} values 32.35, 36.02, 36.89 (µg/ml) compared with standard Galanthamine IC_{50} 8.97 µg/ml. Brine shrimp lethality assay identified presence of cytotoxic constituents of plants and revealed hexane extract of A.roseum to be most toxic possessing median lethal concentration value of 40.04 µg/ml. MTT assay results presented that plants are not cytotoxic against cell lines. In conclusion, the plant extracts exhibited occurrence of bioactive compounds with significant AChE inhibition reinforcing their consumption in the management of AD and supreme effective of all for AD treatment are the extracted BuOH fractions of C.colocynthis and Z.Fabago as they shown no cytotoxicity against both cell lines and maximum inhibition of acetyl cholinesterase enzyme.

CHAPTER 1

1.0. Introduction

According to (Ekor, 2014), plants have always been a necessity for human medicines be it herbal products, nutraceuticals or drugs. They provided the basic source of bioactive compounds against human ailments. (Ravishankar & Shukla, 2007) estimates have shown that the World population still relies about 60-80 percent on traditional supplications for common illnesses. The traditional formulations have rich ingredients of plant secondary metabolites. Likewise traditional system medicine evolved e.g the Unani System for Medication is a combination of traditional medicines from Egypt, Syria, and Iran etc. It also originated and embraced in subcontinent by the Arab, Greeks and Persians (Ansari, 1985). At present times the Unani Medicines are prevalent in the rural areas of subcontinent where it is also the main source of treatment in urban cities as Bangladesh (Izhar, 1989). As a result of their curative actions they have gained the trust of many while being used up every day (Nile, et al., 2017). According to (Cai, et al., 2003), the Unani Medicines consisted of phenolic acids, flavonoids, coumarins, tanins and other essential oils include nitrogenous compounds such as amines, alkaloids, and betalains and carotenoids, are rich for the fragrance and other medical properties. Poly herbal plant preparations are also used over single drug or mixture of drugs which have improved efficiency with no side effects (Ravishankar & Shukla, 2007). These Properties makes plant based medicine being used on a large scale by Pharma. Food industries also use plants for flavors (Kosar, et al., 2008).The complexity of resolving various bioactive compounds derived from crude extract employ use of various extraction and fractionation techniques of chemistry. For the matter of screening, several different models like plant bioassays, tissues, cell culture and assay guided chromatography are used for identification of biologically active compounds (Wang, et al., 2011). As potions, here it is crucial to determine the quality of the supplement as it is given orally and in high dosage ((Nile, et al., 2017).

This study analyses the Alzheimer's disease and the result against the cytotoxic and enzyme inhibitory activity (Acetylcholinesterase Inhibition) in addition it also provides the details of screening program against liver HUH and Breast MCF-7 cancer cell lines.

1.1. Alzheimer Disease

Alzheimer's disease has become a major threat to the world it has overwhelming effects including dementia but still we are unable to find any cure. Survey estimates that around 27.7

– 35 million individuals suffer from AD (2005 – 2010). According to (Wimo, et al., 2010), the treatment of AD amounts to 156 Billion USD to 604 Billion USD. The disease therapeutic study focuses on active interaction of drug to control acetylcholine effects of Alzheimer's through acetyl cholinesterase inhibition. Prosthetic mechanism involving one of the two actions of the drugs to regulate mimic action of acetylcholinesterase while, controlling acetylcholine stimulus. The other action is Acid-transferring to counter the enzyme action. The drug therapeutic aids include treatments of initial stages by increasing levels of acetylcholine which improves the cholinergic neurotransmission (McGleenon, et al., 1999) but however accompany some unwanted effects like hepatotoxicity and gastrointestinal disorder. The drug action can last for a short time period, permanent or irreversible (Nair & Hunter, 2004). The natural remedial supplements comprises plant derived formulations such as alkaloids, rivastigmine and galantamine and some modern Ache inhibitors from natural sources (Ogino, et al., 1997).

1.1.1. Plants being source of Alzheimer's Medicine

Medicine from the plants has always been made use of from the prehistoric era particularly for memory dysfunction. However synthetic drugs have not been able to find a definitive cure of AD and therefore finding another source for the cure had become a necessity having an emphasis on plants (Albanes, 1999). By now the established supplements improve the level of chemicals known as neurotransmitters inside the brain but there is no solution to halt or hinder the demolition of neurons. Presently most effective drug agents for AD is Galanthamine which is alkaloid based originated from Galanthusnivalis L. It originated from Bulgaria and Turkey and extensively used for neurological conditions. Galanthusnivalis L. is one of the plant species that has been studied and tested being in clinical use for more findings. Among the various screening techniques used in medicinal finding ethopharmacological stands in front of all of them.

Searches for discovery of new drugs for AD treatment are rising by modern inventions to specifically aimed as new AChE inhibitors having the least amount of side effects. Numerous plants all around the world have been reported for screening every day for cholinesterase inhibitory target. Plant remains as one of the most significant sources of the plants that is appealing to human nature and effective for drug utilization.

1.2. An Introduction of the Studies

Traditional medicine is used to treat numerous disorders and is a very common practice in Pakistan. To find out the numerous factions from plants extracts bioassay-guided isolation was implemented and these extracts were also studied for having cytotoxic activities by implementing different screening methods. We explored some local plants of tropical region to identify the potential of plants for cure of AD.

Argyrolobium roseum, Zygophyllum fabago, and *Citrullus colocynthis* are being studied for phytochemical research for analysis of bioactive fractions. Presence of cholinesterase inhibitor action in the crude extracts was found in *Zygophyllum fabago* & *Citrullus colocynthis*. In order to find the cytotoxicity of the active extract, brine shrimp assay and MTT assay was used. This report attributes presence of activity referring to Cholinesterase inhibition of constituents of extracts of the plants

A literature study of *Argyrolobium roseum* reveals that comprehensive phytochemical studies were made in 2013 by (Khanum, et al., 2013), it was the first instance when the in-vitro anti-oxidant abilities of organic elements were observed. The inhibition actions caused by acetyl and butyl-cholinesterase were also resorted for plant (*Trifolium angustifolium)* of Leguminosae group. This suggests that A. roseum belonging to same family of Leguminosae may also possess cholinesterase inhibition effects hence, *Argyrolobium roseum* is screened here to find out the AChEI potential. Literature review of plants provides useful information for biological activities.

1.3. Aims

The aim of the present research study is to identify local species of common medicinal plants found in Pakistan and to determine biological profile of their organic extracts.

- To observe the in-vitro anti-cholinesterase actions of several plant extracts and their fractions.
- Identification of the cytotoxic potential of several extracts of solvents:
  - Performing in vitro cytotoxic analysis by Brine shrimp Lethality Assay of several solvent extracts.
  - Performing Cytotoxicity Assay on the cancer cells of human (MTT Assay) using crude extracts.

Chapter 2

2.0. Literature Review

Plant have been consumed by men from historic eras in every discipline of life. Men have healed their wounds through medicines and exploring all horizons to achieve maximum life and a healthy body. Throughout history men have reaped the benefits from plants by observing through trial and error that what kind of plants and animals provide beneficial food and what kinds are detrimental or harmful for us (Gani, 1998). The chemical components and secondary metabolites of maximum plants are unidentified and might result in hazardous consequences on human health. Conversely, some plants may have medicinal potential, which are not stated to be utilized as herbal medicine.

At the present times the use of plants as medicines or drugs have become a necessity and its growing ever strong, estimation says that 70-80% population of the world depend upon medicines primarily derived from plants (Farnsworth & Soejarto, 1991). Mostly all pharmaceutical lines are made from plants as they have become a vital source for drugs, cosmetics, food supplements and functional foods (Benamar, et al., 2010). It can be rightly said that these sources have always played a vital role. However, the rapid growth of population and inadequate allopathic medicines undermine the supply and demand of the herbal medicine. (Lambert, et al., 1997).

2.1. Position of medicinal plants in Pakistan

Pakistan has some wonderful geographical benefits having a total area of 80,943 km^2 situated on latitude of 23° 45’ to 36° 50’ and longitude of60° 55’to 75° 30’, there are 600 to 700 different species of plants used in for different medicinal purposes. This is a result of the variation of the climatic zones and a range of altitude from 0 to 8611m. As a result of the extensive variety of the species many researches are being conducted all over by mainly universities for ethnobotanical listing of resources. On the whole the researches are being done in the current phase on documenting level. For almost all kinds of ailments medicines are made from plants whether it be a minor issue or a major problem, for example wild plants are being used for Ephedra, Artemisia Hippophae etc. (Shinwari, 2010). The plants present in the localities have been studied by ancestors. This knowledge is passed on from centuries and is preserved in the minds. The herbal medicines cure many forms of diseases from migraines to stomach aches or scars (Bhardwaj & Ghakar, 2005). They massive sea of knowledge of Unani,

Ayurveda is threatened of being lost. Currently there are 500 different species used in Pakistan out of which 95 are already being used to cure some form of diseases (Bhardwaj & Ghakar, 2005). There have been abundant studies and findings on usage of these plants in the numerous locations of Pakistan, but knowledge of the plant or specie is limited to the elders of that area (Hocking, 1958).

(Shinwari & Gilani, 2003) have reported that there have been many researches on the basis of geographical locations of the plants. (Hamayun, et al., 2006) have reported the herbal medicines from Northern Pakistan. (Haq & Hussein, 1993) reports 70 different types of plants belonging to Manshera, NWFP. Ethnobotanical researches on the area of Ayubia National Park, Nathiagali, Swat being done in great depth by (Shinwari & Ayaz, 2002) whereas (Ali & Qaisar, 2009) studied 83 taxa being utilized in Chitral district from Hindukush range. (Goodman & Ghafoor, 1992) studied 114 different types having ethnobotanical treatment in Baluchistan province. (Shah, 1996) studied 171 different kinds of plants being utilized as medicines in Kharan district, Baluchistan. On the other hand, the chemical and pharmacological features of the species were observed by (Gilani, et al., 2007). (Gilani & Cobbin, 1986) reported different species extracts of plants made in to drugs called himabicine, being sample of a cardio concerning anti-muscarinic drug. The scientific analysis of the old fashioned use of some plants as medicines was given by (Gilani & Rahman, 2005) and (Shinwari, et al., 2009) as they discovered the availability of AChE inhibitory elements.

Likewise distinctive result of actions were found by (Choudhary, et al., 2005) in Prosopis juliflora and Withanolides from Withania somnifera. (Tariq, et al., 1995) also tested herbal plants looking in to their antimicrobial actions with respect to 25 pathogens or non-pathogens in Karachi, Sindh, Pakistan. Ethnobotanical, pharmacological and pharmaceutical actions should also be considered and explored in endemic species (Shinwari, 2010). The main area of the revolutionary researches has been done in Kashmir, Baluchistan and Chitral called the northern areas of Pakistan. Chitral, Kashmir and northern Baluchistan are the center of evolution in radiation of medicinal plants. (Shinwari, 2010) has reported that pharmacological and ethnobotanical uses of some endemic kinds may also be discovered.

2.2. World Market of Medicinal plants

According to (Larid, 1999), Japan consumes the highest botanical drugs in the world whereas France, Spain, UK, Germany, Japan, China, Italy and the US, are giant consumers of MAPs. (Laird & Pierce, 2002) studies reveal that the Market of herbal Medicines was $6.7 US billion

by Europe, leading the pact, 5.1 US billion by Asia, 4.0 US billion North America, 2.2 US billion by Japan and lastly the remaining world summing to 19.4 US billion dollars. The market in Japan in botanical elements in 1996 approximated to 2.4 billion dollar and in 1995, 5 billion dollars from China. The Major supplier of un processed MAPs is India where overall medicines contributed to 53.219 US dollars and 13.250 US dollars particularly from oils (Lambert, et al., 1997).

2.3. Bioactivity guided Herbs research

There are still many different species of plant that remains unanalyzed, they can be exposed to pharmacologic screening keeping in mind their traditional use. If any real beneficial result ids witnessed, processes like chromatography and spectroscopy are practiced on it for isolating the active element. There are numerous books that listed those plants and species that are not observed. A bioactive based investigation consists of three parts. Starting with observing the biological activity and detecting the active elements that take part in it, bioassay is carried out to remove the inactive element, so isolation is possible. Next, the process of fractionalization is used where the pure elements are produced from the compound being again and again subjected to this process. At the end, the determination of the chemical arrangement of the compound is declared (Goldstein, 1974).

2.4. Approaches to discover new products

There are numerous steps on searching chemical characteristics of plants to have important discoveries or finding regarding useful products. Now the chemical elements are being isolated for example checking of in vitro actions with respect to cell lines. The end product results are analyzed, subjecting to clinical processes, for further development and certification. According to (Hamilton, 2003) there is another way of screening that includes genetic information being extracted from plants.

There is no specific amount of plant included in a local drug or the combination of different drugs produced locally so using an industrial process is not beneficial. The traditional dispensers now are moving towards herbal medicines which yield more profits. So, the authentication or the amount of material being used is again variable and undefined. This leads us to the matter of the traditional techniques which for once resulted in numerous disadvantages needing a correct decisive way for production. It is clear today that the technology available now was not available then. Results are transported in a proper organization of the system to produce operational, constant, measured dosage. Thus, as a result these old-fashioned practices

should be preserved and as being a labor intensive, small scale production so employment can also be offered and knowledge can be passed as well, or low-cost technological methods be introduced. One disadvantage is that using the high-tech methods will detach the traditional makers and he would have no idea of what's going on. Thus, it is vital to have educated practitioner who is able to operate well and can see the long-term benefits of using the sophisticated methods for the production and the quality it guarantees. Another problem that arises is either the product produced after the process is accepted but the original makers who have never used such techniques are likely to not believe in them as well. Here comes the awareness program through which the advantages be taught to all the practitioners. If the medicine be used for foreign exchange it should have been developed in pharmaceutical industries, the raw ingredients used are:

- Formulation of drugs from isolation of pure compounds
- Separate intermediates for the manufacture of half synthetic medicines.
- Formulate uniform galenicals for extracts, powders, tinctures technology and more streamlined processes are used if a pure pharmaceutical drug is to be produced.

The issue of Pollution should not to be ignored and safety precautions should be used. If a plant contains the essential intermediate, then its cultivation can be enhanced in the country from which it is produced so some of the value of the production can be retained. Galencial is a processed product derived from plants, is a uniform fluid/solid extract precipitate. The health care industries also reap the benefits from this uniformity of production. Fresh preparations of new products are required to have some development work, with respect to its behavior or nature. The (Planning Commission, 2000), says that as the extracts are hard to granulate, very susceptible to contamination from microorganisms and a little moisture can affect its activity, thus there have to be extra precautions and well planning of each parameters of processing.

2.5. Alzheimer's disease

Among many neurodegenerative disorders one of the main is (AD) Alzheimer's disease categorized with advanced degeneration of memorial and cognition. Amyloid-B, neurofibrillary tangles and synaptic loss because of lack of the acetylcholine (ACh) with the deterioration of cholinergic neurons are some of the trademarks of the disease. Ach is essential for learning and for the memory and plays the role of a protagonist in the disease. Substrates ACh is formed in cholinergic neurons from processing of synthetic enzyme choline acetyl-transferase which uses acetyl coenzyme-A. It is a neurotransmitter in the nerve endings.

Medicine from the plants has always been made use of from the prehistoric era particularly for memory dysfunction. However, pharmacy medications have not been able to find a definitive cure of AD and finding another source for the cure had become a necessity having an emphasis on plants. (Albanes, 1999). We are still searching nature for some answers specifically Ache inhibitors having the least amount of side effects, numerous plants all around the world are screened every day for cholinesterase inhibitory potential.

2.5.1. Alzheimer Disease Epidemiology and Hazards

The cases of AD are evident in adults of 65 and above. it goes up to 50% over the age of 85.an Estimation from the united nations concludes the alarming rate of growth of AD be enhanced from 25.5 to 114 million in the span from 2000 to 2050. In addition, an expenditure of 70 US billion in US has been on AD alone and a total of 72,914 deaths it has caused (CDC). AD itself does not cause death but its adverse effects on the body such as unable to swallow and immobility causes the death. Thus malnutrition, pneumonia causes the ultimate death (Thies & Bleiler, 2011). As aging is seen as the basis for the cause of AD but there is other factor as well such as reduced brain capacity, overall brain shrinkage, less mental accomplishment and mentally demanding professions and followed by a restricted mental and physical action in daily life. According to (Mayeux, 2003)an accidental head injury is also a serious risk factor.

2.5.2. Phases of AD

The phases underneath deliver an overall structure of how capabilities alter throughout the progression of illness.

Stage One	No Deficiency
Stage Two	Slight Decay
Stage Three	Mild Decay
Stage Four	Reasonable Decay
Stage Five	Reasonably Severe Decay
Stage Six	Severe Decay
Stage Seven	Seriously Severe Decay

These stages are not the same for everyone. The clinical director of the New York University School of Medicine's, Barry Reisberg M.D, serving in Silberstein Aging and Dementia Research Center reported these seven stages based on his work.

2.5.3. AD and its pathology

There are many neurotransmitter and pathophysiologic processes involving in pathology of the disease. The trademarks or the specific identity of the disease is deposits of extracellular plaques containing β-amyloid protein, the depletion of cholinergic neurons and the creation of intracellular neurofibrillary tangles. These are the identifications of Alzheimer disease with respect to pathology (Figure 1).

Plaques and NFTs first being identified by Aloiss Alzheimer for the first time in 1906 were a definite discovery of AD but not the most distinctive. These symptoms also occur with the process of aging and several other neurodegenerative disorders (Lei, et al., 2010). The area controlling the clinical symptoms contain plaques and NFTs.

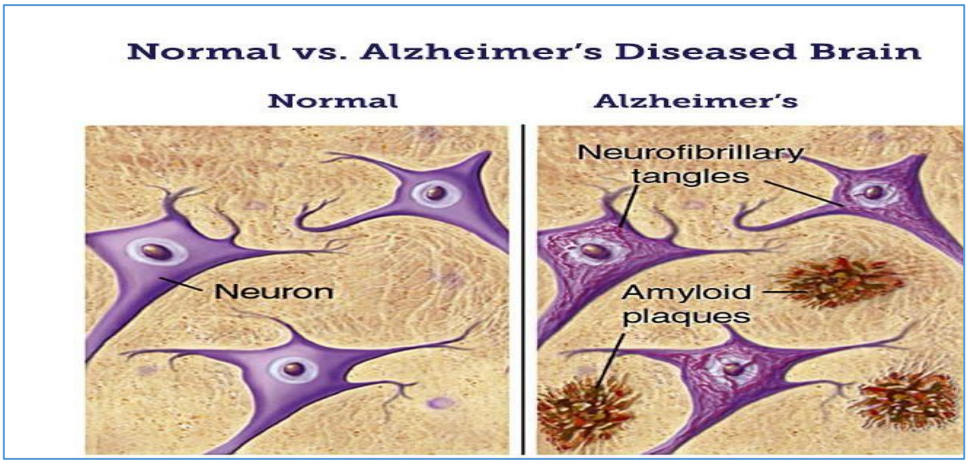

Figure 1: A comparison of a normal neuron and Alzheimer ravaged neuron (Courtesy AHA Foundation, 2000-2009)

2.5.4. B-Amyloid Plaques

According to (Morrison & Lyketsos, 2005), Secretase are a collection of enzymes which degrades the precursor protien. B-Amyloid plaques are separated from the protein having undefined prominence in the organism. The product becomes of no harm when the precursor protein is deteriorated by α-secretase.

2.5.5. Neurofibrillary Tangles (NFTs)

Microtubules in cytoskeleton is stabilized by tau which after being modified destructs the neuronal microtubules resulting in the creation of NFTs (Kao, et al., 2010). Microtubules have many important functions from which one of them proving the path through which the nutrients are transported, and neuronal transmission is spread inside the neuronal axon.

The destruction of neuron transport and its communication system finalizes the neural death of the cell. This happens because of the collapse of microtubule resulting from the tau protein becoming hyper-phosphorylated during the life span of Alzheimer disease. The hyper-phosphorylated tau is said to be present only after the plaque creation, however the connection between the two is ambiguous (Selkoe, 2002).

2.5.6. Decrease of Cholinergic Neurons

It has been studied that in various areas of the brain the number of cholinergic neurons reduces to a staggering 75% which is a dramatic reduction (Perry, et al., 1978). This happens by the late stage of AD causing the reduction of cholinergic neurons.

Acetylcholine bonds the postsynaptic receptor types which are muscarinic and nicotinic. It is also an essential neurotransmitter regarding memory and reduction of cholinergic actions and correlating with various features of cognitive deficiencies (Figure 2).

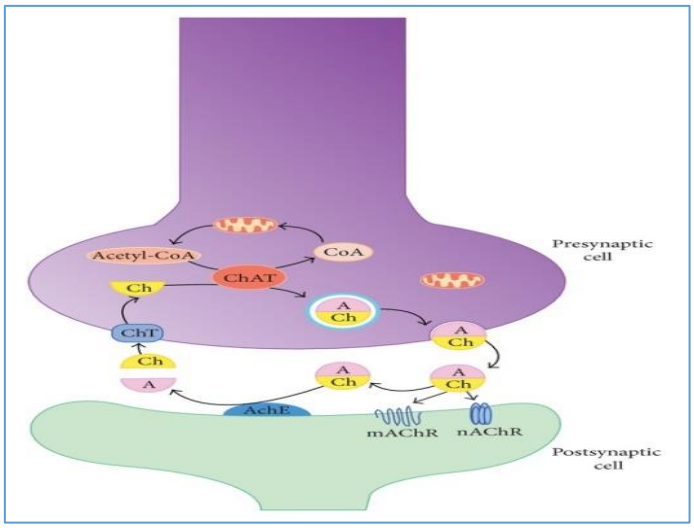

Figure 2: Acetylcholine action mechanism

According to (Morrison & Lyketsos, 2005) in the AD pathology implication have been made for the influence of presynaptic nicotinic receptors which release the neurotransmitter essential for the memory.

The area most affected by the properties of AD are the higher mental functions for example the neocortex and hippocampus (Figure 3).

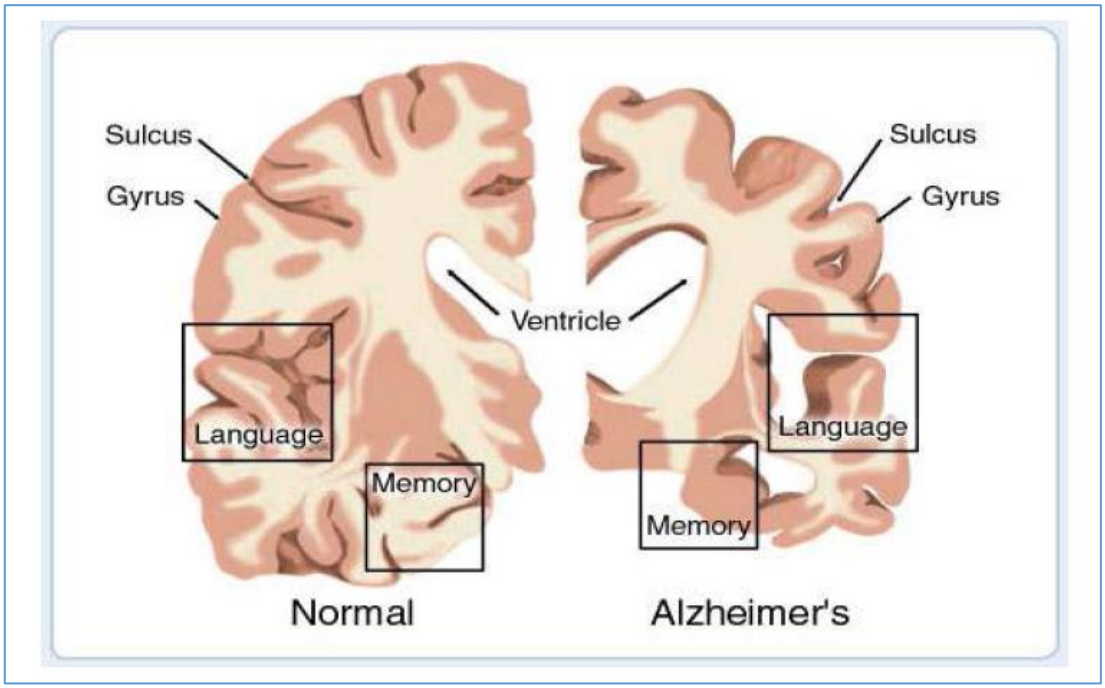

Figure 3: Comparison of a normal human brain and Alzheimer ravaged brain (Courtesy of American Health Assistance Foundation, 2000-2009)

2.6. Acetylcholinesterase (AChE) and its inhibition

2.6.1. Acetylcholinesterase (AChE)

Figure 4 shows that AChE is seen in excitable tissues such as the synaptic junctions and is a membrane bound enzyme. The main part of the AChE is the cancellation of nerve impulse communication in the cholinergic synapses being rapidly hydrolyzing the neurotransmitter ACh (Mukherjee, et al., 2007).

Therefore, AChEIs according to (Rollinger, et al., 2004)encourage a growth in the concentration and extends the activity of synaptic ACh. It belongs to the carboxylesterase chain of enzymes and becomes the main objective of inhibition by organophosphorus compounds for example nerve agents or pesticides.

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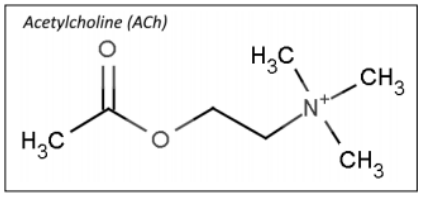

Figure 4: Chemical structure of Acetylcholine

2.6.2. Structure and mechanism of the enzyme

The maximum allowed diffusion is close to 25000 molecules per second which is the rate of degradation of ACh. Particular ACh contain extraordinary catalytic activity (Brown, 1954). There are two sites of AChE, one is the anionic site and the other is esteratic site (Figure 5). The construction and the course of actions taken by AChE are clarified by the crystal structure of the enzyme.

The cationic substrates and inhibitor are accommodated by the anionic substituted site and the positive quaternary amine of acetylcholine that guards the gorge towards the active state residue bounds the cationic substrates by their linkage and not by any negatively charged amino acid in the state. These amino acids are preserved through various species. According to (Chaturvedi, 1985) tryptophan 84 is critical in its replacement along alanine resulting in 3000-fold diminution in reactivity. Being 20 angstroms in length the gorge enters mid-way over the enzyme. From the bottom of the active site the molecule is situated 4 angstroms away.

There is a triad of three amino acids which are quite identical, serine 200, histidine 440 and glutamate 327, they just have one difference of glutamate as the third member rather than aspirate being that member. These three are included in the hydrolyzed acetate and choline. However, this triad is the opposite of chirality of other proteases (Roy, et al., 1987) . Free choline and acyl enzyme are formed by hydrolyzing carboxyl ester (Jena, 1991) histidine 440 group assists the nucleophilic attack on the enzyme which produces acetic acid and redeveloping free enzyme.

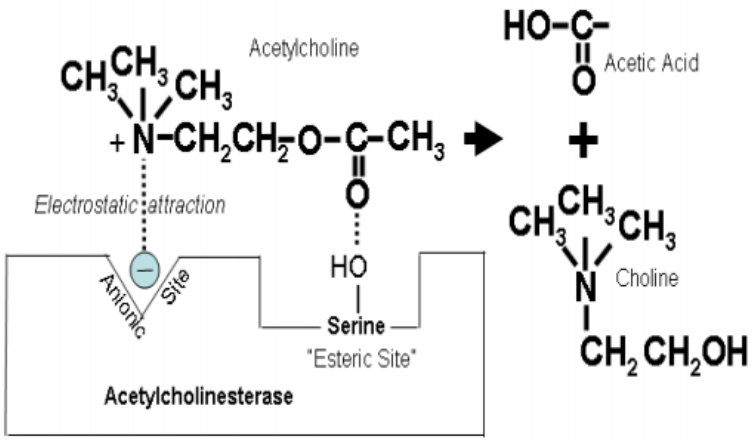

Figure 5: Mechanism of Action of AChE

2.6.3. Inhibitors of AChE (AChEIs)

The cure for AD is still not discovered but there are ways to slow down the disease and its symptoms. The symptoms are divided in two categories the cognitive and behavioral and psychiatric. As AChE is a neurotoxic combination thus they can cause central, peripheral or both central and peripheral cholinergic crisis. According to (Houghton, et al., 2006) there are two main ways to utilize them as pharmaceutical and pesticides. The two types of cholinesterase are reversible and irreversible. For reversible inhibitors they produce transient complexes and compete with acetylcholine in the active state the active state is bounded and blocked by a single group of quaternary alcohols. Some greatly constant phosphor intermediates are formed by irreversible inhibitors covalently attached to theAChE sites (Golan, et al., 2011).

The organophosphates bound and experience early hydrolysis by AChE, however the acyl transition is changed by a phosphoryl moiety that is sliced tremendously slow, successfully and is permanent (Golan, et al., 2011).

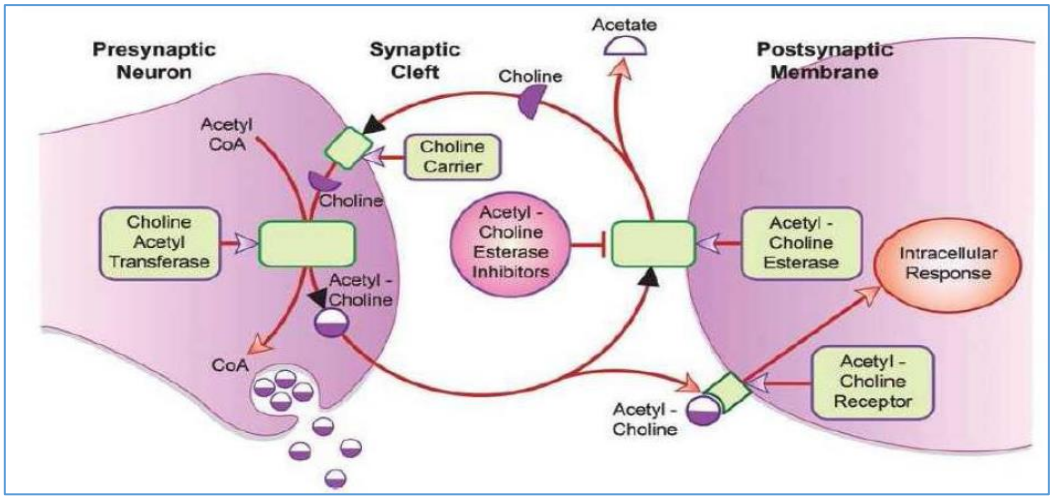

Figure 6: Acetylcholine inhibitors inside cholinergic nerve transmission

The Cholinesterase inhibitors function as follows:

•Stop acetylcholine from breaking down, a chemical communicator essential for learning and memory

•Postponement or deterioration of indications for 6 to 12 months regular, for around half of the people who consume them

•Adverse effects like nausea, vomiting, loss of appetite and improved frequency of bowel activities are included

At this time there existed 4 cholinesterase inhibitors: tacrine, donepezil, galantamine, and rivastigmine.

a. Donepezil (Aricept) is permitted for the treatment of all stages of Alzheimer's.
b. Rivastigmine (Exelon) is permitted for the treatment of minor to major Alzheimer's.
c. Galantamine (Razadyne) is permitted for the treatment of slight to modest Alzheimer's.
d. Tacrine (Cognex) is the first cholinesterase inhibitor permitted.

2.7. The research of selected Medicinal plants

To authenticate the efficiency of traditional medicine we chose 3 different plants from a variety of locations on basis of ethnopharmacological information

1. *Argyrolobium roseum*
2. *Zygophyllum fabago*
3. *Citrullus colocynthis*

Table 1: Medicinal plants selected for cytotoxicity & Acetylcholinesterase inhibitory activity

S. No.	Botanical Name	Common Name	Family	Ethnobotanical Use
01	*Argyrolobium roseum*	Makhnibooti /Broom	Papilionaceae	Anti-diabetic Anti-bacterial Anti-inflammatory Cooling agent Aphordiasic
02	*Zygophyllum fabago*	Syrian Bean Caper	Zygophyllaceae	Anti-rheumatic Anti-helmintic Anti-asthmatic Anti-tusive Cathartic Expectorant
03	*Citrullus colocynthis*	Bitter Apple	Cucurbitaceae	Constipation Diabetes Leprosy Edema Fever Asthma Bronchitis Joint pain Cancer Mastitis Jaundice

2.7.1. Argyrolobium roseum(Jaub. &Spach)

2.7.1.1. Overview of *Argyrolobium roseum (Jaub. &Spach)*

Kingdom: Plantae

Subkingdom: Angiosperm

Super division: Eudicots

Division: Tracheophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Fabales

Family: Fabaceae

Genus: *Argyrolobium*

Species: *Argyrolobium roseum*

Sub Species: *Ornithopodioides* (Jaub. &Spach) Jafri & Ali

2.7.1.2. *Genus*: Argyrolobium

The genus Argyrolobium contains over 178 different species which are used in old fashioned medicine in numerous countries being abundant in Africa, Madagascar the Mediterranean area and southern Europe, and to the east of Indian subcontinent (Polhill, 1968). It is derived from a large family Leguminoseae or Fabaceae which can be easily found in Pakistan. This is the 3rd largest families from all the plants that produce flowers. Papilionaceae includes 482 genera and 7200 different species it also contains A.roseum (cambers Jaub and Spauch).

The medicinal characteristics present in these plants include hallucinogenic and analgesic potential being used as astringent in cure for lecuoderma or leprosy (psordeacorylifortia). Overall, they cure different diseases which include yellow Dhal a combination of leaves of cajanuscajan L, anemia is treated by pigeon pea, hepatitis, diabetes, urinary infections and yellow fever. An alkaloid present in it is known to be utilized in cigarette cessation is Cytisine.

Species: *Argyrolobium roseum*

Synonym: *Argyrolobium roseum* ssp. *Ornithopodioides* (Jaub. &Spach) Jafri & Ali*Cytisusroseus*

Author: Cambers Jaub and Spauch

Local Name: Makhnibooti/ Broom

Figure 7: ***Argyrolobium roseum***

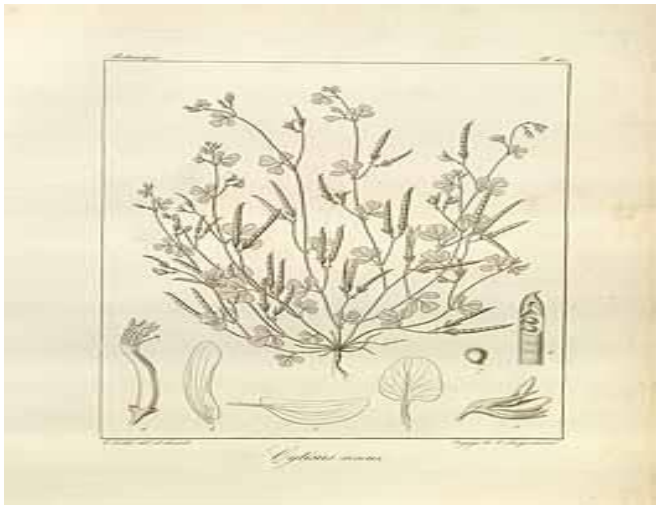

Figure 8: Whole plant ***Argyrolobium roseum***

2.7.1.3. Habitant & Habitat

Figure 7 shows a prostate herb, furry, much branched yearly herb, originate in dehydrated areas in lower hills and plains of sub-tropical regions. Located in the Indian subcontinent and Pakistan, Iraq, Iran, Afghanistan, India and Nepal, is a unique herb growing in areas of tropical and sub temperate tracts. The stem of the plant is erect, branched, herbaceous and green. It contains leaves that are composite, round at the peak and tapering at base represented in figure 8. Compound leaves with leaflets, trifoliate, obovate, curved at the apex and tapering on the base, even, glossy and white green.

2.7.1.4. Taxonomic features

Stem terete, hairy and is much branched. Stipules 1.5-2 mm, free lateral. Leaves trifoliate, petiole 3-10 mm long. Petioule less than 11 mm. Leaflets 4-13 mm in length, obovate, obtuse,

glabrous overhead, pilose underneath. Inflorescence 1-4 flowered peduncle raceme, peduncle 0.4-4.0 cm. Bract 1.5 mm in length. Calyx 4.5-5.0 mm in length, bilabiate and pilose. Corolla 5 mm in length, red pod 0.8-3.5 cm in length, 2.5-3 mm broad, 3-15 seeded, pilose (Ali, 1966).

2.7.1.5. Flowers and Foliage

It blossoms from April to October with yellow and pinkish-red flowers as in Figure 9 and 10. Fruit is a pod consuming an amount of black seeds.

Figure 9: Flowers of A.roseum (Yellow)

Figure 10: Flowers of A.roseum (Purple)

2.7.1.6. Existence in Pakistan

The herb is very unique and is found in tropical and sub moderate areas of north western Himalayas in Indian sub-continent and Pakistan including Azad Kashmir, Meerpur, Kotly, Peshawar, Balakot, Abbottabad, Haripur, Punjab, Jehlum, Rawalpindi, Islamabad, Murree and Baluchistan (Hussain, et al., 2014).

2.7.1.7. Traditional Uses

For the cure of liver, stomach, and bladder inflammation, a fresh herb grown in water is utilized. A significant alkaloid cytosine is removed from the plant that was used for cigarette cessation.

2.7.1.8. Medicinal Importance

Utilized an as anti-diabetic, antibacterial, hypoglycemic, anti-inflammatory, cooling agent, aphrodisiac diuretic & sedative. Also, have uses in Gleets, skin diseases, skin lesions and pimples and curing jaundice in Pakistan.

2.7.1.9. Reports of natural products

Natural products reported of *Argyrolobium roseum* are terpenes, phenols, saponins, flavonoids, alkaloids, tannins, cardiac glycosides and reducing sugars.

2.7.1.10. Phytochemicals

According to (Hussain, et al., 2014) there is report of two phytochemicals, Vitexin and D-Pinitol.

2.7.1.11. Activities reported

There has been no kind of studies or report identification or determination of active extracts. In 2013 it was the first instance when in vitro and oxidant abilities of organic fractions and aqueous excerpts were analyzed.

2.7.1.12. Research Aim

Trifolium Angustifolium var.is related from the exact family of Leguminosae that holds acetyl- and butryl- cholinesterase inhibition actions (Ertas, et al., 2015). On the basis of the study, screening of *Argyrolobium roseum* with Cholinesterase assay will be done for the very first instance to identify occurrence of active compounds. In addition, any other biological action can also be identified.

2.7.2. Zygophyllum fabago L.

2.7.2.1. Overview of *Zygophyllum fabago L.*

Kingdom: Plantae

Subkingdom: Tracheobionta

Super division: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Sapindales

Family: Zygophyllaceae

Genus: *Zygophyllum L.*

Species: *Zygophyllum fabago L.*

Communal Name: Syrian bean-caper

2.7.2.2. The genus Zygophyllum

The Authors that have contributed their works in systematics of the genus has accepted the genus Zygophyllum being primary identified as Linnaeus, there was a classification of 6 classes internally Zygophyllum, which are named as Z. fulvum L., Z. sessilifolium L, Z. coccineum L, Z. fabago L. Z. morgsana L, and Z. spinosum L. defined by (Alzahrani & Albokhari, 2018). The leader amongst them is Zygophyllum, it is a plant with flowers belonging from Zygophyllaceae. The name is taken from the origin of Greek words Zigong which mean yoke or phyllo which meant leaf. This portrayed that the leaves had two leaflets50 different types and more of the genus contains were given in dry and less dry areas of the Mediterranean Basin, Africa, central Asia and Australia. The countries Afghanistan, Pakistan, South Africa, Australia, Iran and central Asia contains plants of the genus. In Pakistan two provinces i.e Sindh and Baluchistan has this variety. Six types of Zygophyllum can be found in Pakistan namely *Zygophyllum eurypterum*, *Zygophyllum megacarpum*, *Zygophyllum propinquum*, *Zygophyllum fabago* and *Zygophyllum simplex* the genus is full of saponins and various other components like flavonoids, alkaloids, tannins etc.

2.7.2.3. *Zygophyllum fabago L.*

Zygophyllum fabago L. (also known as Syrian Beancaper) is also an essential part of genus Zygophyllum. It includes tropical shrubs with foul smelling buds. It is a hairy herb with leaves and stems dying at the time the growing season finishes. It is measured as a harmful weed of financial prominence in United States (Bellstedt, et al., 2008).

Figure 11: Zygophyllum fabago flowers and leaves

Figure 12: *Zygophyllum fabago* seeds and seed pods

2.7.2.4. Habitant & Habitat

It is mostly found in arid and less arid and saline deserts being a perennial herbaceous plant in south western and central parts of Asia tropical, Indian subcontinent Pakistan, south of Europe and Africa. Its consumers include Turkey, Mediterranean regions, South, north and north east Africa and center parts of Asia (Lefevre, et al., 2010). *Zygophyllum fabago* is produced alone or in bunches in Iraq. It has always been found plenty on roadsides on the Middle East and

west of Iraq. It can also be said that it's a perennial glabrous upright herb. It is a perennial glabrous upright herb. Branches divaricate, striate, basal sproutsdispersion, higher one climbing. The Leaves are pinnately bifoliolate, petiole 12 to 15 mm in length, slightly compressed; and stipules are ovate-elliptic which is about 5 to 10mm in length (Bellstedt, et al., 2008).

2.7.2.5. Taxonomic Features

Glabrous, hairy, split from a thick wooded crown, vertical or scattering to 1 m at least in width. Leaves opposed, composite, having a single pair of dense, waxy, rectangle to obovate leaflets 1 to 4 cm in length. A minor stems preads from the middle of the leaflets. Stipules three-sided. Taproot is sprout, deepening, splitted, often long, fleshy adjacent roots which produce new sprouts. Flowers are alone like on the stalks, one or two in leaves axils. Five Petals, distinct, coppery, 7 to 8 mm in length. Five sepals, distinguished, rectangle, 6 to 7 mm in length. Ten stamens, protracted outside petals. Ovary larger, bony, sessile, five chambered, with one seed per chamber. Capsules that are oblong to cylindrical, five sided, mildly winged, 25-35 mm in length, with an insistent thread style. Stalk mostly bent down. Seeds are elliptical, compressed, and gray to brownish, uneven, 2 to 3 mm in length.

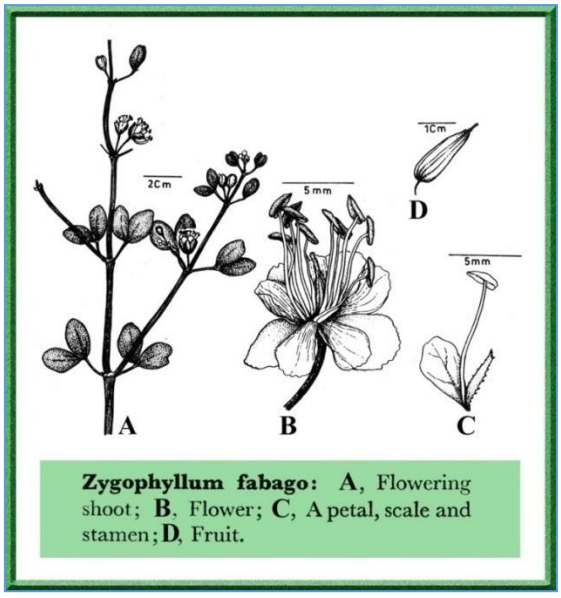

Figure 13: Taxonomic features of Z.fabago

2.7.2.6. Flowers & Foliage

A bushy herbaceous perennial acting as an annual herb in areas with harsh winter conditions. Its flower comes starting in May to August, growing up to a half foot high. Leaves slightly

succulent, conflicting, composite having two elliptical, one-inch leaflets. Stems are even and branching with a thickened woody crown. The flowers are minor; dense groups having five petals with protruding stamens, salmon to beige, with pink veins, three quarters of an inch. Flower buds have uses of being a substitute for capers. It replicates by seed and vegetative from lateral creeping roots.

2.7.2.7. Occurrence in Pakistan

It is herbaceous plant native to Pakistan, found in arid and saline deserts in Baluchistan.

2.7.2.8. Traditional Uses

In Baluchistan, which is one of the Province of Pakistan, a huge amount of plants has in history been used in treating great variety of diseases (Rehman & Ahmad, 1986). In the rural areas, where health services are not adequate, people depend on locally available medicinal plants for curing various illnesses. *Zygophyllum fabago L.* (Zygophyllaceae) is one of such indigenous medicinal plants of Baluchistan. The plant is being used locally for the cure of a number of diseases. Native people use this plant by boiling 15 to 20 g of leaves with shoots in water for few minutes and consumed it as a warm drink two times a day.

2.7.2.9. Medicinal Importance

Zygophyllum fabago is being utilized in the folk style of medicine in various countries as it has numerous biological effects (Khan, et al., 2014). For example, its uses contain anti-inflammatory agents, expectorant, pain relaxer , anti-tussive, anti-rheumatic, anti-asthmatic and anti-helminthic as a part of a drug for gout & rheumatism, and is also used for the cure of skin conditions and wounds, as a stupe (Tareen, et al., 2002). *Zygophyllum fabago* is being utilized as a drug for gout & rheumatism. It's again being utilized on the surface for the cure of skin diseases, wounds and kinds of injuries and is known to have some antiseptic properties. This plant's fruit has been used from very long times as a medicine in Iraq, being utilized as for controlling various diseases of skin and for relief of colic pain. On the whole, the plant extract is used to treat hemorrhoids and various infectious diseases by herbalists (Banno, 2010).

2.7.2.10. Phytochemicals

Some work on the isolation of natural compounds and their biological activities has been done on some species of this plant. Only two tri-terpenoidal saponins being reported in the above sea level parts of the same species of Z. fabago (Zaidi, et al., 2012).

2.7.2.11. Natural products reported

Zygophyllum fabago's literature survey showed presence of following natural products: Fatty Acid, Saponins, Terpenoid, Steroids, and Quinazoline Alkaloids Harrmin & Harmol.

2.7.2.12. Activities reported

Crude extracts of *Zygophyllum fabago* possess moderate activity for Butryl Cholinesterase and Acetyl Cholinesterase.

2.7.2.13. Research Purpose

In spite of the wide-ranging use in traditional medicine of *Zygophyllum fabago*, there is none safety information for its accessible usage. The plant was cytotoxic on brine shrimps and Candida albinos when used at high concentrations (Alhaddad, 2015). Crude extract has been reported to possess the Cholinesterase Assay. Our aim will be to identify the most active fraction in which the activity resides. Solvent- solvent extraction will be used to fractionate the crude methanol extract.

2.7.3. Citrullus colocynthis (L.) Schrad.

2.7.3.1. Overview of *Citrullus colocynthis (L.) Schrad.*

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Dilleniidae

Order: Violales

Family: Cucurbitaceae

Genus: Citrullus Schrad.

Species: Citrullus colocynthis (L.) Schrad.

Common Names: Watermelon, Bitter Apple, Kurtuma

2.7.3.2. The Genus Citrullus

The Citrullus is the genus of Cucurbitaceae family belonging to the Benincaseae tribe. It is a genus holding desert vine's four species, whose essential crop is *Citrullus lanatus*. C. P. Thunberg in 1773, made new molecular data along with sequences from *Citrullus lanatus*

demonstrating that this plant is a bitter melon and not a watermelon, as described in original description of the species by Thunberg's (Hai, 2016).

2.7.3.3. *Citrullus colocynthis (L.) Schrad*

Citrullus colocynthis is a valuable cucurbit plant, extensively found in the world in the desert areas, including Pakistan. Its fruit is widely known for nutraceutical prospective and pharmaceutical uses. (Asyaz, et al., 2010). The fruit is commonly called Colocynth or Bitter Apple in English, Hanjal in Urdu, Anedri in Sanskrit, Rakhal in Bengali, Kattuvellari in Malayalam, Indrayan in Hindi, and Pcitummatti in Tamil (Amamou, et al., 2011). This plant has a history to be utilized as traditional medicine, and is most famously known for management of diabetes, asthma and jaundice. In recent times, many studies have been performed on the pharmacology, phytochemistry and toxicology and (Hussain, et al., 2014). For *Citrullus colocynthis* rate of research papers publishing is increasing according to website of Australian New Crop's data. (Australian New Crops Website, 2009).

Figure 14: *Citrullus Colocynthis*

2.7.3.4. Habitant& Habitat

It is a desert vinyl plant, perennial trailing herb with somewhat woody tuberous root. The plant is originally from tropical Asia & Africa and is widely available in the Southern part of Asia and Sudan including Pakistan and India, Arabian and Sahara deserts, in Africa and Mediterranean region, and Southern Islands (Gurudeeban, et al., 2010).

2.7.3.5. Occurrence in Pakistan

In Pakistan it is found in sandy soil areas of Muzaffargarh, Layyah and Jhang and known in native language as "Kurtuma".

2.7.3.6. Taxonomic Features

The fruit was initially introduced in the mid ages to the Cyprus and Spain by the Arabs. It is a perennial herbaceous vine which produces small flowers. Bony, uneven and having rough hairy leaves is the description of the stems which are one after the other settled on the petioles, 5–10 cm in length, 1.5 to 2 cm wide, 3 to 7 lobed, pale yellow blossoms. The flowers present on the axils of the leaves and are yellow in color. It is single, monecious and pedunculated. The produced fruits become yellow when dry is generally 15 to 30 in number, 7 to 10cm wide, greenish having yellowish stripes. The fruit of *Citrullus colocynthis* has kind of taste as unpleasant and globular having an even consistency. It is solid having a rind around it containing 200 to 300 seeds (Figure 15). Seeds are smaller being 6mm long, ovoid, compressed, brownish when ripe and smooth. Around seventy five percent of the main weight was possessed by seeds. It is often the case that the mass of the fruit is 506 g and the pulp has a mass around 50% of it and the seed contents is 71.8g (Hussain, et al., 2014).

Figure 15: Citrullus colocynths (a) Un-ripened fruit (b) Fully ripened fruit (c) Seeds

2.7.3.7. Flowers & Foliage

The flowers present are yellow in color present on the axes and have yellow or green peduncles. Every one of them contains a sub-campanulated 5-lobed corolla with a 5-parted calyx. In various flowers of the very same plant, the male and female reproductive parts (pistils or ovary) are borne. The male calyx is smaller than the corolla, having 5 stamens, in which one is single with monadelphous anther and 4 are coupled whereas the female flowers contains a 3 carpel ovary and 3 staminoids. The 2 sexes are distinguishable by detecting the female ovary (Lloyd, 1898).

Figure 16: A *Citrullus Colocynthis* Female Flower

2.7.3.8. Traditional uses

A famous source of seed oil in the country of Israel goes by the name of *Citrullus colocynthis*, the fruit of which is utilized as a laxative (Schafferan, et al., 1998). In the UAE, *Citrullus colocynthis* is an important medicine belonging to folk styled medicines and has anti-inflammatory function (Wasfi, et al., 1995). An irritant Activity subjected towards enteric mucosa softens the bowel contents and stimulates the intestinal peristalsis. Rheumatic types of pain can be treated by it when been a decoction of various plants, it also treats asemantic cancer and hepatic protective agents (Asyaz, et al., 2010). In countries like Africa and Tunisia or more Mediterranean countries these plants and more of its derivations are utilized in the treatment of urinary infections. It produces a strong hydragogue and catharsis in human. Another important function is that it acts as a blood cleanser and cures tumors and largamente of the (Baquar, 1989).

2.7.3.9. Medicinal Importance

A number of illnesses are cured from *Citrullus colocynthis* for example diabetes, mastitis and cancer, constipation, asthma, leprosy, jaundice, joint pain and bronchitis (Asyaz, et al., 2010). It's utilization as medicines or herbs is done in Asia and Africa including Pakistan, China and India. These countries utilize the plants in the treatment of gut disorders such as colic pain and gastroenteritis, dysentery, indigestion, cough, cold, injuries and toothache (Amamou, et al., 2011). According to (Asyaz, et al., 2010) the fruits are utilized for curing of bacterial infections, intestinal disorders, cancer in human and animals and diabetes in the subcontinent Pakistan and its neighbor India.

2.7.3.10. Natural products reported

Natural products reported in literature for *Citrullus colocynthis* are flavanoids, carbohydrates, essential oils, fatty acids, phenolic compounds, glycosides and alkaloids (Salama, 2012).

2.7.3.11. Phytochemicals

Studies has shown *Citrullus colocynthis* fruit's main component as Cucurbitacins. It has to be noted that only some studies have been on the identification and isolation of chemical constituents.

2.7.3.12. Activity reported

The Literature survey of *Citrullus colocynthis* for Acetylcholinesterase assay shows the Crude extract being moderately active.

2.7.3.13. Research Aim

Bioassay directed fractionation will be achieved so that identification of the major bioactive secondary metabolites can be done.

2.8. AChE Inhibition Assay Method

2.8.1. Ellman's Assay (Microtitre plate method)

Out of many steps of nerve transport one of the most essential steps is enzymatic hydrolysis of acetylcholine (Giacobini, 2000) and the defects in its procedure is undermined as one of the reasons for Alzheimer's disease. For the identification of cholinesterase actions and in addition for the monitoring of ACH hydrolysis by acetylcholinesterase ATCH or butyryl cholinesterase (BuChE) in vitro, Ellman's procedure is used (Ellman, et al., 1961). In this Process the disposal of ATCH occurs as its similar qualitative kinetic activities helps in on line observing the hydrolysis. the process is also applicable for identification og cholinsterase actions of tissue exerpts, homogenates, cell suspension etc. howeveer the high hemoglobin quanitutu at 412 nm disturbs the process. Thus more advnanced techniques were discovered by (Worek, et al., 1999). The authentic Ellman's colorimetric process has its bases on the action of thiocholine (a component of ennzumatic hydrolysisof ATCH) having 5,5dithiobis-2-nitrobenzoic acid resulting in a yellow compound (5-mercapto-2-nitrobenzoic acid and its dissociation types) at pH 8. The mainly amount of absorbed coefficient was found to be 412 nm. As the extract is hydrolyzed there is an increase in the absorption. And the activities of the enzyme as reaction rate is to be analyzed having a gradient of time dependence of the absorption. It is a fast reaction and that's why there is no rate limiting in the enzymatic hydrolysis (Komersova, et al., 2007).

2.9. Cytotoxicity testing methods

2.9.1. The Brine Shrimp Lethality test (BSLT)

The realization of the chemistry, biology and pharmacology in the greatest of details is the motivation and desire to use multidisciplinary approaches on traditional medicines. One of these approaches included the monitoring of the cytotoxicity of the extract of fractions regarding nauplii, *Artemi salina*. The vulnerability of *Artemia salina* or the brine shrimp larvae to the procedure against medicinal plants extracts is to measure toxicity in chemicals and also natural products (Logarta, et al., 2001). This test is a simple bench top bioassay which is used to search plant extracts for biological actions and produces great results (Ajaiyeoba, et al., 2006). A huge variety of chemicals and natural components ar toxic against brine shrimp nauplii. This destruction of the organism when exposed to variable concentrations is the base of the toxicity test. Bioactive components are often more toxic in higher concentration and the toxication can be determeined as pharmacology in high doses, this evidence gives the reasons to be implied for the analysis of plant extracts . The process can detect a vareity of bioactive elements available. The basic scope of the process is to provide a screening test that supports more expensive bioassays once the active compononets is in isolation pahse. (Psitthanan, et al., 2004) It has been studied to produce great correlation with cytotoxicity of tumor cell lines of colon carcinoma cells (Wagensteen, et al., 2007). In addition, according to Mackeen, et al 2000, cytotoxicity, photo toxicity, pesticide and trypanocidal activities can also be easily predicted through the same process.

2.9.2. Benefits of *Artemia salina* as a toxicity test

The test can be carried out anywhere in the world as the cyst is easily available. In addition, the cyst also can be stored easily for years in a dry place and can be used at any time, the amount required of it is also very small. The importance of yearly maintenance of stock cultures, having the biological and technical difficulty and the obvious financial consequences is wholly eradicated. Large amount of testing organisms with the same age and physiological illnesses are effortlessly gained to initiate the test

2.9.3. List of principles for a standard Artemia toxicity test

The nauplii test has much sensitivity, it must be hatched in strictly maintained condition of atmosphere, Ph., salinity, aeration and light. It is important for the larvae to be of the exact size for the test and should not turn in to instar stage (Figure 17). The details should immaculate with the cyst being from the same place of the origin and the conditional factors be the same

following the details. Another test known as the control test regarding the toxicant chemical be performed with parallel sensitivity of larvae and following all the standards of the test (Vanhaecke, et al., 1981).

Figure 17: Brine shrimp (larvae): Newly Hatched

2.9.4. In vitro assay for cytotoxicity – MTT assay

The first description of this assay was done by Mosmann in 1983 and much modification is done. The assay is used for biological and biomedical research being first initialized to determine the practicality of adherent animal cells. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is utilized in the assay that is a yellow dye which turn to a purple insoluble formazan because of the dehydrogenase system. According to (Wang, et al., 2010) the crystals formation is quantifiable by spectrophotometrically, dissolving in organic solvent, the amount of metabolically active cells in a culture a proportional to the concentration. A multi well plate reader performs the discovery and quantification of the crystals. The quantity of viable cells has a relation with the sum of mitochondrial activities and the conversion of MTT to formazan crystals by existing cells identifies the mitochondrial actions. (Van Meerloo, et al., 2011) revealed that the process is utilized to analyze the in vitro cytotoxicity of medicines or drugs in the cell lines.

Chapter 3

3.0. Materials and methods

3.1. Chemicals, Reagents & Instruments

A thermo pH-meter, EZ read Microplate reader (Biochrom 800plus), Elmasonic S180 H ultrasonic bath and a vortex (IKA GENIUS 3) and Galapagos software was used for the measurement of acetylcholinesterase assay activity. For purification and extraction of plant extracts all chemicals consumed were of commercial grade and were used without further purification. Methanol, Butanol and Di-sodium hydrogen phosphate dihydrate ($Na_2HPO4.2H_2O$) and 20 cm × 20 cm aluminium sheets of silica gel plate 60 F254 for thin layer chromatography were purchased from Merck Darmstadt Germany. Other organic solvents such as Butanol, Ethyl acetate, Hexane, Dichloromethane were of commercial grade and purchased from DAEJUNG Korea. For evaporation and condensation of extracts Buchi-R-120 rotavapor was used. Fractionation was made through column chromatography with packing column with DIAION HP-20 gel. S-Acetylthiocholine iodide 98% was purchased from (abcr GmbH) Germany. DTNB-5,5'Disulfanediylbis (2- Nitrobenzoic acid) known as Ellman's Reagent was purchased from Ark-pharm-USA. Buffered aqueous solution of Acetylcholinesterase enzyme (AChE) (EC 3.1.1.7) C0663-50 UN Lot # 125M4054V (≥500Units/mg Protein BCA) from human erythrocytes and Sodium dihydrogen phosphate dihydrate($NaH_2PO4.2H_2O$) was procured from Sigma Aldrich Steinheim Germany. Brine shrimp eggs were purchased from San Francisco A..eef tanks. Chemicals used for MTT Cytotoxic Assay and human carcinoma cell lines HUH & MCF-7 were procured from KRL

3.2. Plant materials and extract Preparations

3.2.1. Plant Material

In March, 2010 *Argyrolobium roseum*, whole plant, was gathered from Kotly, Azad Jammu and Kashmir. Mr. Muhammad Ajaib a Taxonomist recognized it, from Department of Botany, GC University, Lahore. It has been deposited in the herbarium of GC University and has been consigned a specimen voucher number of (GC. Herb.Bot.1397).

In June 2002, *Zygophyllum fabago,* whole plant was gathered in Ankara Turkey. The plant was recognized by Bilge Sener. A specimen voucher number of (GUE# 2312) was consigned and

deposited at Faculty of pharmacy Gazi University herbarium of Ankara Turkey. We acquired Z.fabago from Sargodha University.

Citrullus colocynthis, whole plant was collected from Karachi University and identified by Surriya Yasmeen and deposited in herbarium of Khi University.

3.2.2. Extraction and Separation

Crushed and air-dried whole plant of *Argyrolobium roseum, Zygophyllum fabago* and *Citrullus colocynthis* were percolated three times (at interval of one week) with MeOH at room temperature.

The shade-dried, whole plant, A. roseum, material was crushed into fine powder which was extracted with methanol (5L × 3) at ambient temperature. The methanolic extract was concentrated on rotary evaporator (Buchi-R-120 Rotavapor) to obtain the dark green residue (1.52 g).

Similarly, *Zygophyllum fabago* and *Citrullus colocynthis* plants were gourd and soaked in methanol for 72 hours. The extracts, crude methanolic, were concentrated by working on a rotary evaporator in a 3L round bottom flask. After the concentration, the liquid samples were placed in screw top bottle and stored at -20°C until liquid-liquid extraction was performed.

3.2.3. Thin layer chromatographic Analysis

To identify polar or non-polar nature of crude plant extracts, thin layer chromatography (TLC) was executed on (20×20cm) aluminuim sheets of silica gel plate 60 F254. On a TLC plate, all crude extract samples were spotted. The TLC plate was placed in a closed jar and a small volume of mobile phase was added in the container. TLC experiment was performed to investigate 100µg of the extract positioned in a band of 1 cm, utilizing three solvent systems of differing polarities. The solvent systems consumed as mobile phase were:

1. Hexane: Ethyl Acetate
2. Methanol: Chloroform
3. Ethyl Acetate: Chloroform

TLC plates were examined under ultraviolet light having wavelengths of 254 & 360 nm and marked visible bands. Then, TLC plates were sprayed with KMNO4 & Drangdorf Reagent. Fractions were isolated by Column chromatography and then examined.

3.2.4. Liquid-Liquid Extraction

Based on TLC results further extraction and fractionation was performed. Plants were extracted using different organic solvents like butanol, hexane and ethyl acetate by solvent-solvent extraction method and subjected to vacuum filtration and dried on rotary evaporator (Buchi-R-120 Rotavapor) at 45 °C, under reduced pressure and stored in freezer.

Concentrated crude MeOH extracts were removed from the freezer and a 200gram aliquot that was re-suspended in 700mL distilled water. This mixture was shaken vigorously for 2min to ensure the complete mixing. This suspension was placed in a 2 L separatory funnel where 1 L of hexane was added for A.roseum crude extract. With stop-cock closed and glass stopper in place, the funnel was shaken for 3min then placed in a holder where layers were able to separate. The top layer (hexane) was removed into a 2 L bottle. This process was repeated two more times. Again, similar extraction was repeated with two other individual solvents, i.e. ethyl acetate and butanol. After the last layer of butanol was removed the aqueous fraction left in the separatory funnel was also retained. Similar method was followed for rest of the two plants. All fractions were concentrated in 3 L round bottom flasks by rotary evaporators. Dried fractions were stored at -20°C.

3.2.5. Fractionation of crude extracts of plants

The MeOH crude extracts were subjected to solvent-solvent extraction and further divided into sub-extracts using various solvents based on their preferential solubility. The methanolic extract was concentrated and dissolved in 100% H_2O. The water layer was then extracted successively using hexane, EtOAc and butanol for sub-fractionation thrice with each solvent. These extracts were vacuum concentrated (Figure 18).

The aqueous extract and butanolic extract were fractionated based on lipophilicity by column chromatography (DIAION HP-20 gel) using water-MeOH with increasing ratios of MeOH (50% to 100%, ca. 2x 750ml each of the five fractions), and finally with Methanol (2x 1 L) (Figure 18).

The filtrates of the respective fractions thus obtained were concentrated in rotary evaporator at 37°Cunder reduced pressure. Then respective concentrates were weighed and stored in labeled airtight vials in a refrigerator.

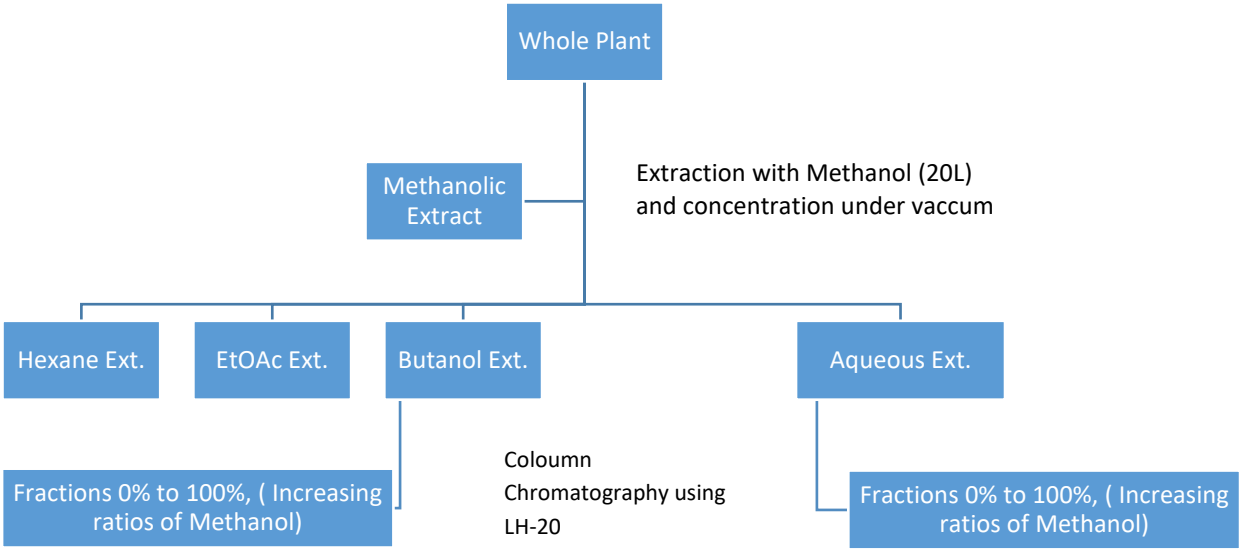

Figure 18: General Fractionation Scheme from the crude extract of Plants

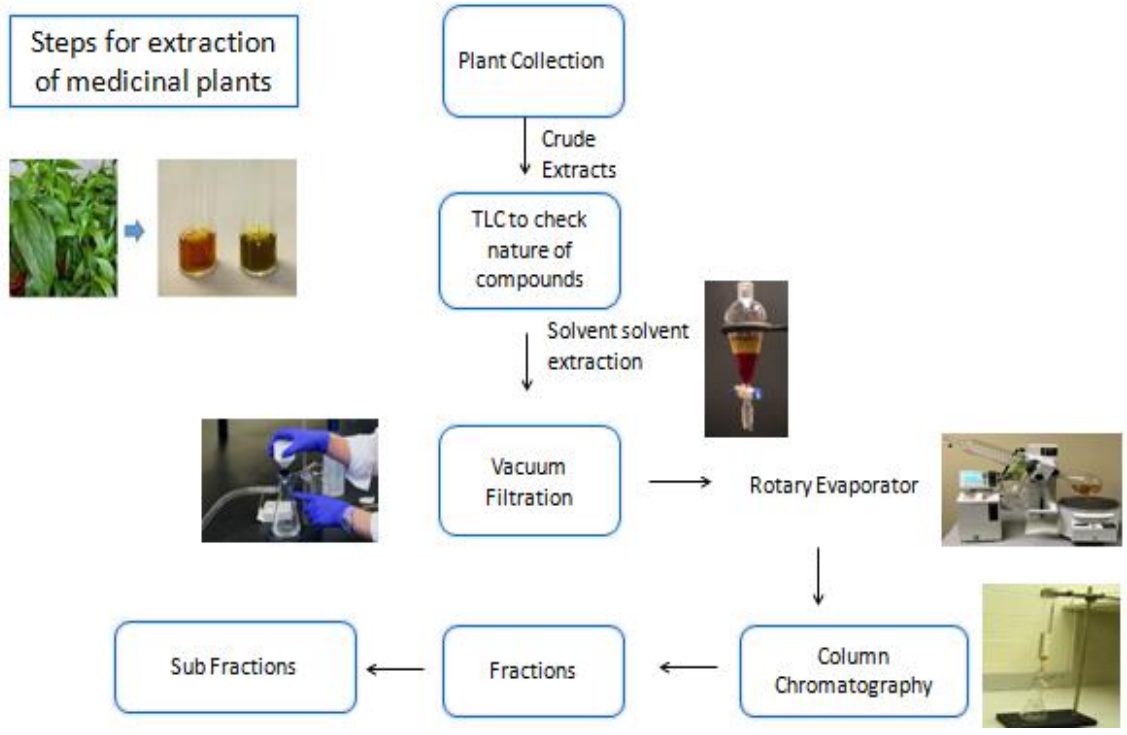

Figure 19: Summary of methodology of plants extraction & fractionation

3.3. Quantitative evaluation by Microplate Assay

3.3.1. Reagents required for AChE Assay:

1. Sodium phosphate Buffer 100 mM
2. Acetylthiocholine iodide (ACTI)
3. DTNB (Ellman's reagent)
4. Galanthamine
5. Enzyme Solution

3.3.2. Buffer preparation

For preparation of sodium phosphate buffer dihydrate dibasic and monohydrate monobasic solutions are required and prepared 0.2 M Stock solutions of these two respectively.

a) Dihydrate Dibasic Stock Solution (0.2 M):

Added 35.61g $Na_2HPO4.2H_2O$ to 800 ml double distilled H_2O (Slowly), brought up to 1000ml. Stored at room temperature.

b) Monohydrate Monobasic Stock Solution (0.2 M):

Added 31.21g $NaH_2PO4.H2O$ to 800ml double distilled H_2O (slowly), brought volume up to 1000 ml. Stored at room temperature.

c) Sodium Phosphate buffer 100 ml (0.1 M pH=8)

To make sodium phosphate buffer of 0.1 M we added 47.35 ml of dibasic stock solution and 2.65 ml of monobasic stock solution in 100 ml reagent bottle. Then added 45 ml double distilled H_2O. It made 95 ml of solution. Adjusted its pH to 8 (if it is below pH 8 add base sodium hydroxide NaOH and if it is above 8 add phosphoric acid H_3PO4 respectively. Finally added double distilled H_2O to bring the volume of the solution up to the mark and stored the prepared buffer in refrigerator.

3.3.3. Preparation of DTNB solution

DTNB-5,5'Disulfanediylbis (2- Nitrobenzoic acid)

Molar Mass= 396.34 g/mol.

First stock solution of DTNB was prepared and then serial dilutions of required concentrations were made from this stock solution.

a) 50 ppm Stock solution:

50 ppm stock solution of DTNB was made by weighing 1 mg of DTNB in a vial. Brought up to 20 ml with 100 mM sodium phosphate buffer and stored in freezer in aliquots for thawing on the day of assay as per requirement. DTNB was covered properly with aluminuim foil to protect from light.

b) Serial Dilution: 6.25 ppm (0.015 mM)/(15µM)

For 5 ml volume: Formula= Low concentration/high concentratiom * required volume

6.25/50*5 = 0.625 ml

On the day of assay prepared the buffer DTNB solution by adding 0.625ml of DTNB from stock solution and 4.375 ml of sodium phosphate buffer already prepared was added into it. It made 0.02 mM DTNB Solution. Put for 10 minutes in water bath at 37 ℃and used this chromogenic solution for assay (Plate loading).

3.3.4. Substrate Solution Preparation

Acetylthiocholine Iodide (ACTI)

Molar mass= 289.18 g/mol

Stock solution of ACTI was prepared and then serial dilutions of required ppm concentration were made from stock solution.

a) 50 ppm Stock Solution:

50 ppm stock solution was prepared by weighing 1 mg of Acetylthiocholine Iodide (ACTI) in a vial and added double distilled H_2O to bring up the volume up to 20 ml. Stored in freezer in aliquots and thawed on the day of assay as per requirement.

b) Serial Dilution: 12.5 ppm (0.04 mM) / (40 µM)
   For 5 ml volume:
   Formula= Low concentration/high concentratiom * required volume
   12.5/50*5 = 1.25 ml

ACTI was diluted by adding 1.25ml of ACTI from stock solution and 3.75 ml of double distilled H_2O. It made 0.04mM ACTI solution and was stored in freezer.

3.3.5. Inhibitor Stock Solutions Preparation (Plant Extracts)

Stock solutions of 1000 ppm of plant extracts were prepared, and further serial dilutions were made from this stock solution.

1 mg/ml=1000 ppm

1 g/L =1000 ppm

Stock Solutions: 1000 ppm

Weighed 10 mg of dried plant extracts on analytical balance and dissolved it in 10 ml of respective solvent in which the extracts were soluble (such as methanol) in a glass vial. Vortex mixer was used for complete mixing, and sonicator for further dissolving the extract completely, if required. Stored the prepared extracts solutions in refrigerator.

Serial dilutions Formula:

Low concentration / high concentration * required volume

Dilutions	Calculations
500	500/1000*10=5
250	250/1000*10=2.5
100	100/1000*10=1
25	25/1000*20=0.5

500 ppm: Took 5µL of respective plant extract from stock solution in an eppendorf and added 5 µL of methanol into it to make serial dilution of 500ppm.

250 ppm: 2.5 µL from stock solution + 7.5 µL from Methanol

100 ppm: 1 µL from stock solution + 9 µL from Methanol

25 ppm: 0.5 µL from stock solution + 19.5 µL from Methanol

3.3.6. Preparation of Standard Inhibitor (Galantamine) Positive Control

a) 1000 ppm stock:

Weighed 1mg of Galantamine on analytical balance and transferred it to a glass vial or Eppendorf, 1ml of methanol was added to make a stock solution of 1000 ppm. Stored in refrigerator.

b) Serial dilution of 0.1 ppm:

$$0.1/1000*1 = 0.1 \text{ ml}$$

1. Took 0.1ml from 1000ppm stock solution of Galantamine.
2. Added 0.9ml methanol in it to make Galantamine solution of 0.1ppm.

3.3.7. Preparation of enzyme solution (0.5 U/mg)

Enzyme Source: Electrophorus Electricus (Powdered)

Packaging: 1000 U/mg

For each well in micro plate 10 ul of enzyme solution is required and is prepared freshly. To prepare 0.5 U/mg enzyme solution, Tris HCl Buffer is required with pH maintained at 8.

a) 20 mM Tris HCL Buffer Preparation:

0.315g of Tris HCl was transferred to a reagent bottle then, added double distilled H_2O and raised volume up to 100 ml. Maintained its pH at 8 and stored in refrigerator.

b) 10 U/mg Enzyme stock solution:

0.1 mg of powdered enzyme was weighed in an Eppendorf and 999.9µL of prepared Tris-HCl Buffer (pH 8) solution was added and stored in freezer in aliquots and thawed on the day of assay as per requirement.

c) Dilution solution: 0.5 U/mg

Formula= Low concentration / High concentration * required volume

$$0.5/10*100 = 5 \text{ µL}$$

On the day of assay prepared the enzyme dilution by adding 5 µL of acetylcholinesterase from stock solution in an eppendorf and added 95µL of 20mM Tris HCL Buffer (pH 8). Vortex it

for 1 minute for complete mixing of the enzyme with buffer. Kept the prepared solution on ice bath while loading the wells during assay and stored in freezer.

3.3.8. Microplate Assay for AChE measurement

AChE inhibitory action of the extracts was measured by using an adaptation of spectrophotometric 96 well microplate assay method developed by (Ellman, et al., 1961) by utilizing EZ read Microplate reader (Biochrom 800plus). Enzyme source was buffered aqueous solution of Human erythrocytes acetylcholinesterase (EC 3.1.1.7, Sigma), whereas substrate for the reaction was acetylthiocholine iodide (abcr GmbH- Germany). For the measurement of AChE activity,5,5'Disulfanediylbis (2- Nitrobenzoic acid) Ellman's Reagent (DTNB, Ark-pharm-USA) was used for the reaction. Galanthamine was employed as standard drug for the study.

The assay is based on extremely sensitive method which measures change in absorbance by acetylthiocholine hydrolysis at 405nm. AChE hydrolyses the acetylthiocholine to produce thiocholine and acetate. Acetylthiocholine hydrolysis is measured by thiocholine production by monitoring the formation of the yellow color which is formed when thiol reacts with 5,5' Disulfanediylbis (2- Nitrobenzoic acid) catalyzed by enzyme AChE liberating 5-thio-2nitrobenzoate anion which is absorbed at 405nm.

Hundred millimolar Sodium phosphate (pH 7.7) was utilized as buffer throughout assay. Enzyme solution was made in buffer to get stock solution of 0.025 U/ml. This solution was placed at 2-4 ℃. DTNB was dissolved in buffer and ACTI solution was prepared in de-ionized water.

In 96 well micro titer plate,10 µl of 100 mM sodium phosphate buffer (pH 7.7), 10 µl of 0.031 mM DTNB (Ellman's Reagent), 10 µl of sample solution and 10 µl of AChE solution (0.025 U/ml) were inserted by pipette in each well and incubated at 30 ℃ for 30 minutes. The reaction was then originated by adding of 10 µl of 0.04 mM acetylthiocholine iodide. The reaction of Ellman's reagent with thiocholines resulted in the formation of yellow color which was supervised at a wavelength of 405 nm utilizing an EZ read Microplate reader. The measurements and calculations were done using Galapagos Software. By comparing rates of reaction of samples relative to blank samples determined inhibition percentage. Methanol in sodium phosphate buffer (pH 7.7) was used as a blank during the experiment. Methanol was utilized in place of test compound as a negative control. The standard anticholinesterase

alkaloid drug Galanthamine dissolved in methanol at varying concentrations different from samples was used as a positive control. During this study, the Galanthamine concentrations tested were 1000, 1 and 0.1 µg/ml to calculate its IC_{50}. Following equation was used for the calculation of percentage inhibition:

$$Inhibition\% = \frac{absorbance\ of\ negative\ control\ -\ absorbance\ of\ test\ compound}{absorbance\ of\ negative\ control} * 100$$

Where absorbance of negative control is activity of enzyme without inhibitor and absorbance of test is activity of enzyme with test sample.

The experiments were performed in triplicate (n=3) for calculation of mean values and standard deviation. Table curve 2D v5.01 software was employed for IC_{50} calculation.

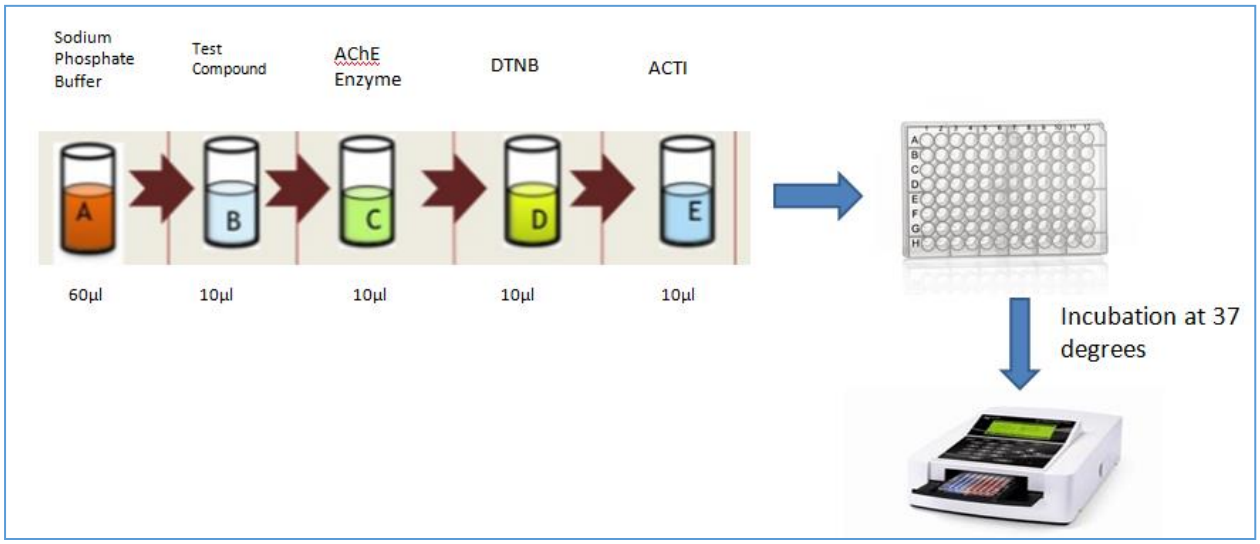

Figure 20: In-vitro Ellman's Assay procedure for evaluation of acetylcholinesterase inhibitory potential of Plant Extracts

3.4. Qualitative evaluation by bio-autography method

The AChEI effectiveness of crude extracts was evaluated by TLC bio-assay detection procedure using bio-autographic assay. Stationery phase a 20 cm × 20 cm Aluminium sheet of silica gel plate 60 F254 was utilized. TLC plate was spotted with crude extracts dissolved in methanol at a 1 mg/ml concentration. Optimized mobile phase of methanol: water (3:1) was used to develop TLC plate. TLC plate after being developed, was then dried out at room temperature. 1 mM DTNB (Ellman's Reagent) and 1 mM ACTI solutions were freshly prepared in sodium phosphate buffer and were sprayed on dried TLC plates spotted with methanolic crude plant extracts. Plates were then dried for 25 seconds and AChE enzyme

solution (3U/ml) prepared in sodium phosphate buffer was sprayed over the respective plates. Plates were kept at room temperature for five minutes to develop colorless spots against a yellow background. The plate was observed under visible light and appearance of colorless spots indicated zone of inhibition of AChE. A positive spot indicating AChE inhibitor gets disappear faster and needs to be photographed immediately. In a similar manner another TLC plate was developed called as false positive without spotting the extract to confirm the true inhibition. Appearance of white spots on the TLC plate at the similar place as that of the extract was considered as false inhibition. Galanthamine (1mg/ml) as a positive control was put on TLC plate which was developed following same conditions as described above. After elution, AChEI activity was analyzed by positive white spot against yellow background as zone of inhibition of AChE.

3.5. Brine Shrimp Lethality Assay

3.5.1. Serial Dilutions Preparation for Cytotoxic assay

Dried plant extracts of 10 mg were weighed by an analytical balance and transferred to glass vial. For preparing 1000 ppm stock solution added 10 ml organic solvent in which extracts were soluble such as in methanol in the glass vials containing 10mg of extract. Test tubes rinsed with acetone and labeled. In each test tube added ten larvae. 1 ml of crude extract's solution was then inserted into the respective test tube having1 ml of seawater and 10 larvae. After 24 hours, the viability of shrimps was noticed.

3.5.2 Brine Shrimp Hatching Methodology

The first description of the methodology was given by (Meyer, et al., 1982). Eggs are placed in brine and hatched within 48 hours. For hatching Brine shrimp eggs, measured one liter of water and poured into 1000 ml beaker. Then weighed 27 g of commercial grade sodium chloride by analytical balance and added it into the beaker containing 1000 ml water. For maintaining proper aeration in the beaker, at the bottom of the beaker was inserted the tip of an airline from an air pump. 1 g of brine shrimp eggs were poured into beaker containing artificial seawater. A 40Watt bulb, placed a few inches away, was used to provide the required light for incubation. After 48 hours, the hatched nauplii were collected from the beaker. For separating the hatched nauplii from eggs turned off the lamp and air supply. Using this technique, the empty eggs will glide while the brine shrimps will distillate in the water column. Making use of a Pasteur pipette,10 nauplii were transferred to each labeled test tube.

Figure 21: Brine Shrimps Hatching

3.5.3 Toxicity testing

The hatched brine shrimps were then exposed for toxicity testing to plant extracts of different concentrations prepared (i.e 1000, 100, 10 and 1µg/ml). Each serial dilution was tested in triplicate. After 24 hours, the number of viable nauplii were counted and percentage of death was calculated

3.5.4 Calculation

After 24 h, the viability of larvae was observed and the number of mortal nauplii was noticed. This bioassay's mortality endpoint is defined as the absenteeism of precise forward motion during thirty second of surveillance. Nauplii were considered dead when they were immobile and stayed at the bottom of the test tubes. To conclude the % mortality, counted the number of mortal and number of viable nauplii, in each test tube. The percent lethality of brine shrimp was calculated with the formula given below for each concentration and control.

$$\%\, mortality \;=\; \left(\frac{Number\, of dead\; nauplii}{Number\; of\; dead\; nauplii \;+\; Number\; of\; live\; nauplii}\right) * 100$$

3.6. Invitro toxicity assessment MTT Assay

The methylthiazole tetrazolium (MTT) assay

This experiment was performed in KRL Laboratories using the standard operating procedures.

3.6.1. Test sample preparation for MTT Assay

5000ppm of plant extract's stock solution was made by weighing 10 mg of plant extract on Analytical Balance and dissolved in 2ml of autoclaved distilled water in an eppendorf. To avoid contamination whole procedure was performed in bio-safety cabinet. All eppendorf were labeled respectively. Eppendorf were vortexed containing stock solution to completely solubilize the extracts. Extracts were centrifuged for two minutes at 8000 rpm. Supernatant was

collected carefully in another eppendorf and the pellet containing un-dissolved particles was discarded. Then used syringe filter of 0.2µm to remove any bacterial contamination.

3.6.2. Cell Culture conditions

Two cell lines i.e breast cancer (MCF-7) and liver cancer (HUH) were used for toxicity testing of the plant extracts. Both cell lines were grownup in DMEM (Invitrogen). The medium possessed fetal bovine serum (10 % FBS) accompanied with 1% of antibiotics comprising of Streptomycin (100µg/ml) and Penicillin (100U/ml). In CO_2incubator, cell cultures were incubated at 37 °C.

3.6.3. MTT Assay

3.6.3.1. Day 01: Media Preparation for feeding cell lines

In fume hood sterilized with 70% ethanol DMEM media containing glucose, glutamine, sodium -pyruvate and sodium bicarbonate was added in autoclaved distilled water in measuring cylinder. DMEM was filtered with syringe filter (0.2µm) to remove any bacterial contamination. 50ml of 10% FBS was added in beaker containing filtered DMEM. Streptomycin and Penicillin were added in corning flasks containing 10 ml of autoclaved distilled water each to make 1% antibiotic solutions. Then 5 ml of each antibiotic was added in beaker containing DMEM and FBS. Media is ready to feed the cell lines. Continuous monitoring is required to check any contamination in cell lines under microscope. Flask containing cell culture cell lines are taken out from incubator and transferred to fume hood to feed the cell lines with proper growth medium to survive. Flasks media is removed and then washed with 1X PBS. Freshly prepared media was then added in flask and stored cell cultures at 37 °C in CO2 incubator with 5% CO_2.

3.6.3.2. Day 02: Loading Plate

Cells concentration required in each well is 100µl. For 24 hours, cells were sowed in 96-well plate. Plates were labeled appropriately for both cell lines LHUH & MCF-7. Dismantled cell lines by mechanical method and then added 100µl of cells in 96-well plate. Then required concentration of each test sample was added in labeled wells. Plates were then stored in CO_2 incubator for 24 hours.

3.6.3.3. Day 03

After 24 hours of incubation with crude extracts 10µl MTT was inserted in each well, the plate was covered and kept again in incubator for around 2 hours. After 2-4 hours of incubation the purple crystals of Formazan (MTT metabolites) (as shown in Fig.) were dissolved with 100 µl

of 15% DMSO was added into the wells. Mix all wells using pipette to ensure complete solubilization. After complete mixing, absorbance was read at micro-plate reader (FLUOstar Omega) at 570nm. According to the plant screening program NCI U.S, a crude extract is usually deemed to have in vitro cytotoxicity having an LC_{50} value less than or equal to 20 µg/ml and considered as promising crude extract for further purification. (Potchanapond graidist, 2015)

The experiments were executed in triplicate. % Viability of cells was computed using the formula:

$$\%\ viability = \ Sample/\ Control\ * 100$$

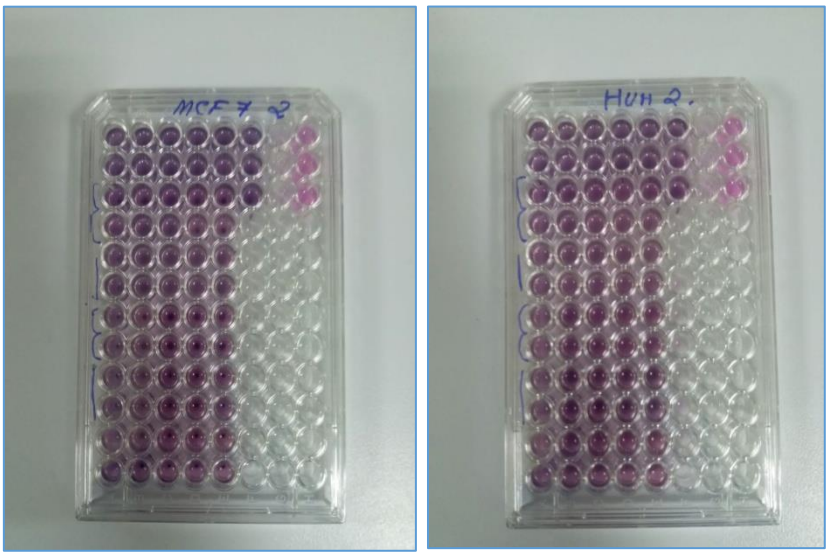

Figure 22: Microwell plates for Cytotoxicity testing against cell lines: (a) MCF-7 (b) HUH

3.7. Statistical Analysis

In this study, all the assays were executed in triplicate and the data acquired was expressed as mean ± SD. Traditional statistical methods were employed to compute means and standard deviations of three replicates. Statistical significance was ascertained by one way analysis of variance followed by Holm-Sidak test, to determine the variability of test groups vs control by SigmaPlot 14.0.Ink. Table curve 2D V5.01 was used to determine the IC_{50} values of AChE Inhibition assay. $P < 0.05$ was regarded as statistically significant. Graphs were generated by using the SigmaPlot 14.0.Ink software. Statistical analysis was performed on raw data, even when graphs are presented in percentage. P value of less than 0.05 was considered as statistically significant.

Chapter 4

4.0 Results

The present study investigated the cytotoxic and anticholinesterase potential of 24 extracts of three plant species, namely *Argyrolobium roseum* (AR), *Zygophyllum fabago* (ZF) and *Citrullus colocynthis* (CC)in vitro, which were reported to have medicinal properties (Table: 1).

4.1 Extraction and Fractionation Results

Prepared by liquid-liquid extraction and vacuum filtration crude methanolic extracts of plants were initially partitioned by hexane, ethyl acetate, butanol and aqueous extracts. Thin layer chromatography (TLC) was performed to further fractionate the fractions according to their polar or non-polar nature. TLC extraction and fractionation of plant extracts was used to access the fractions. The crude fractions were divided in to arbitrary fractions for subsequent separation. The fractions were either sub-divided by column chromatography on DIAION resin gel with mounting proportions of water and methanol for separation of secondary metabolites in to different organic crude extracts thus forming sub fractions of extracts. The sub-fractions were then concentrated and evaporated on rotary evaporator (Buchi-R-120 Rotavapor) at 45 °C, under reduced pressure. Extraction and fractionation scheme adopted for the plants is shown in figure 23-25.

4.1.1 *Argyrolobium roseum*

Shade dried, whole plant of *Argyrolobium roseum* was crushed into fine powder, plant contents were then extracted with methanol. 1.51 g of this methanolic extract (dark green semi solid substance) was fractionized into non-polar to polar solvents gradually. This was then dissolved into distilled water and fraction of 1.387 g of n-hexane, fraction of 0.752 g of ethyl acetate and 1.372 g of aqueous extract were obtained, as represented below.

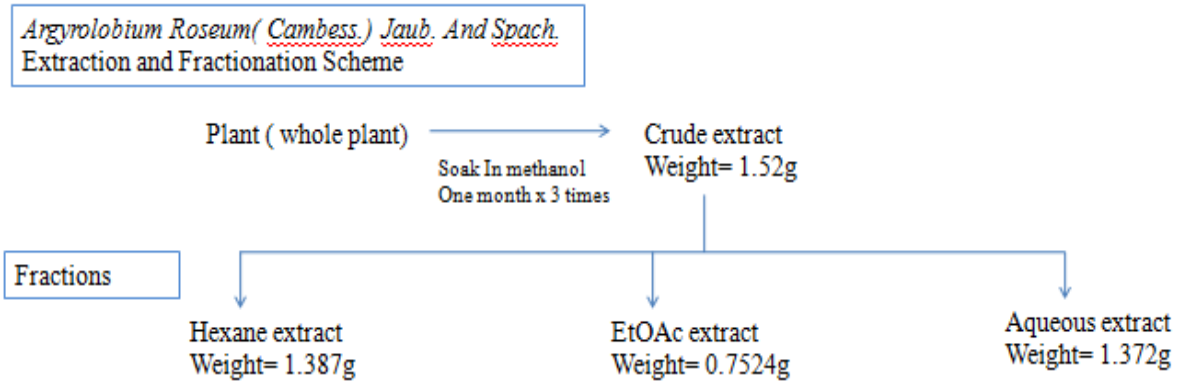

Figure 23: Extraction & Fractionation Scheme of *Argyrolobium roseum*

4.1.2 *Zygophyllum fabago*

Similarly, the whole plant material of Z.fabago at room temperature was dried up in shade and was crushed into fine powder which was extracted with methanol exactly in the same way as described earlier. 1.0285 g was obtained as crude methanolic extract which was fractionated using solvent-solvent extraction of immiscible solvents with crude extract dissolved into aqueous extract into 1.585 g of ethyl acetate fraction, 0.264g of n-butanol fraction and 0.4877 g of aqueous extract. Aqueous and butanol fractions were further sub-fractionated by column chromatography with increasing proportions of water and methanol as represented below.

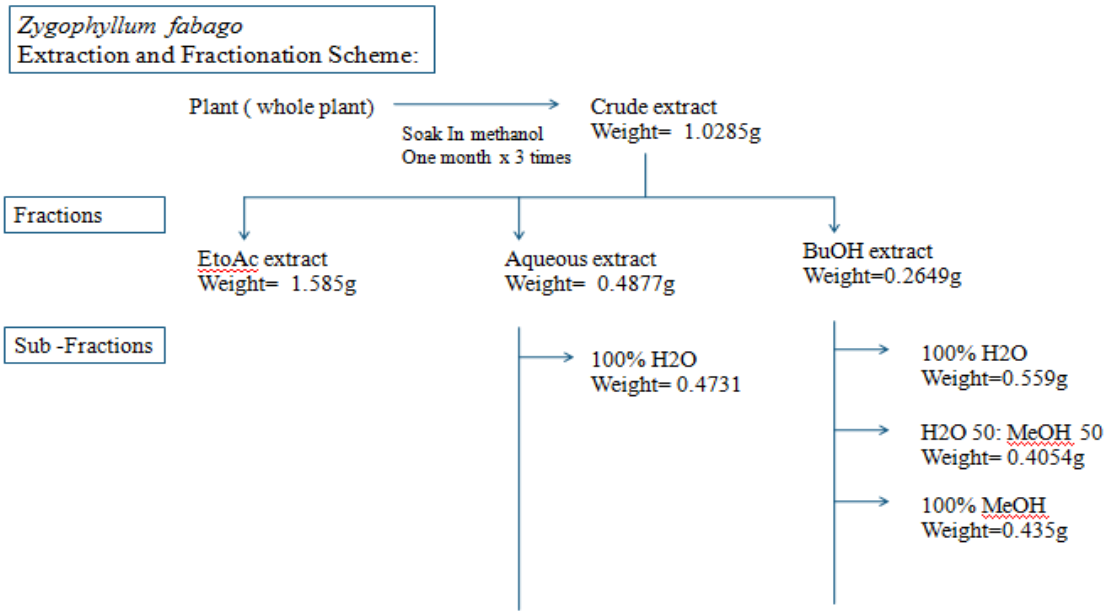

Figure 24: Extraction & Fractionation Scheme of *Zygophyllum fabago*

4.1.3 *Citrullus colocynthis*

Citrullus colocynthis whole plant was ground into fine powder and 0.981g crude methanolic extract was obtained after soaking the plant part into methanolic solvent. The methanolic extract was dissolved in water and aqueous solution was partitioned into non-polar to polar solvents gradually obtaining of 0.3322g of ethyl acetate fraction, 1.134g of butanol fraction and 2.339 g of aqueous extract, as represented below. These fractions were then eluted with increasing proportions of water-methanol in HP-20 to obtain sub-fractions. Solvents used were distilled in rotary evaporator in order to remove the impurities. Weight of each sub-fraction obtained by column chromatography is given below in figure 25.

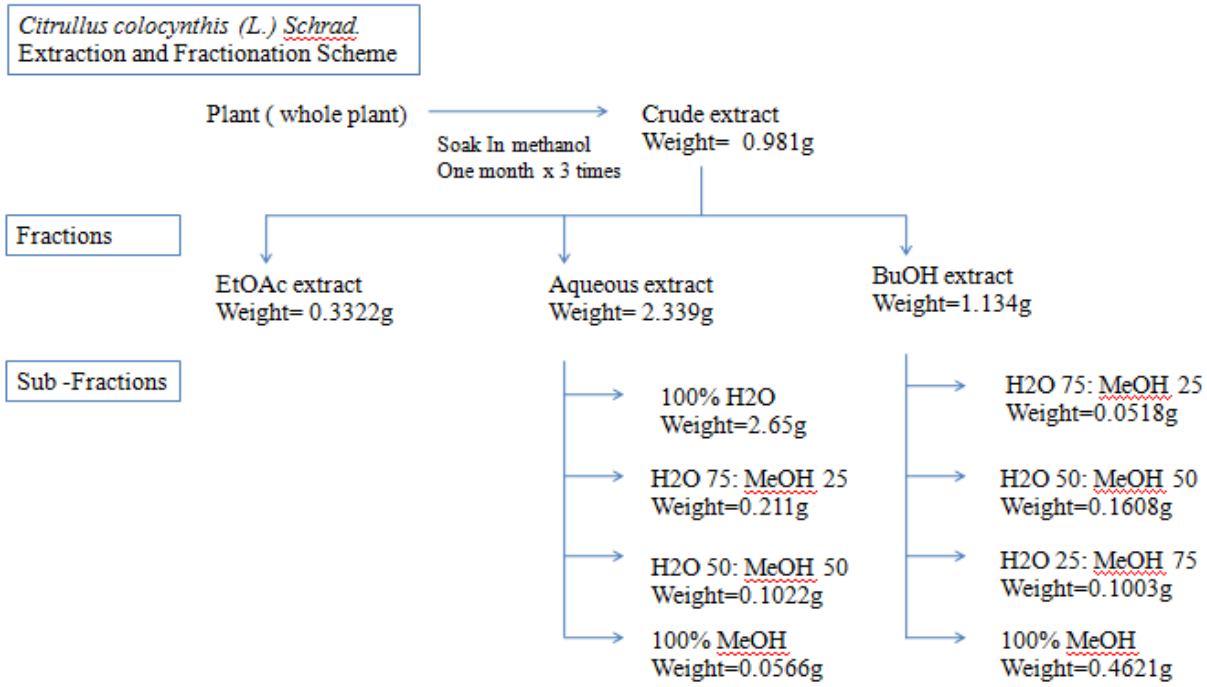

Figure 25: Extraction & Fractionation Scheme of *Citrullus colocynthis*

4.2 Screening of plant extracts for acetylcholinesterase inhibitory effect:

Using Ellman's assay, inhibition effect of plant extracts from 24 different fractions on AChEI activity was assessed. Screenings were performed at concentrations ranging from 25-1000 µg/ml. The results obtained by Ellman's assay for inhibition activities of all plant extracts. Fractions were measured with the standard Galanthamine used as positive control and is shown in table 2 & 3.

Table 2: Percent of Acetylcholinesterase enzyme inhibition of different plant extracts at four different concentrations

S.No.	Botanical Name	Type of Extract (Solvent)	AChE Inhibition (%)			
			100 µg/ml	250 µg/ml	500 µg/ml	1000 µg/ml
1	*Argyrolobium roseum*	Crude	45.78±0.33	64.17±0.33	66.66±2.07	69.92±0.87
2		Aq.	44.83±9.25	72.22±2.02	85.82±6.04	95.78±2.02
3	*Zygophyllum fabago L.*	Crude	38.44±3.97	49.19±1.61	68.01±0.93	76.61±4.84
4		EtOAc	45.33±1.53	49±4.36	77.66±2.08	94.35±0.80
5		Aq.	48.11±2.83	54.84±0.81	69.89±1.23	82.01±2.24
6		Aq. (100% H2O)	6.989±3.05	46.91±0.62	63.98±0.931	66.12±0.81
7		BuOH	36.56±6.52	55.91±2.46	59.41±3.05	86.83±0.46
8		BuOH (100% H2O)	38.98±1.23	59.95±2.03	89.52±0.81	92.20±0.93
9	*Citrullus colocynthis (L.) Schrad.*	Crude	45.53±1.09	49.53±0.63	85.84±1.09	87.92±1.30
10		Aq.	36.46±3.82	44.44±1.89	53.15±4.36	80.68±2.63
11		Aq. (100% H2O)	32.36±2.42	72.29±2.42	81.20±2.42	88.18±2.93
12		Aq. (H2O 75 : MeOH 25)	7.16±4.08	26.42±5.25	59.01±1.13	63.46±1.86
13		Aq. (H2O 50 : MeOH 50)	6.42±0.43	18.52±2.67	59.75±1.13	62.22±0.74
14		Aq. (100% MeOH)	8.148±2.22	20.98±5.60	37.78±5.92	53.83±1.86
15	Galanthamine (Positive Control)		67.89±1.43	75.41±1.47	80.73±1.64	83.91±0.57

Table 3: Percent of Acetylcholinesterase enzyme inhibition of different plant extracts at five different concentrations

S.No.	Botanical Name	Type of Extract (Solvent)	AChE Inhibition (%)				
			25µg/ml	100 µg/ml	250 µg/ml	500 µg/ml	1000 µg/ml
1	*Argyrolobium roseum*	Hexane	19.48±1.29	60.92±5.48	72.41±1.52	76.24±1.45	90.23±4.14
2		EtOAc	35.83±2.34	54.40±2.32	59.77±2.29	63.79±1.15	86.97±3.16
3	*Zygophyllum fabago*	BuOH (H2O 50 : MeOH 50)	38.73±8.80	65.32±1.61	73.65±0.93	77.15±1.23	80.38±1.23
4		BuOH (100% MeOH)	45.24±3.15	63.76±6.22	70.56±1.25	83.83±2.28	89.15±1.74
5	*Citrullus colocynthis (L.) Schrad.*	EtOAc	41.27±1.82	62.52±1.64	69.63±1.48	85.63±1.46	90.12±3.80
6		BuOH	23.01±3.64	83.70±1.48	89.87±1.54	95.31±1.13	94.81±0.74
7		BuOH (H2O 75 : MeOH 25)	10.12±3.31	72.84±3.73	83.95±1.54	92.09±1.13	95.80±1.54
8		BuOH (H2O 50 : MeOH 50)	26.17±2.26	64.94±5.60	82.72±10.7	95.80±1.13	97.53±1.54
9		BuOH (H2O 25 : MeOH 75)	24.44±5.92	52.59±4.86	61.48±1.96	72.59±0.74	77.53±0.43
10		BuOH (100% MeOH)	34.93±1.57	44.69±2.38	55.06±1.13	68.89±2.22	75.80±5.61
11	Galanthamine (Positive Control)		45.61±1.76	67.89±1.43	75.41±1.47	80.73±1.64	83.91±0.57

Values are presented as mean values of % of inhibition ± standard deviation of three independent experiments. The AChEI action of plant extract is characterized according to (Vinutha, et al., 2007)claiming as weak inhibitors below 30% inhibition, (30-50% inhibition) as moderate inhibitors and more than 50% inhibition as potent inhibitors.

4.2.1 *Argyrolobium roseum* AChE Inhibition

Crude methanolic extract of *Argyrolobium roseum* 1.51 g was prepared and fractionated using solvent-solvent extraction into hexane, EtOAc and BuOH fractions. The ethyl acetate extract 0.752 g of powder, hexane extract 1.387 g while the aqueous extract 1.371 g were obtained. Each of these extracts were subjected to bioactivity screening tests. The activity of the plant extracts with acetylcholinesterase enzyme in the inhibition studies at series of concentrations 100, 250, 500 and 1000 µg/ml.

Crude extract of the *Argyrolobium roeum* showed the moderate inhibition activity in the assay, and a mild activity was observed in the aqueous extract. Results showed that EtOAc and Hexane extract displayed extraordinary inhibitory activity on AChE at 100 µg/ml, above 50% inhibition rate. The assay results are shown in figure 26. Dose dependent inhibitory activity of highly active extracts was additional tested, to compute the IC_{50} values, at lower concentration, 25 µg/ml.

Results of IC_{50} values are shown in figure 27. Activities with lower IC_{50} values are suggestive of valuable inhibition potential with the enzyme. The assay result data demonstrates that less polar fraction hexane extract of *Argyrolobium roseum* possessed the strongest activity, out of all the tested fractions, having an IC_{50} value of 63.01 µg/ml followed by EtOAc 109.6 µg/ml and Aq. extracts 113.3 µg/ml compared with standard Galanthamine (IC_{50}= 8.97 µg/ml). The increase of activity is suggestive of the fact that the principal constituents responsible for the activity compared to the crude extract were contained in the hexane fraction.

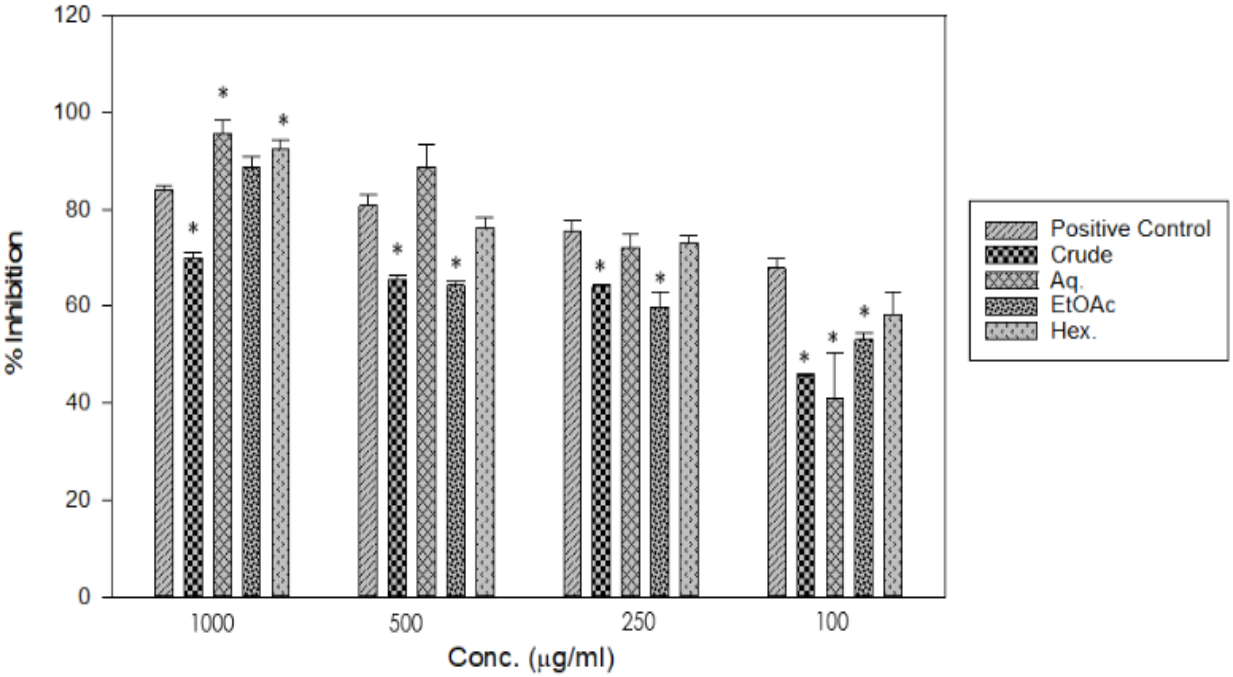

Figure 26: Percentage Inhibition of Acetylcholinesterase of different extracts of *Argyrolobium roseum*.

Each value is symbolized as mean ± SD (n=3). Means with sign (*) in the graph are significantly ($p<0.05$) different from Control group.

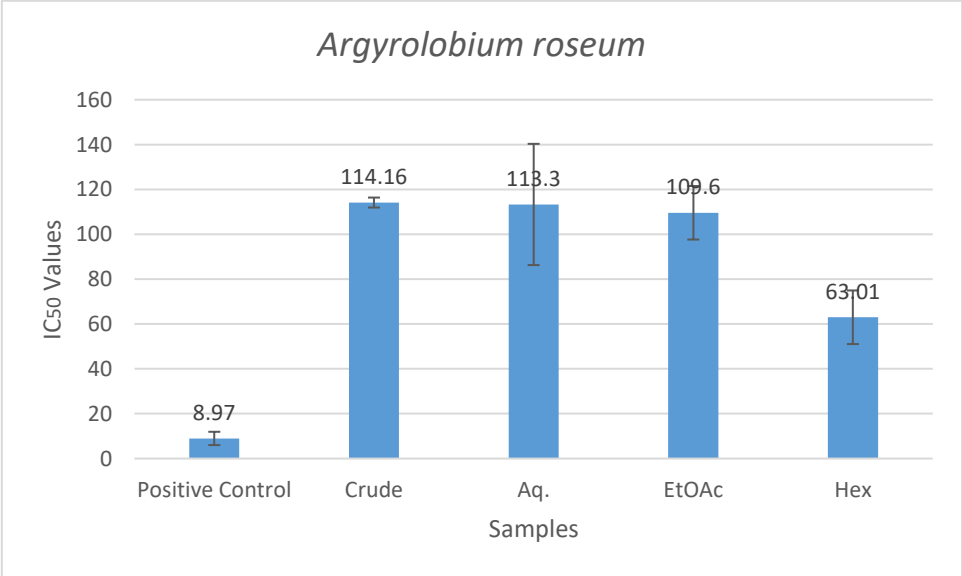

Figure 27: IC_{50} values of acetylcholinesterase enzyme inhibitory activity of different extracts of *Argyrolobium roseum.*

Each value is denoted as mean ± SD (n=3).

4.2.2 *Zygophyllum fabago* AChE Inhibition

Crude methanolic extract of *Zygophyllum fabago* was similarly fractionated and sub-fractionated. Through column chromatography with HP-20 eluted with increasing proportions of methanol and water. Each fraction was subjected to bioactivity screening tests. The activity of the plant extracts was tested against acetylcholinesterase enzyme at series of concentrations ranging from 25-1000 µg/ml. Crude extract of *Zygophyllum fabago* showed weak inhibition activity (IC_{50} =213.18 µg/ml) in the assay, while a mild activity was detected in the ethyl acetate extract (IC_{50} =188.58 µg/ml) and aqueous extract (IC_{50} =130.66 µg/ml).

Presence of activity in hydrophilic fractions indicated that the activity resided mainly in polar compounds. The fact also related to being active compounds possibly phenolics for the bioactivity in accordance with the reported literature. The enzyme inhibition activity in butanol fraction comparable to the crude extract was devoid of any activity (IC_{50} =234.36µg/ml). Based on these observations the butanol fraction containing the polar compounds was further fractionation attributed on hydrophillicity property of constitution butanol.

Compare to galantamine (IC_{50} of 8.97 µg/mL) used as standard AChE inhibitor; the tested fractions of *Z.fabago* at 100 µg/ml had moderate inhibitory activities, in except being two fractions of butanol eluted at (50 H2O:50 MeOH) and (100% MeOH) showed potent inhibitory activity at this concentration. At 1000 µg/mL, the inhibitory potential of fractions ranged from 66-94 % respectively as shown in figure 24. BuOH fraction eluted at (50 H2O:50 MeOH) and (100% MeOH) exhibited outstanding inhibitory activity on AChE at 100 µg/ml, above 50% inhibition rate. At the lowest tested concentration of 25 µg/mL, the AChE inhibitory activity of the two fractions dropped to 38.73% & 45.24% for BuOH eluted at (50 H2O:50 MeOH) and (100% MeOH) respectively. Concentration dependent inhibition was also observed and the IC_{50} values in ranges from 37.79 µg/mL for 100% eluted MeOH fraction of butanol extract, to 272 µg/mL for 100% H_2O fraction of aqueous extract.

Results of IC_{50} values are shown in figure 29. 100% MeOH fraction of butanol extract possessed strongest AChEI effect possessing IC_{50} = 37.79 µg/ml followed by H2O-MeOH (50:50) fraction of butanol having IC50 value 37.98 µg/ml. However, all the samples had lower inhibitory activities compare with that of galantamine (IC_{50} 8.97 µg/mL).

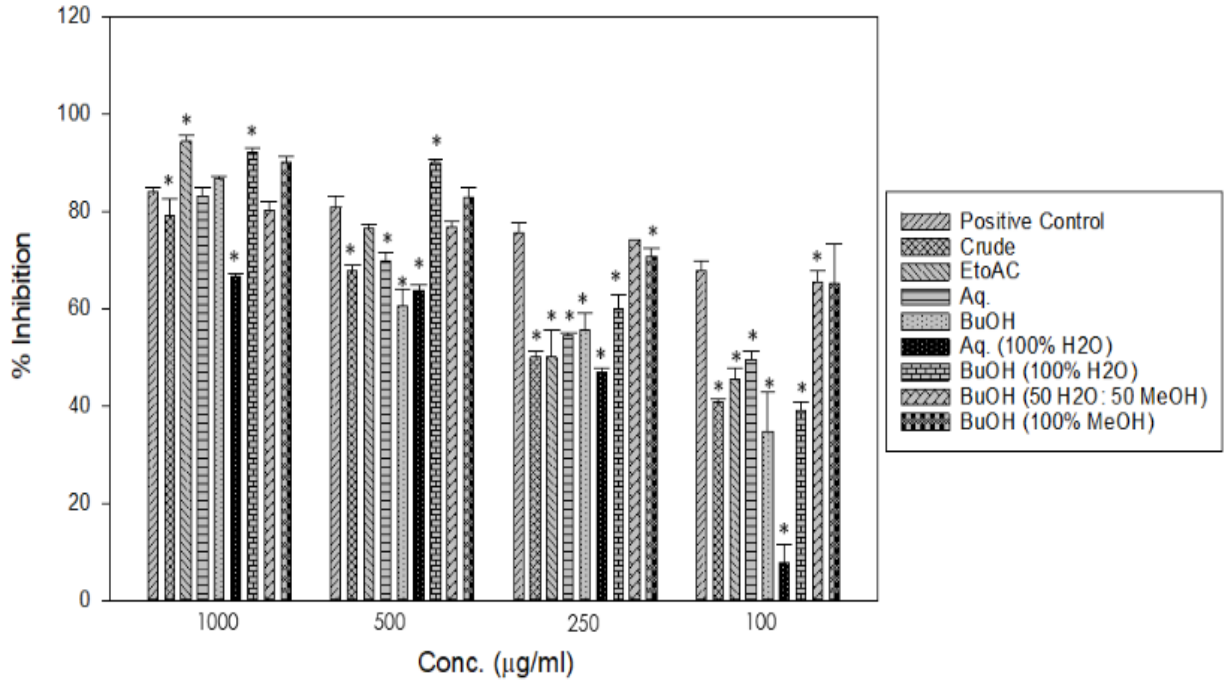

Figure 28: Percentage Inhibition of Acetylcholinesterase of different extracts of *Zygophyllum fabago*.

Each value is symbolized as mean ± SD (n=3). Means with sign (*) in the graph are significantly ($p<0.05$) different from Control group.

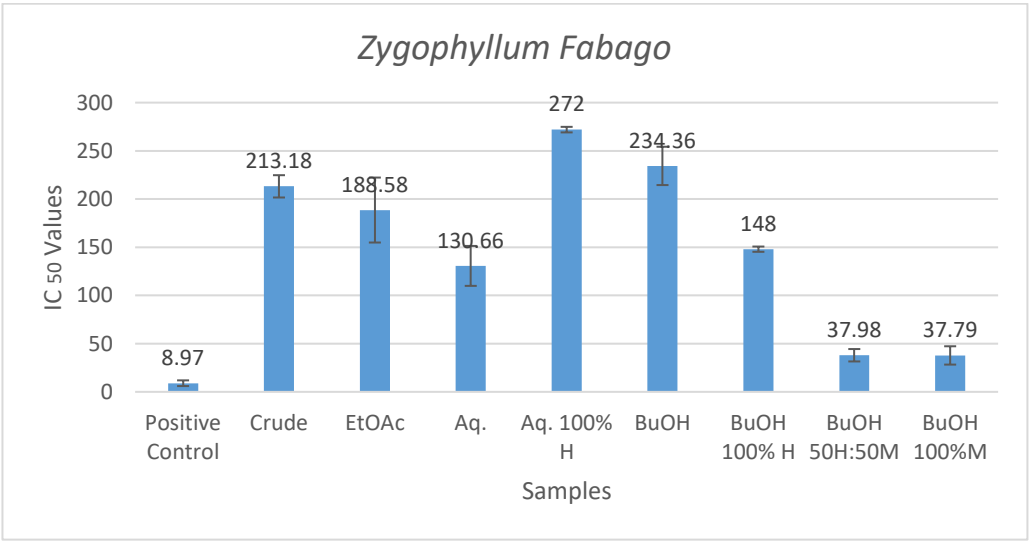

Figure 29: IC_{50} values of acetylcholinesterase enzyme inhibitory activity of different extracts of *Zygophyllum fabago*.

Each value is denoted as mean ± SD (n=3).

4.2.3 *Citrullus colocynthis* AChE Inhibition

Crude methanolic extract of *Citrullus colocynthis* was fractionated into EtOAc, aqueous and BuOH fractions. These fractions were further exposed to sub-fractionation and eight sub-fractions were made. All fractions were tested for their efficacy against acetylcholinesterase enzme.

The results of Ellman's assay performed for AChEI activity of *Citrullus colocynthis* fractions is shown in figure 30. It demonstrates that butanol extract and its two sub-fractions which we obtained after elution (H_2O:MeOH 75 : 25) and (H2O : MeOH50 : 50) showed highest inhibitory activity against acetylcholinesterase enzyme at different tested concentrations compared to galanthamine standard used as a positive control. At 1000µg/ml all the tested fractions showed potent inhibitory activity against AChE ranging from lowest 53.83% to highest 97.53%. At a lower concentration, five fractions including EtOAc and BuOH eluted at (75 H2O:25 MeOH), (H2O: MeOH50:50) and (H2O: MeOH 25:75) presented notable inhibitory activity on AChE at 100 µg/ml more than 50 percent inhibition rate.

These fractions with above 50% inhibition rate were then further tested at 25 µg/ml to determine IC_{50} values of the extracts. Activity results of measured IC_{50} values are shown in figure 31. Graph demonstrates that the BuOH extract of C.colocynthis showed highly significant IC50 value of 34.84 µg/ml compared with standard Galantamine IC_{50} 8.97 µg/ml, followed by EtOAc extract 44.78 µg/ml and of BuOH extract's sub-fractions eluted on (75 H2O : 25 MeOH) and (50 H2O : 50 MeOH) showed highest inhibition of AChE with IC_{50} values of 45.39& 53.67 µg/ml respectively.

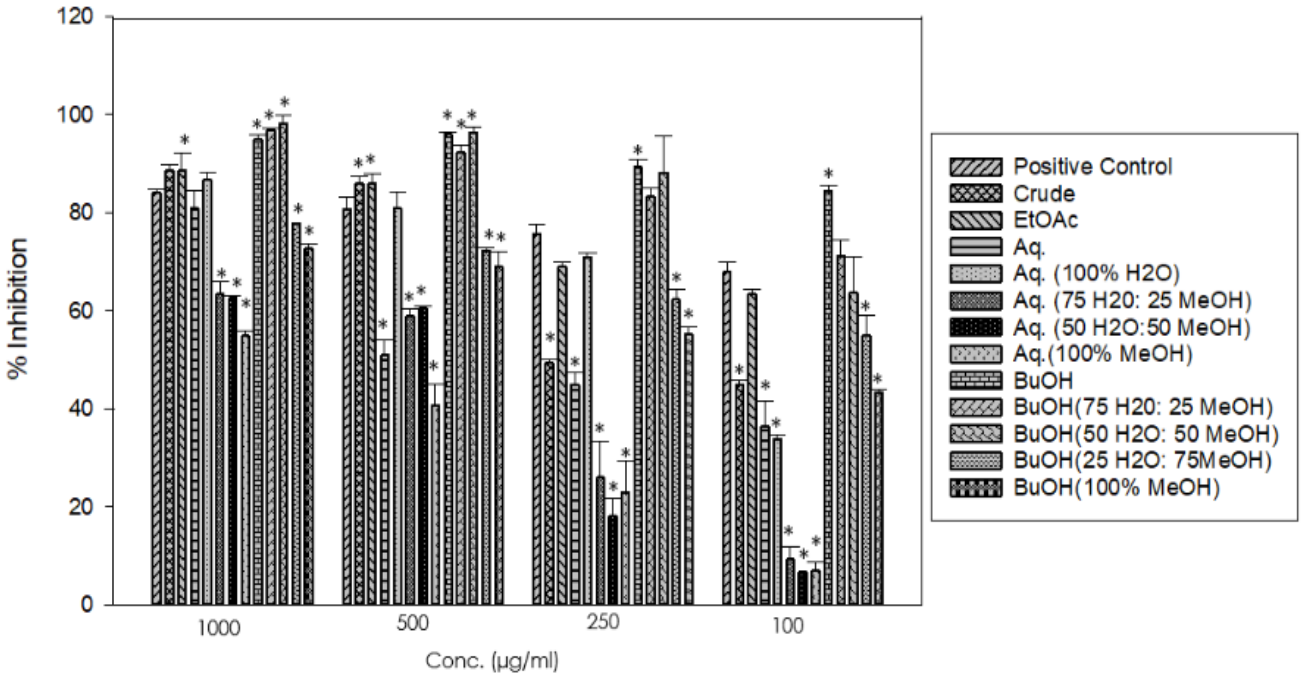

Figure 30: Percentage Inhibition of Acetylcholinesterase of different extracts of *Citrullus colocynthis*.

Each value is symbolized as mean ± SD (n=3). Means with sign (*) in the graph are significantly ($p<0.05$) different from Control group.

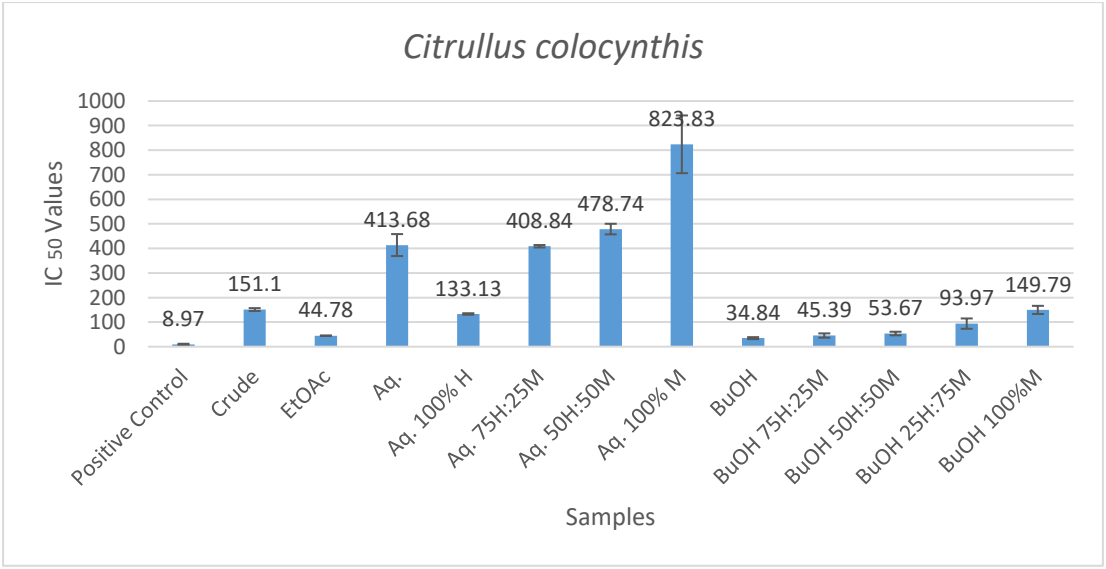

Figure 31: IC_{50} values of acetylcholinesterase enzyme inhibition of various extracts of *Citrullus colocynthis*.

Each value is denoted as mean ± SD (n=3).

4.2.4 Enzyme Inhibition (IC_{50}µg/ml) of fractions in the Acetyl Cholinesterase assay

A low IC_{50} value is suggestive of valuable inhibition of the enzyme. Measured IC50 values of all fractions in the Acetyl cholinesterase assay are shown in Table-4. Each value is denoted as mean ± SD (n=3).

The hexane extract of *Argyrolobium roseum* possessed the strongest inhibition out of all the tested fractions, possessing an IC_{50}=63.01µg/ml contrasted with standard Galanthamine IC_{50}=8.97 µg/ml.

The sub-fraction of Butanol extract eluted with 100% MeOH and H2O-MeOH (50:50) in HP-20 column chromatography of *Z.fabago* crude extract possessed strongest AChEI effect with IC_{50} value of 36.02 µg/ml and 36.89 µg/ml correspondingly.

The BuOH and EtOAc extract of *Citrullus colocynthis* revealed highest inhibition effect with IC_{50}=32.35 µg/ml and 45.97 µg/ml. Similarly, the BuOH extract sub-fractions eluted on H2O-MeOH (75:25) and (50:50) showed utmost inhibition of ACHE having IC_{50} Values of 42.31 µg/ml & 58.32 µg/ml.

Table 4: Enzyme Inhibition (IC_{50} µg/ml) of fractions in the Acetyl Cholinesterase assay

Extracts	***A.roseum***	***Z.fabago***	***C.colocynthis***
80% MeOH-H2O	114.16±2.97	213.18±11.6	151.1±5.94
Hexane Extract	63.01±11.95	N.P	N.P
EtoAc Extract	109.6±11.93	188.58±33.7	47.78±0
Butanol Extract	N.P	234.36±19.8	34.84±4.00
MeOH-H2O (0:100)[a]	-	148±2.73	N.P
MeOH-H2O (25:75)[a]	-	N.P	45.39±8.81

MeOH-H2O (50:50)[a]	-	37.98±6.42	53.67±7.27
MeOH-H2O (75:25)[a]	-	N.P	93.97±21.04
MeOH-H2O (100:0)[a]	-	37.79±9.49	149.79±16.62
Water Extract	113.3±27.02	130.66±20.7	413.68±44.68
MeOH-H2O (0:100)[a]	N.P	272±2.88	133.13±3.06
MeOH-H2O (25:75)[a]	-	N.P	408.84±5.01
MeOH-H2O (50:50)[a]	-	-	478.74±21.59
MeOH-H2O (100:0)[a]	-	-	823.83±117.5
Galanthamine	8.97±2.97		

[a]Fractions obtained from Partition chromatography with DIAION HP-20 Dion Resin

[b]Standard

4.3 Brine Shrimp Lethality Assay Results

The in vitro cytotoxicity assay was conducted on three medicinal plants fractions and sub-fractions. Various crude extracts were obtained using methanol, hexane, ethyl acetate, butanol and water. The Lethal concentration (LC_{50}) values were determined using Microsoft Excel, 2013. The percentage mortality vs. concentration of the extracts was plotted. The trend line was used to calculate the LC_{50} values. An LC_{50} value is that concentration which kills 50% of the larvae and LC_{50} value more than 100 µg/mL is deemed to signify a non-toxic extract in brine shrimp assay (Moshi *et al*., 2010).

4.3.1 Cytotoxicity of *Argyrolobium roseum*

Table 5: Brine Shrimp Cytotoxicity assay results of *Argyrolobium roseum* indicated as percentage mortality at various concentrations

Extracts	**Conc. µg/ml**	**Number of Surviving brine shrimps After 24 hours**			**Entire Number of Survivors**	**Average Mortality %**	**LC_{50}**	**Remark**
		T1	T2	T3				
Crude	1	7	5	7	19	36.66%	183.03	Non-toxic
	10	7	5	6	18	40%		
	100	5	5	6	16	46.67%		
	1000	0	0	0	0	100%		
Ethyl acetate	1	6	6	5	12	43.33%	27.37	Toxic
	10	6	5	5	11	46.67%		
	100	5	2	3	10	66.66%		
	1000	0	0	0	0	100%		
Aqueous	1	8	7	7	15	26.67%	277.75	Non-toxic
	10	7	7	6	14	33.33%		
	100	7	6	5	13	40%		
	1000	0	0	0	0	100%		
Hexane	1	7	5	5	12	43.33%	24.48	Toxic
	10	6	4	4	10	53.33%		
	100	6	4	3	9	56.67%		
	1000	0	0	0	0	100%		

The extracts of *Argyrolobium roseum* showed maximum toxicity against brine shrimps as 100% of the shrimps were killed at high concentration in 24h. The hexane extract (LC_{50}=24.48 µg/ml) was most active out of all the four fractions tested for cytotoxic potential followed by the EtOAc extract (LC_{50}=27.37 µg/ml). Overall two organic extracts of *A.roseum* were out of four tested were toxic towards the brine shrimp nauplii. Crude methanolic extract was not active as its LC_{50} value observed is 183.0 µg/ml and aqueous extract also showed no activity possessing LC_{50} value of 277.7 µg/ml. It is directly correlated with increasing concentrations as well. Dose-response curve of percentage mortality is shown in figure 32. Experiment was performed in triplicate and each value is denoted as mean ± SD (n=3).

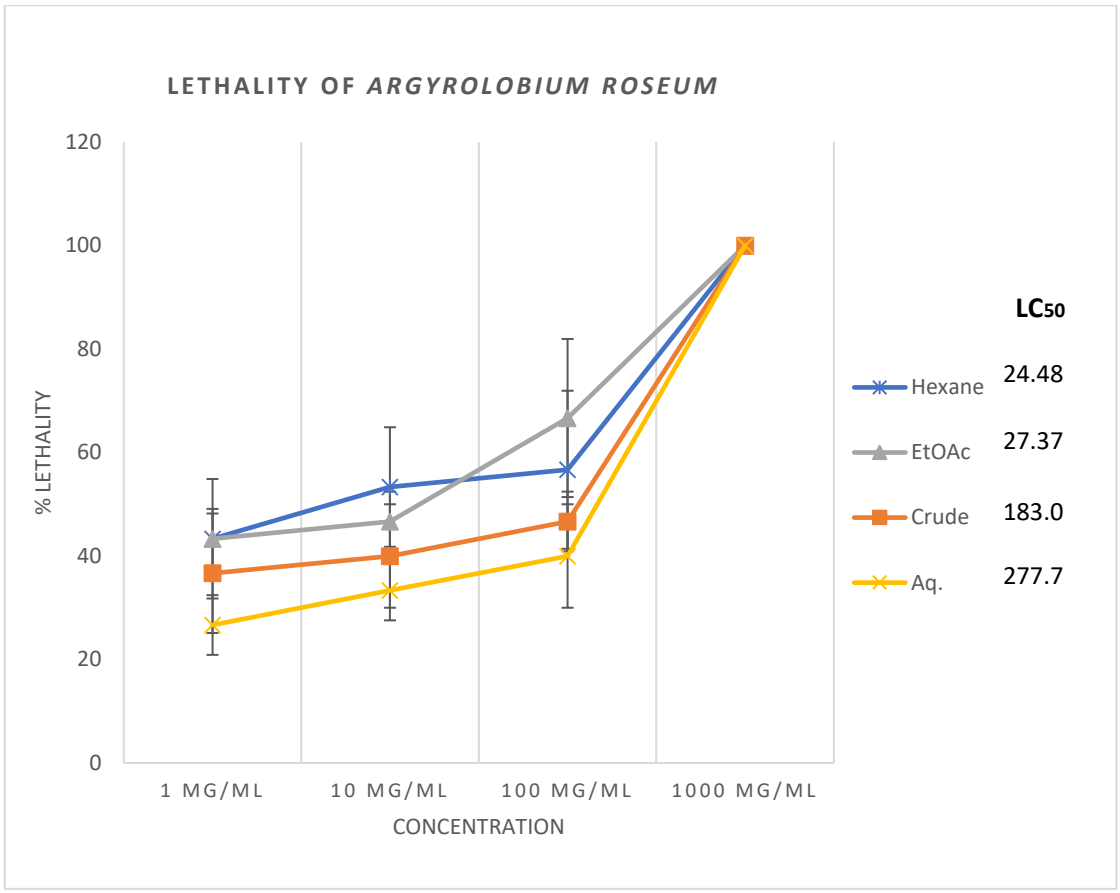

Figure 32: Brine shrimp cytotoxicity dose response curves of *Argyrolobium roseum*

4.3.2 Cytotoxicity of *Zygophyllum fabago*

Table 6: Brine Shrimp Cytotoxicity assay results of Zygophyllum fabago indicated as percentage mortality at various concentrations

Extracts	Conc. (µg/ml)	Number of Surviving Brine Shrimps After 24 hours			Entire Number of Survivors	Average Mortality %	LC_{50}	Remark
		T1	T2	T3				
Crude	1	9	7	8	13	35%	234.15	Non-toxic
	10	7	6	6	12	40%		
	100	4	5	4	11	45%		
	1000	0	0	0	0	100%		
Ethyl acetate	1	6	8	8	10	50%	70.82	Toxic
	10	3	3	2	9	55%		
	100	2	1	1	4	80%		
	1000	0	0	0	0	100%		
Aqueous	1	7	5	6	10	50%	84.08	Toxic
	10	7	3	5	9	55%		
	100	6	4	4	8	60%		
	1000	0	0		0	100%		
Butanol	1	5	7	6	12	40%	32.52	Toxic
	10	4	5	5	10	50%		
	100	4	5	3	10	50%		
	1000	0	0		0	100%		

The cytotoxicity brine shrimp assay effects of crude methanolic medicinal plant *Zygophyllum fabago* extract's percentage lethality at several concentrations and LC_{50} values are revealed in Table 6. At 1000 µg/mL, all the extracts revealed 100% mortality of brine shrimp. LC_{50} values fluctuated from 32.52 to 234.15 µg/mL, with butanol extract having the lowermost value of LC_{50}=32.52 µg/ml (most potent); this was followed by ethyl acetate extract (LC_{50}=70.82 µg/mL), then by aqueous extract (LC_{50}=84.08 µg/ml) and lastly crude methanolic extract (234.15 µg/ml). The percentage lethality of nauplii increased with an increase in concentration. Figure 33 is showing dose-response curves of *Z.fabago* extracts against percentage lethality of the brine shrimps. Each value is denoted as mean ± SD (n=3).

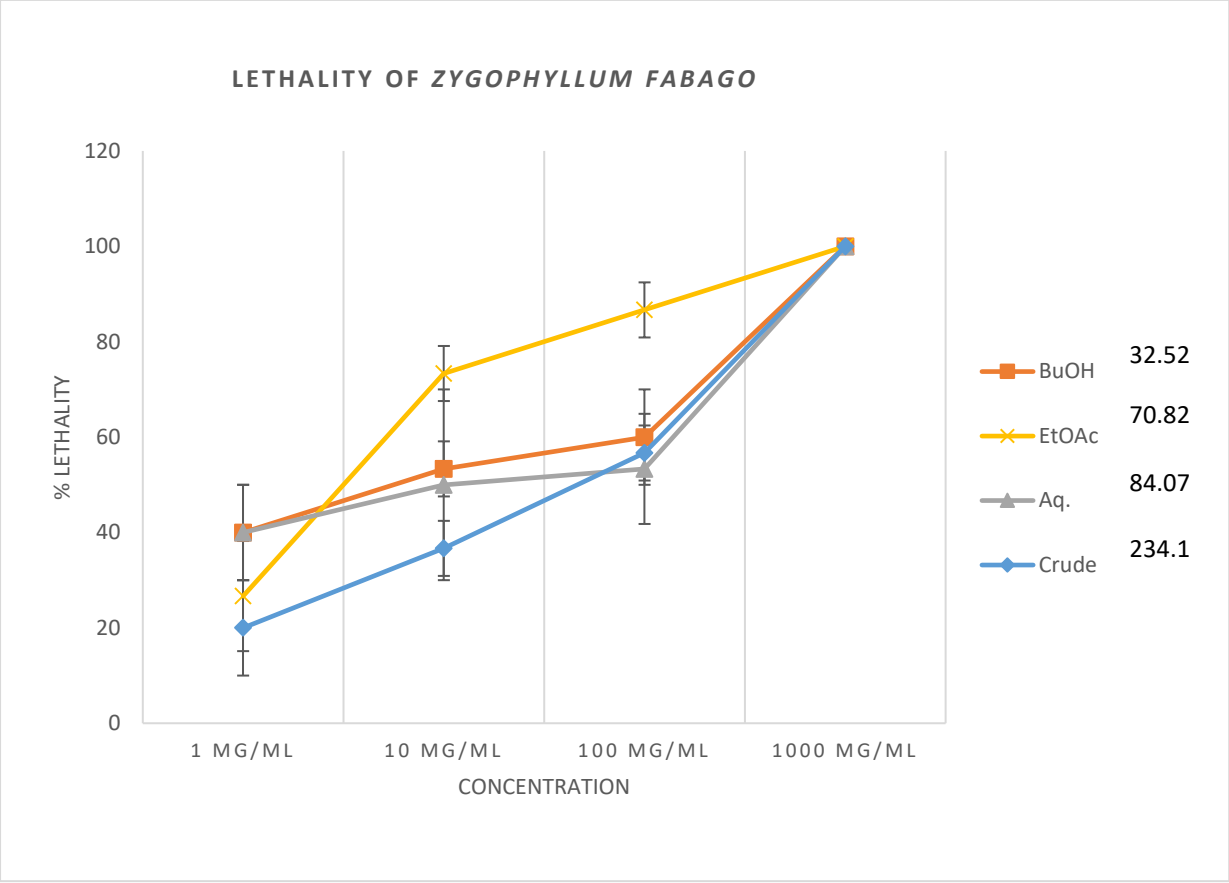

Figure 33: Brine shrimp cytotoxicity dose response curves of *Zygophyllum fabago*

4.3.3 Cytotoxicity of *Citrullus colocynthis*

Table 7: Brine Shrimp cytotoxicity assay results of *Citrullus colocynthis* indicated as percentage mortality at various concentrations

Extracts	Conc. ug/ml)	Number of Surviving Brine Shrimp After 24 hours			Entire Number of Survivors	Average Mortality %	LC_{50} µg/ml	Remark
		T1	T2	T3				
Crude	1	7	8	7	15	26.67%	135.31	Non-toxic
	10	6	4	5	10	50%		
	100	4	4	3	8	63.33%		
	1000	0	0	0	0	100%		
Ethyl acetate	1	8	7	6	15	30%	227.18	Non-toxic
	10	7	6	5	13	40%		
	100	6	5	6	11	43.33%		
	1000	0	0	0	0	100%		
Aqueous	1	8	8	6	16	26.66%	228.63	Non-toxic
	10	6	6	6	12	40%		
	100	6	5	5	11	46.67%		
	1000	0	0	0	0	100%		
Butanol	1	7	8	8	15	23.33%	254.76	Non-toxic
	10	6	7	7	11	33.33%		
	100	5	5	5	10	50%		
	1000	0	0	0	0	100%		

The extracts of *Citrullus colocynthis* showed toxicity against brine shrimps at higher concentrations, as 100% of the shrimps were killed in 24h. However, the measurement of lethal concentration (LC_{50}) showed that overall *Citrullus colocynthis* extracts were not cytotoxic. The crude methanolic extract was the most active of the four extracts (LC_{50}=135.3 µg/ml) followed by the Ethyl acetate (LC_{50}=227.18 µg/ml), aqueous (LC_{50}=228.63 µg/ml) and butanol extract (LC_{50}=254.76 µg/ml). Overall all four organic extracts of *C.colocynthis* were not toxic towards the brine shrimp nauplii as only the extracts having LC_{50} values less than 100 µg/ml are considered cytotoxic. Dose-response curve of percentage mortality is shown in figure 34. Experiment was performed in triplicate and each value is denoted as mean ± SD (n=3).

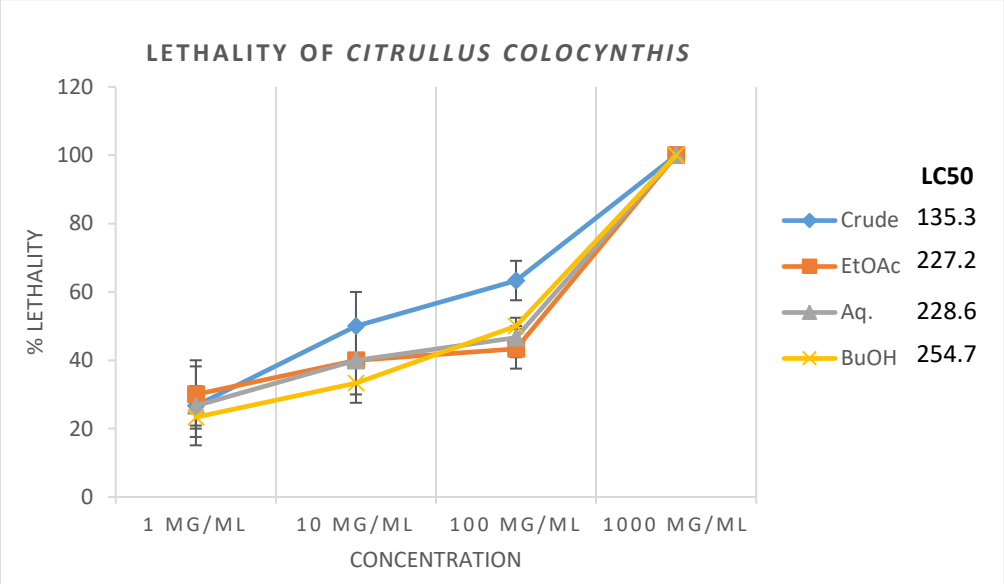

Figure 34: Brine shrimp cytotoxicity dose response curves of *Citrullus colocynthis*

4.4 Cytotoxicity Results of MTT Assay

To identify that crude extracts and their fractions for potential as a therapeutic source of selective nerve transmission mechanism action, cytotoxicity studies were performed to check the cytotoxic potential by MTT assay along with brine shrimp lethality assay. This study was designed for in vitro confirmation of cytotoxic effects of the crude extracts of the plants selected on cell viability, HUH and MCF-7 cell lines were incubated with various plant extract concentrations. Cell viability was determined after 24 hours. The viable cells percent was computed as the absorbance of treated cells (Sample) ratio to untreated cells (Control). Values given represent the mean ± standard deviations of the experiments carried out in triplicate. Absorbance values that are lesser than the control cells specify a fall in the rate of cell proliferation. Contrariwise, a greater absorbance rate specifies a surge in cell proliferation.

Hardly, rise in proliferation might be offset by cell death; proof of cell death may be concluded from morphological changes. The cytotoxic concentration CC_{50} is the concentration which kills 50% of the cells, was determined against the plant extracts which had shown less than 70% cell viability. An extract with CC_{50} greater than 30 µg/mL is believed as non-toxic (Fadeyi, et al., 2013).

4.4.1 Cytotoxic effects of *Argyrolobium roseum* on HUH cell line

The cytotoxic potential of the crude extracts of *Argyrolobium roseum* on the proliferation of liver cancer cells were observed by MTT Assay. Results of different concentrations of *Argyrolobium roseum* including 100,200,300,400 and 500 µg/ml tested against the liver cancer cell lines. The activity results are presented in Table 8, and graphically presented in figure 35. Dose response graphs constructed in the range 100-500 µg/ml represents decreasing number of viable cells with increased extracts concentration.

MTT assay of *Argyrolobium roseum* extracts shows no significant effects on liver cancer cell in concentration range between 100 µg/ml to 500 µg/ml compared with control. Only the hexane extract showed some levels of cytotoxicity (very weak) against liver cells causing cell death 34.08% and 34.89% at 400 and 500 µg/ml concentrations. Whereas the crude, ethyl acetate and aqueous extract demonstrated no cytotoxic effects on HUH cell line. Hence, overall the *Argyrolobium roseum* extracts were non-cytotoxic to liver cancer cells. It was found that the percentage of viable cells was decreasing with rising concentration of tested samples. Lowest value of CC_{50} obtained from HUH cell line assay was 558.78 µg/ml for hexane extract which is considered as non-cytotoxic.

Table 8: Cytotoxic activity of the *Argyrolobium roseum* extracts on HUH Cell Line

Extracts	**% Cell Viability at several Concentrations**					**CC_{50} µg/mL**
	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL	
Crude	100.64	94.85	90.19	87.14	84.73	–
EtOAc	92.12	89.55	81.67	76.53	68.81	821.28
Aqueous	105.63	113.98	113.02	138.26	160.77	–
Hexane	79.26	71.86	67.85	65.92	65.11	558.78

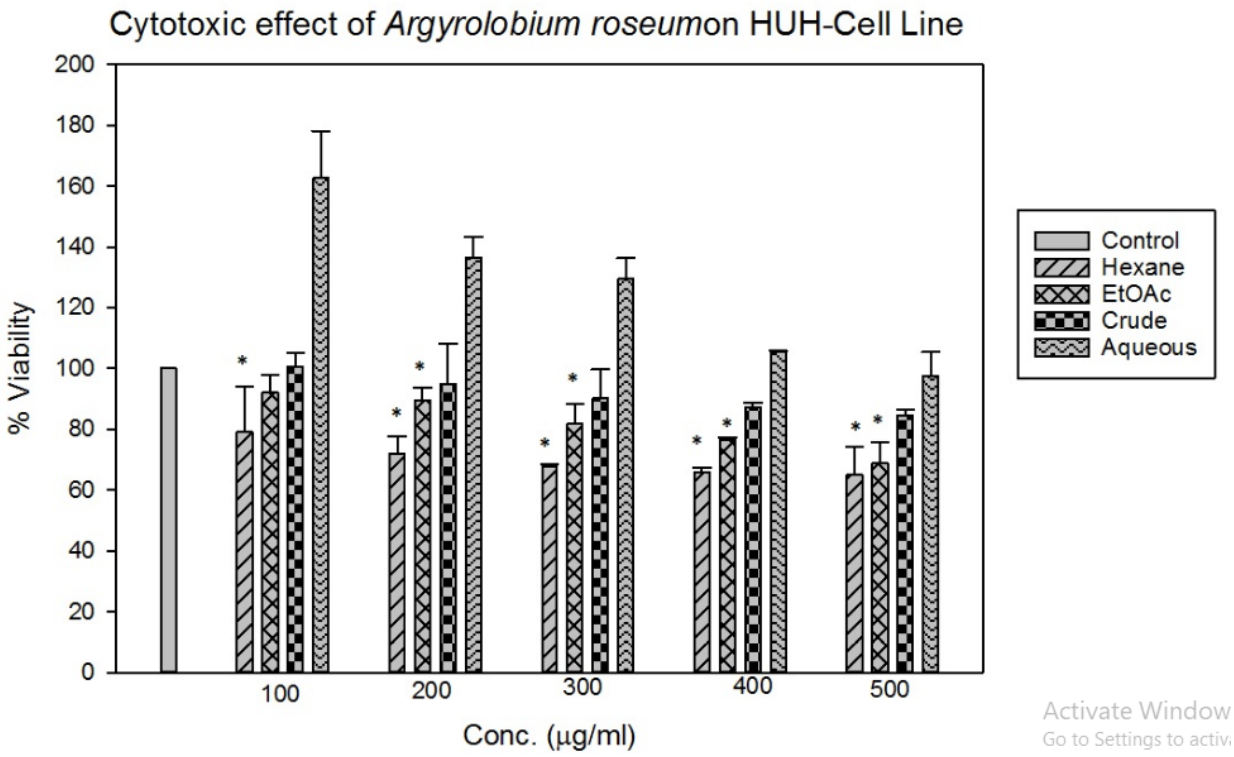

Figure 35: Cytotoxic effect of *Argyrolobium roseum* extracts against HUH cell line

Each value is denoted as mean ± SD (n=3). Means with sign (*) in the graph are significantly ($p<0.05$) different from Control group.

4.4.2 Cytotoxic effects of *Argyrolobium roseum* on MCF-7 cell line

MTT Assay of plant extracts on the MCF-7 cell line revealed no significant cytotoxic effect at all the concentrations ranging from 100-500 µg/ml of *Argyrolobium roseum* extracts. *Argyrolobium roseum* was slightly toxic due to 500 µg/ml concentration.

No cytotoxicity effect on MCF-7 cell line was recorded for aqueous and ethyl acetate extract of *Argyrolobium roseum* at 100 µg/ml concentration whereas the higher concentrations of 200, 300, 400 and 500 µg/ml cytotoxic effect was found significant. At 500µg/ml the viability of cells for aqueous and ethyl acetate extract decreased to 65.28% and 51.59% respectively. Thus, both aqueous as well as ethyl acetate extract of *Argyrolobium roseum* showed very weak cytotoxicity and are non-toxic in nature.

The cytotoxicity study against MCF-7 cell line exhibited the viability percentage of the methanolic crude and hexane extracts was lower as compared to other extracts. Both of these extracts exhibited cytotoxicity greater than 50% for breast cancer cells; at the concentration of 500 µg/ml, utmost 64.84% and 58.66% of the cells were not viable. Whereas, Ethyl acetate and

aqueous shown less than 50% cytotoxicity towards MCF-7 (breast cancer cells). Cytotoxic concentration of crude and hexane extract is CC50=301.64µg/ml and CC50=305.36µg/ml respectively. Results are tabularized in Table 9, and graphically presented in figure 36.

Table 9: Cytotoxic activity of the *Argyrolobium roseum* extracts on MCF-7 Cell Line

Extracts	% Cell Viability at several Concentrations					CC_{50} µg/mL
	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL	
Crude	69.61	65.28	38.52	36.93	35.16	301.64
EtOAc	68.55	57.24	55.65	53.79	51.59	373.36
Aqueous	90.19	82.95	79.95	79.06	65.28	758.48
Hexane	61.39	52.38	46.64	43.82	41.34	305.36

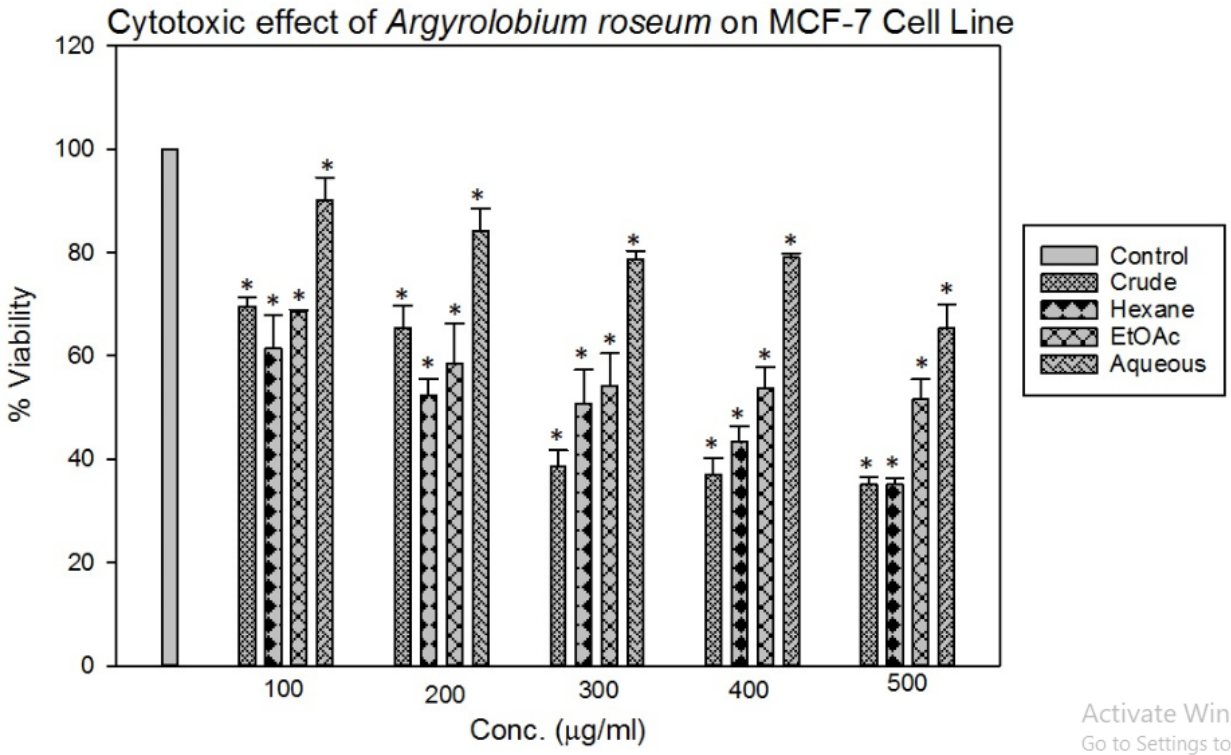

Figure 36: Cytotoxic effect of *Argyrolobium roseum* extracts against MCF-7 cell line

Each value is denoted as mean ± SD (n=3). Means with sign (*) in the graph are significantly ($p<0.05$) different from Control group.

4.4.3 Cytotoxic effects of *Zygophyllum Fabago* on HUH cell line

The cytotoxic effects of the crude extracts of *Zygophyllum Fabago* on different concentrations including 100,200,300,400 and 500 µg/ml on liver cancer cell lines are tabularized in Table 10, and graphically presented in figure 37. Dose response graphs constructed in the range 100-500 µg/ml represents decreasing number of viable cells with increased extracts concentration.

MTT assay of *Zygophyllum Fabago* extracts shows no significant effects on liver cancer cell in concentration range between 100µg/ml to 500µg/ml compared with control. Only the ethyl acetate extract showed some levels of cytotoxicity (very weak) against liver cells causing cell death 32.96% and 48.08% at 400 and 500 µg/ml concentrations. Whereas the crude, butanol and aqueous extract demonstrated no cytotoxic effects on HUH cell line. Hence, overall the *Zygophyllum fabago* extracts were non-cytotoxic to liver cancer cells. In general activity increased with rising concentration of tested samples. CC_{50} value calculated by plot of percent viability and concentrations was 548.27 µg/ml for ethyl acetate extract which renders this plant extract as non-cytotoxic.

Table 10: Cytotoxic activity of the *Zygophyllum Fabago* extracts on HUH Cell Line

Extracts	**% Cell Viability at several Concentrations**					**CC_{50} µg/mL**
	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL	
Crude	97.18	95.83	90.44	82.48	76.35	−
EtOAc	87.29	84.24	73.31	67.04	51.92	548.27
Aqueous	93.38	93.99	92.28	88.48	86.89	−
Butanol	136.34	98.07	151.61	140.19	131.99	−

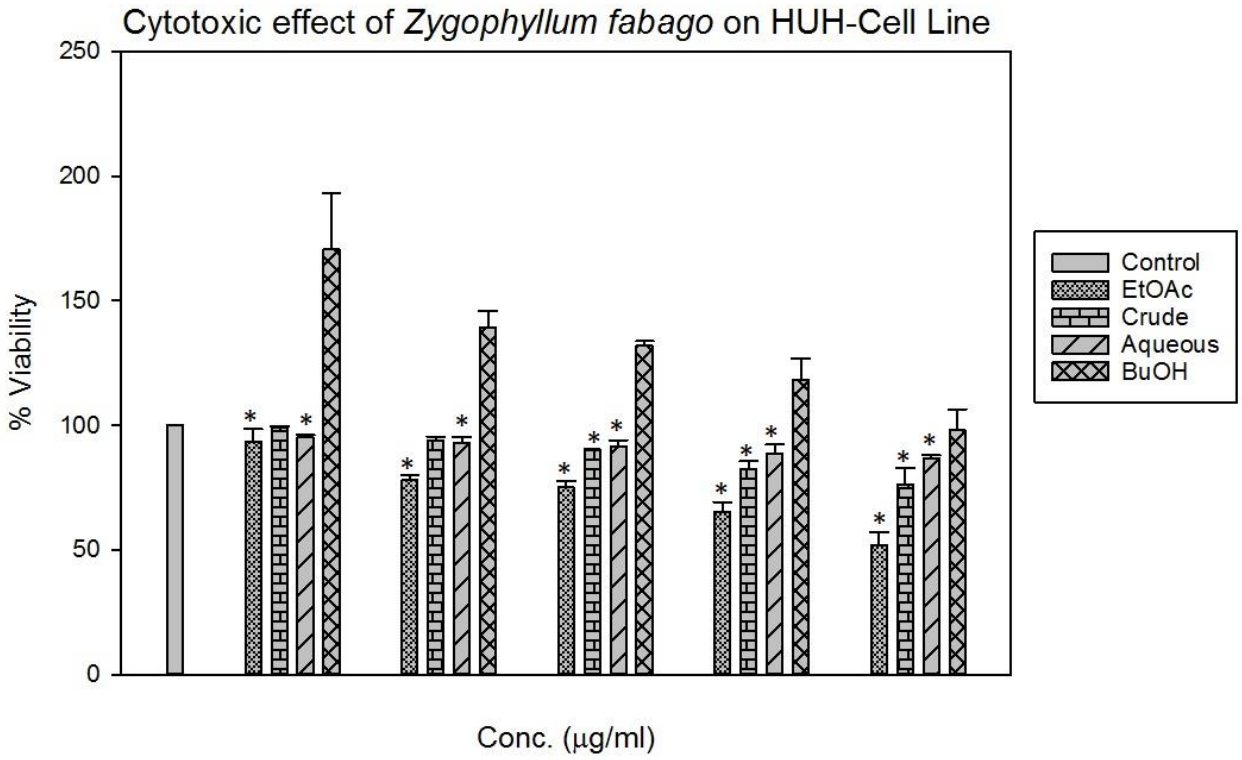

Figure 37: Cytotoxic effect of *Zygophyllum fabago* extracts against HUH cell line

Each value is denoted as mean ± SD (n=3). Means with sign (*) in the graph are significantly ($p<0.05$) different from Control group.

4.4.4 Cytotoxic effects of *Zygophyllum fabago* on MCF-7 cell line

MTT Assay of plant extracts on MCF-7 cell line revealed no significant cytotoxic effects at the concentrations ranging from 100-500 µg/ml of *Zygophyllum fabago* extracts. The cytotoxic effects of the crude extracts of *Zygophyllum fabago* with concentrations ranging from 100,200,300,400 and 500 µg/ml were prepared and tested against breast cancer cell line as tabularized in Table 11, and graphically presented in figure 38.

Zygophyllum Fabago aqueous and butanol extract revealed no cytotoxic activity on MCF-7 cell line on all concentrations tested. At even 500µg/ml the viability of cells for butanol and aqueous extract was more than 80%. Thus, both butanol as well as aqueous extract of *Zygophyllum Fabago* are non-toxic in nature.

However, crude methanolic and ethyl acetate extracts of Z.fabago showed some levels of cytotoxicity towards breast cancer cell lines. Both extracts, for breast cancer cells exhibited greater than 60% cytotoxicity; utmost 74.12% and 59.46% of the cells were not viable at 500

µg/ml concentration. Cell viability decreased to 25.88% and 40.54% at this concentration. Cytotoxic concentration (CC_{50}) of crude and ethyl acetate extract calculated is 296.03 µg/ml and 354.33 µg/ml respectively.

Table 11: Cytotoxic activity of the *Zygophyllum fabago* extracts on MCF-7Cell Line

Extracts	% Cell Viability at several Concentrations					CC_{50} µg/mL
	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL	
Crude	65.09	62.16	43.14	43.63	25.88	296.03
EtOAc	71.11	66.61	52.12	45.93	40.54	354.33
Aqueous	97.35	94.60	89.80	89.60	87.15	–
Butanol	96.91	96.64	94.87	91.25	89.58	–

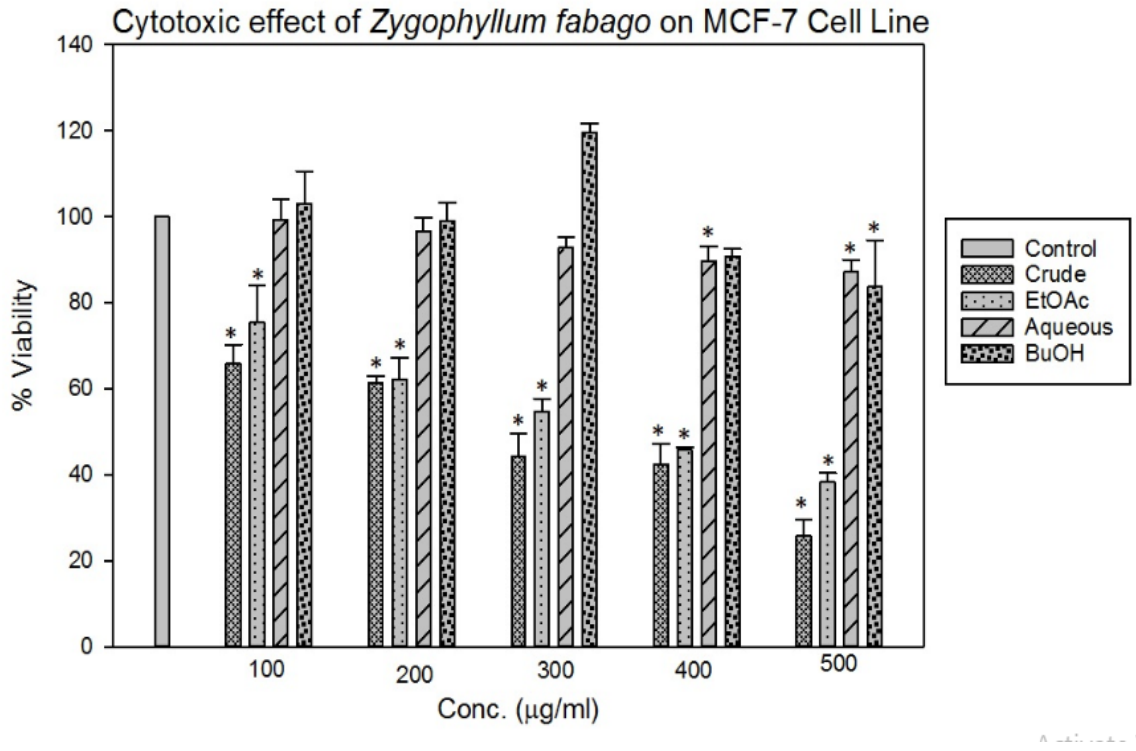

Figure 38: Cytotoxic effect of *Zygophyllum fabao* extracts against MCF-7 cell line

Each value is denoted as mean ± SD (n=3). Means with Sign (*) in the graph are significantly ($p<0.05$) different from Control group.

4.4.5 Cytotoxic effects of *Citrullus colocynthis* on HUH cell line

The cytotoxic effects of the crude extracts of *Citrullus colocynthis* on different concentrations including 100,200,300,400 and 500 µg/ml on liver cancer cell lines are tabularized in Table 12, and graphically presented in figure 39. Absorbance values that are lesser than the control cells specify a decrease in the rate of cell viability. Whereas, a greater absorbance rate specifies arise in cell propagation. Dose response graphs constructed in the range 100-500 µg/ml of *Citrullus colocynthis* extracts showed increased viability of cells compared with the control.

MTT assay of *Citrullus colocynthis* extracts shows no cytotoxic effects on liver cancer cell in concentration range between 100 µg/ml to 500 µg/ml compared with control. Overall the *Citrullus colocynthis* extracts were non-cytotoxic to liver cancer cells. Only the ethyl acetate extract inhibited by 21.45% of the cell's growth at a maximum concentration of 500 µg/ml.

Table 12: Cytotoxic activity of the *Citrullus colocynthis* extracts on HUH Cell line

Extracts	**% Cell Viability at several Concentrations**					**CC_{50} µg/mL**
	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL	
Crude	108.95	94.85	111.15	107.96	110.54	−
EtOAc	99.39	87.62	85.42	85.05	78.55	−
Aqueous	104.53	101.47	102.69	98.77	106.86	−
Butanol	112.5	116.91	129.41	122.43	119.12	−

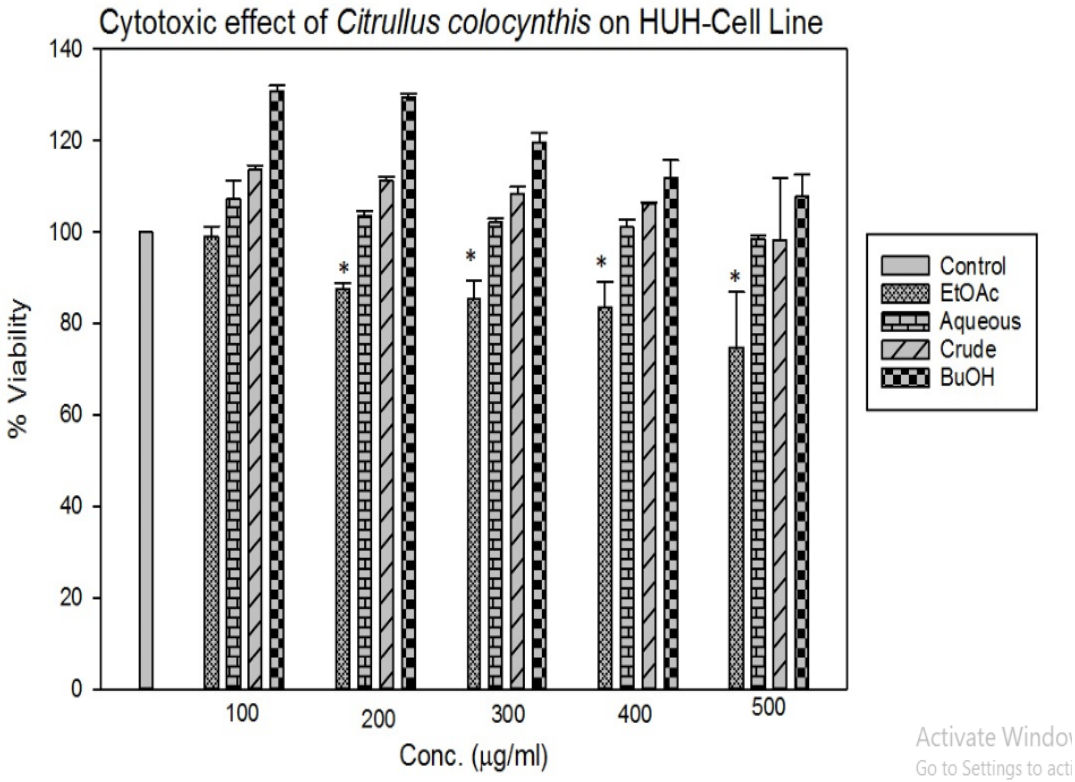

Figure 39: Cytotoxic effect of *Citrullus colocynthis* extracts against HUH cell line

Each value is represented as mean ± SD (n=3). Means with superscript (*) in the column are significantly ($p<0.05$) different from Control group

4.4.6 Cytotoxic effects of *Citrullus colocynthis* on MCF-7 cell line

Cytotoxicity testing on MCF-7 cell line revealed no significant cytotoxic effects when tested on 100-500 µg/ml concentrations. The cytotoxic effects of the crude extracts of *Citrullus colocynthis* on various concentrations are shown in table 13, while graphically presented in figure 40.

Citrullus colocynthis crude methanol and aqueous extract showed no cytotoxicity effect on MCF-7 cell line. At maximum concentration of 500 µg/ml the viability of cells for butanol and aqueous extract was more than 70%. Thus, both methanol as well as aqueous extract of *Citrullus colocynthis* are non-toxic in nature.

However, ethyl acetate and butanol extract of this plant showed some levels of cytotoxicity towards breast cancer cell lines, both extracts for breast cancer cells proliferation presented greater than 50% inhibition; utmost 61.08% and 55% of the cells were not viable at 500 µg/ml concentration, respectively. While percentage of viable cells decreased for EtOAc was 38.92% and BuOH extract was 45% at the concentration of 500 µg/ml. Cytotoxic concentration values

(CC_{50}) of ethyl acetate and butanol extract calculated were 374.20 µg/ml and 518.67 µg/ml respectively. Hence, attributed as non-toxic and safe for use in Alzheimer's drug formulation. Since both of these extracts also showed promising activity against AchE.

Table 13: Cytotoxic activity of the *Citrullus colocynthis* extracts on MCF-7 Cell Line

Extracts	% Cell Viability at several Concentrations					CC_{50}µg/mL
	100 µg/mL	200 µg/mL	300 µg/mL	400 µg/mL	500 µg/mL	
Crude	90.49	88.23	87.84	80.29	79.41	–
EtOAc	82.15	65.88	60.19	45.19	38.92	374.20
Aqueous	94.61	93.33	87.55	75.09	72.16	–
Butanol	95.69	91.76	90	76.27	45	518.67

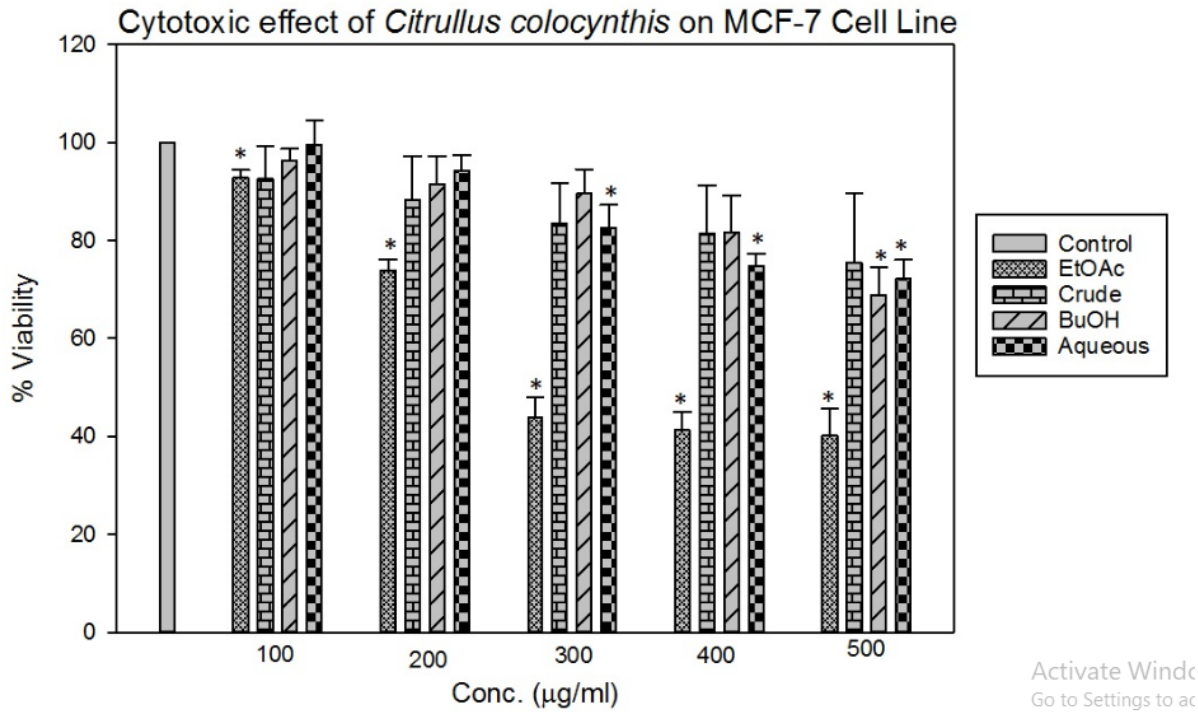

Figure 40: Cytotoxic effect of *Citrullus colocynthis* extracts against MCF-7 cell line

Each value is denoted as mean ± SD (n=3). Means with sign (*) in the graphs are significantly ($p<0.05$) different from Control group.

Chapter 5

5.0 Discussion

Alzheimer's disease categorized with advanced degeneration of memory and cognition requires AChE inhibitors for its treatment and scientists all over the world are screening numerous plants for cholinesterase inhibitory potential. In the present study, in vitro bioactivity screening was performed for various plant extracts obtained through detailed fractionation of crude plant extracts. Prepared by liquid-liquid extraction and vacuum filtration crude methanolic extracts of plants were initially partitioned by hexane, ethyl acetate, butanol and aqueous extracts. Thin layer chromatography (TLC) was performed to further fractionate the fractions according to their polar or non-polar nature. The fractions were further sub-divided by column chromatography on DIAION resin gel with mounting proportions of water and methanol for separation of secondary metabolites in to different organic crude extracts thus forming sub fractions of extracts. Anticholinesterase potential of 03x plants extracts using Ellman's assay from 24 different fractions was evaluated and cytotoxic activity was assessed by Brine shrimp lethality assay and MTT (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) cell proliferation assay using 2 human cancer cell lines (HUH-liver & MCF-7-breast).

Already available pharmaceutical drugs cause unwanted side effects along with the treatment of disorder, this includes systematic complications leading to hepatotoxicity and gastrointestinal selective action from the plant extract is one of the most significant characteristics of the plant derived medicine. Potential of plants for treatment as a drug is therefore preferred. The anti-tumor resistance for liver cancer and breast cancer cell lines are also investigated during this study. The already establishes supplements improves the level of chemicals known as neurotransmitters inside the brain but there is no solution to halt or hinder the demolition of neurons.

The AChE inhibition effect of 03x plants extracts using Ellman's assay from 24 different fractions showed that the extracts of the plants exhibited interesting biological activities in Acetyl Cholinesterase Inhibition and Cytotoxicity Assay. Several extracts possessing most potent activities were identified during this study.

Twelve crude extracts from three plants (medicinal in nature) which belongs to three different families of plants were assessed for cytotoxic activity against brine shrimps and against human carcinoma cells (HUH & MCF-7 cells).

The brine shrimp cytotoxicity assay displayed that 7 extracts out of the 12 extracts assayed had LC_{50} values more than the cut-off point i.e 100 μg/ml. Among these, 5 extracts had LC_{50} more than 200 μg/ml, while the remaining had LC_{50} ranging from100 – 200 μg/ml. Just five extracts exhibited LC_{50} value <100 μg/ml, and as a result classified as cytotoxic in nature.

The results of MTT Assay revealed that all the extracts assayed were non-toxic and showed CC_{50} >30μg/ml; above the cut-off point. Out of these, only 6 extracts displayed CC_{50} values less than 400 μg/ml, the highest concentration tested against breast cancer cells. Only one out 12 extracts was found to be moderate cytotoxic. The extracts were from *Zygophyllum fabago* crude methanolic (296.0282 μg/ml).

As a result of this study, it can be concluded that maximum extracts of the plants screened herein presented inhibitory activity in opposition to the enzyme in a dose-dependent mode and were not toxic. Hence, they could be investigated for additional studies in the management of AD. Since, majority of the AChE inhibitors are acknowledged to hold nitrogen, the extracts showing greater activities might have the reason that they are loaded with rich alkaloid content. (Orhana, et al., 2004).

5.1. *Argyrolobium roseum*

Argyrolobium roseum (Camber), was not found in any form to be reported for management of any disorder of mankind as well as Alzheimer's disease, though reported in several texts on flora of Pakistan and Indian Subcontinent's north-western Himalayan region (Nasir, 1977). We were the first to investigate this herb for anti-cholinesterase potential. To the best of our acquaintance, no exhaustive effort has been done on several polar and non-polar fractions of this whole plant. In vitro anti cholinesterase potency of aqueous extract and organic fractions of *A. roseum* are presented in this study for first time. As the rationale of this research was to establish natural products newer potential sources, bioassay guided screening was performed to identify anti cholinesterase and cytotoxic potential by conventionally used standards methods.

The methanolic extract (80%) of the *Argyrolobium roseum* was found to exhibit enzyme inhibition potential against Acetyl Cholinesterase enzyme. Crude extract, aqueous and ethyl

acetate extract showed moderate Inhibition activity. While the less polar fraction hexane, possessed the strongest activity. The increase of activity in this fraction is suggestive of the fact that the principal constituent, responsible for the activity in the crude extract were contained in the hexane fraction. This research study has verified that the anti-AChE activity of methanolic extract of A. roseum is primarily attributable to the fraction and the compounds extracted in hexane.

Hexane extract of *Argyrolobium roseum* was the most toxic possessing LC_{50}= 24.48 µg/ml, followed by extract of ethyl acetate (24.48µg/ml). The high toxicity of extracts of hexane and ethyl acetate on brine shrimps might be due to the consequences of saponins. Earlier studies exhibited that the hexane and ethyl acetate fractions of A.roseum have traces of saponins (Khanum, et al., 2013) and (Apers, et al., 2001) reported these compounds (Saponins) to have high hemolytic and molluscicidal activities.

The extract from A. roseum hexane and ethyl acetate fractions were ranked as highly cytotoxic on brine shrimp lethality assay but ranked as non-cytotoxic on HUH cells, whereas mild toxicity was observed in MCF-7 cells. These annotations propose that the two models which are used in this research complement each other for the discovery of cytotoxic compounds that might be accredited to different toxicity mechanisms; while more sensitive bioassay was regarded as to be brine shrimp assay sensitive in exploring extracts which are cytotoxic in nature as compared to carcinoma cells.

The differentiation may be explained to some extent by brine shrimp assay's non-specificity in identifying cytotoxic substances and the variances in the standards employed to define a toxic compound, though in a number of studies brine shrimp cytotoxicity assay has been stated to exhibit some association with cell line results for identifying cytotoxic extracts (Meyer, et al., 1982).

In brains of AD patients, oxidative damage exists therefore treatment with antioxidants is considered a promising approach for decreasing disease advancement. Latest research has observed a correlation among antioxidant ingestion and demoted frequency of dementia (Grundman & Delaney, 2002). In addition, some of the manmade medicines utilized e.g. rivastigmine, donepezil and tacrine have been described to result in gastrointestinal disorders and complications allied with bioavailability (Schulz, 2003). Refined work from (Marwar & Khan, 2009) shown that the plant *Argyrolobium roseum* contains flavonoids and glycosides,

which are well-known for their antiulcer, anti-inflammatory, antioxidant and hepatoprotective effects. There is a wide-ranging body of literature gathered in the current years signifying the contribution of d-pinitol and vitexin into liver function (Gupta, et al., 2011). Moreover, vitexin had been earlier revealed to portray as an antioxidant agent impeding nearly seventy percent superoxide radicals (Kim, et al., 2005) and d-pinitol had been stated to cause lipid lowering, reducing hepatic lipid droplets and cholesterol (Choi, et al., 2009). On the basis of these studies it can be assumed that *Argyrolobium roseum* is a potential candidate for Alzheimer's disease as it possesses both inhibitory activity of AChE and no cytotoxicity and is reported to have antioxidant activities and also has hepatoprotective capabilities which can minimize the side effects of already available drugs for AD. The hexane extract, presenting the maximum AChE inhibition, is the extremely potential extract to be further studied for detection of the phytochemical constituents.

5.2. *Zygophyllum fabago*

In Baluchistan, a province of Pakistan, a large number of therapeutic plants have historically been consumed to treat a wide range of ailments (Atta-ur-Rehman & Ahmad, 1986). In the rural areas, where health services are not adequate, people depend on locally available medicinal plants for curing various illnesses. *Zygophyllum fabago L.* (Zygophyllaceae) is indigenous medicinal plant of Baluchistan. The medicinal plants of Baluchistan were found to have biologically active compounds, which explain their use for the treatment of cancer, infectious diseases, injuries, wounds and boils, by locals and in folk medicine (Zaidi & Crow, 2012).

(Orhana, et al., 2004) have reported some anti-cholinesterase effect for *Zygophyllum fabago L.* However, to the best of our acquaintance, no exhaustive effort has been done on several polar and non-polar fractions of this whole plant. In vitro anti cholinesterase potency of aqueous extract and organic fractions of Z.fabago are presented in this study for first time. Hydrophilic fractions (aqueous extract) were devoid of any potential activity. Crude extract of *Zygophyllum fabago* showed the weak inhibition activity (IC_{50}= 213.18 µg/ml) in the assay, while a mild activity was observed in the aqueous extract (IC_{50}=130.66 µg/ml).

Presence of activity in the hydrophilic fractions indicated as polar compounds being active compounds for the Bioactivity. The enzyme inhibition activity in butanol fraction comparable to the crude extract was devoid of any activity (IC_{50}=234.36 µg/ml). Based on these

observations, the butanol fraction containing the polar compounds further fractionation based on hydrophilicity was performed for the butanol extract.

The sub-fraction of butanol eluted with water- methanol (1:1), and 100% methanol possessed the strongest activity i.e (IC_{50}=37.98 and 37.79 µg/ml). Butanol extract showed no cytotoxicity against both cell lines HUH and MCF-7 hence, very potent candidate to be formalized into AChE inhibition drug.

Previous studies of (Zaidi & Crow, 2012) showed the Z.fabago extract was greatly efficient against Escherichia coli and Candida albicans proposing toxic nature of this plant against fungal agents. Results of our study conducted for cytotoxicity testing showed that Z. fabago butanol extract was the most toxic with LC_{50}= 32.52 µg/ml, followed by the ethyl acetate extract (70.82 µg/ml) and aqueous extract (84.08 µg/ml).

Some work on the isolation of natural compounds and their biological activities has been done on other species of this plant family, but not on this specie. Just two triterpenoidal saponins were stated from Z. fabago aerial parts. (Attia, 1999). Hence, the high toxicity of butanol and ethyl acetate extract on brine shrimp might be due to the influence of saponins and (Apers, et al., 2001) stated these compounds (Saponins) to have high hemolytic and molluscicidal activities. Our study showed high toxicity in Z. fabago and same effects were also described by (Skim, et al., 1999).

The extract from Z. fabago aqueous, ethyl acetate and butanol fractions were ranked as the most toxic on brine shrimp assay but ranked as non-toxic on HUH cells test, whereas mild toxicity of methanolic crude and ethyl acetate extract was observed in MCF-7 cells. These annotations propose that the two models which are used in this research complement each other for the discovery of cytotoxic compounds that might be accredited to different toxicity mechanisms; while more sensitive bioassay was regarded as to be brine shrimp assay sensitive in exploring extracts which are cytotoxic in nature as compared to carcinoma cells. The differentiation may be explained to some extent by brine shrimp assay's non-specificity in identifying cytotoxic substances and the variances in the standards employed to define a toxic compound (Meyer, et al., 1982).

It was concluded that Ethyl acetate soluble fraction revealed decent AChE effect due to the existence of such bioactive substances while methanol soluble fraction exhibited mild activity and butanol sub-fractions showed strongest AChE effect, hence these fractions are considered

to be significant resources of bioactive compounds besides also reported as noble antioxidants. Alkaloids are also existing in substantial extents. This plant might be an important source of disease anticipation and control, because of existing antioxidant potential in its constituents. Two sub fractions of BuOH extract showed higher acetyl cholinesterase inhibition and no cytotoxicity on both cell lines tested hence this is considered as most potent for drug designing of Alzheimer's disease.

5.3. *Citrullus colocynthis*

Data of the work done by (Shahat1, et al., 2015) showed the moderate inhibitory effect of *Citrullus colocynthis* on acetyl cholinesterase. However, for this plant, no work has been reported for polar and non-polar fractions for AChE inhibitory activity. We were the first to report AChE inhibition potential of fractions and sub fractions of *Citrullus colocynthis*. Outcomes of our study disclosed that the crude extract of the *Citrullus colocynthis* also showed comparable strongest inhibition activity (IC_{50}= 151.1 µg/ml) in the assay. Various extracts namely EtOAc, butanol and water extracts obtained as a result of solvent-solvent extraction were screened in acetyl cholinesterase assay. The ethyl acetate extract showed most potent activity (IC_{50}= 45.97 µg/ml). Amongst the hydrophilic fractions butanol extract also showed strongest activity (IC_{50}= 34.84 µg/ml) to further investigate the presence of the activity in sub fractions, the Butanol fraction was partitioned to on DIAION HP-20 with increasing proportions of Methanol. The sub-fractionate eluted with Water-Methanol (50:50), possessed the strongest activity (IC_{50}= 53.67 µg/ml) while Water-Methanol (75:25) possessed the strongest activity (IC_{50}= 45.39 µg/ml). The increase of activity in these fractions as compared to the crude is suggestive of the fact that the principal constituent, responsible for the activity in the crude extract were contained in the ethyl acetate and butanol fraction.

The various extracts their fractions and sub-fractions investigated showed good inhibitory activity towards AChE using the spectrophotometric method. The results obtained from this study indicate that this type of activity is not only subject to plants containing alkaloids, but rather a diverse class of compounds may exhibit this kind of activity. The AChE inhibitory effect observed for extracts of this plant, may explain a possible mechanism in which the *Citrullus colocynthis* may exert an effect on the CNS. *Citrullus colocynthis* fruit's stated bioactive substances in the literature are flavonoids, carbohydrates, glycosides, alkaloids, essential oils & fatty acids (Hussain, et al., 2014). One of the major classes of compounds observed in herbal sources are flavonoids, with a wide variety of biological activities.

Flavonoids since known as polyphenolic substances have been acknowledged to have robust antioxidant effects, which is a leading point in the management of AD (Khan et al., 2009).

Even though *Citrullus colocynthis* fruit has extensive history as involved in medication, since there are rare reports on safety assessment and systematic toxicity. The study of the saponin by (Diwan, et al., 2000) isolated from *Citrullus colocynthis* whole plant on mice for histopathological effect and acute toxicity testing exhibited LD_{50} value of 200 mg/kg and according to the results reported by (Sharma, 1998) the assayed plant constituent (Saponin) is not toxic, when related with recent bioactive pharmaceutical's LD_{50} values employed in therapeutics. Results of our study correlates with this study as in both assays performed to evaluate cytotoxicity potential of C. colocynthis extracts exhibited no cytotoxicity against brine shrimps as well as in liver cancer and breast cancer cell lines. The EtOAc extract of *Citrullus colocynthis* exhibited very mild cytotoxic effect with CC_{50} value of 374.21 µg/ml on MCF-7 cell line. The butanol and aqueous extracts showed no activity on HUH Cell line as well as against MCF-7 Cell line.

In light of these findings, we conclude that *Citrullus colocynthis* ethyl acetate and butanol extract and its sub fractions possessed higher efficacy against Acetyl cholinesterase enzyme as compared to other extracts having a robust potential to be established into Alzheimer's medicine as they showed no cytotoxicity.

Conclusion

Pakistani flora has exhibited to be a rich resource for AChEIs, especially those which are also reported to be antioxidant in nature. The extracts of *Argyrolobium roseum, Zygophyllum fabago, Citrullus colocynthis* were proved to have a great potential as acetyl cholinesterase inhibitors.

The whole plants were extracted and screened for biological activities. Brine shrimp lethality assay revealed that hexane is most toxic extract of AR plant having an LC_{50} = 40.04 µg/ml and rest all were mostly non-toxic. MTT assay results shown AR-Hexane, ZF-EtOAc, CC-EtOAc fractions to be weakly cytotoxic against only MCF-7 cell line with CC_{50} values of 301.64, 354.33, 374.21 (µg/ml) respectively, hence possessing no cytotoxic effects and can be further processed for AD drug designing. In conclusion, the plant extracts exhibited presence of bioactive compounds with significant AChE inhibition supporting their use in the management of AD.

The draw-back of Alzheimer's disease management include side effects of alkaloids and their toxicity which are commonly utilized as potential sources for inhibition of acetylcholinesterase. In preliminary trials, C.colocynthis and Z.fabago's butanol extracts exhibited no toxicity at concentrations assayed in the present study .The outcomes of the study represented here provided verification to confirm the remedial potential of C.colocynthis and Z.fabago butanol fraction relying on their strongest activities of AChEI in correlation with least off-target effects as they showed no cytotoxicity.

In accordance with the results stated above, we can conclude that these plant extracts can be employed as sources of anti-cholinesterase agents which are easily accessible, with subsequent health benefits. Over all such plants provide promising sources for alternatives to current therapies for AD and further neurodegenerative disorders. In addition, it is concluded that these plants may be helpful for the management of diseases like dementia, Parkinson's disease, glaucoma and myasthenia gravis which are interconnected with the data on AChE inhibitors utilization as a promising therapeutic strategy.

Future Prospects

For cholinesterase inhibitory activity, a huge number of species of plants from various parts of the world have been investigated. There is still a necessity to explore the nature for newer effective and durable AChE inhibitors with minimum side effects.

This study suggests that the tested extracts of three plant species are rich in biologically active secondary metabolites, enlightening that comprehensive phytochemical studies on these plants are advisable. Thus, additional studies are robustly suggested to disclose the bioactive phytochemicals and fundamental mechanism accountable for the observed activities in this research.

Further works, to identify bioactive phytochemicals detailed mechanism which contributes to the biological properties and by employing bioassay-directed fractionation, the separation of the active constituents is strongly recommended.

Though, additional in vivo and clinical studies ought to be carried out for recognition and classification of active phytochemicals which are accountable for these biological properties. Hence, on the basis of this research study we have to say that the assayed plants of these three families might be employed against several diseases and neurology disorders related with ROS generated stress or free radicals, inhibition of acetyl cholinesterase and cell cytotoxic studies.

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