# STUDIES ON THE LEAVES OF *Punica granatum* LINN. AND ITS INVITRO INHIBITION OF AChE FOR POTENTIAL USE IN ALZHEIMER'S TREATMENT

***Dissertation submitted to***

***The Tamilnadu Dr. M.G.R. Medical University, Chennai***

*In partial fulfillment of the requirement for the award of Degree of*

## MASTER OF PHARMACY

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# CERTIFICATE

This is to certify that the dissertation entitled **"Studies on the leaves of *Punica granatum*, Linn and its invitro inhibition of AChE for Potential use in Alzheimer's treatment"** was done by **Mr. N. Ramasamy,** in Department of Pharmacognosy, Madurai Medical College, Madurai – 20, in partial fulfillment of the requirement for the Degree of Master of Pharmacy in Pharmacognosy. This dissertation is forwarded to the Controller of Examination, The Tamilnadu Dr. M.G.R. Medical University, Chennai.

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Date :

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## “I Owe to the helping hands of the almighty”

Indeed my project is a small work done with the help of primitive persons at heart. So, it is my bounded duty to promulgate them individually.

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Last but not least, thanks are to God for my life through all tests in the past two years. Lord, You have made my life more beautiful. May your name be exalted, honoured and glorified.

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# INTRODUCTION

Man's existence on this earth has been made possible only because of the vital role played by the plant kingdom in sustaining his life. Without the variety of living organism that makes up the world of plants, animal life would not survive and our planet would have been a barren and life less world of deserts[1].

Plants are the only economic source of a number of well established and important drugs. In addition they are the source of some chemical intermediates needed for the production of a number of drugs[2].

**Medicinal plants global view**

The universal role of plants in the treatment of disease is exemplified by their employment in all the major systems of medicine. There is a great wealth of knowledge concerning the medicinal, narcotic and other properties of plants that is still transmitted from generation to generation to tribal societies. The knowledge of tribal (folk) medicines are enormous but has been lying dormant due to various reasons and is worthy exploration[3].

By studying the folk medicine not only the Materia Medica of different systems will be enriched, but the economic status of the country will also become sound. As the medicinal plants are considered the natural wealth of the country, for internal consumption as well as for earning valuable foreign exchange[4].

**WHO views on Medicinal Plants**

The world health organization has estimated that nearly 80% of the population of the developing countries meet their primary health care needs through plant based traditional medicine. Towards the end of the 20th century there began a revival of interest in traditional medicine not only in developing countries, but also in the developed countries. The revival of interest in herbal drugs started mainly because of the widespread belief that green medicine is healthier than synthetic products. Now the increasing popularity of plant – based drugs are felt all over the world leading to fast growing market for herbal drugs, pharmaceuticals, nutraceuticals, functional foods and even cosmetics[5].

**Status of India**

India has an ancient heritage of traditional medicine, perhaps largest producer of medicinal herbs and is rightly called **"THE BOTANICAL GARDEN OF THE WORLD".** Medicinal herbs have been in use for thousand of years, in one form to another under the indigenous system of medicine[6].

India was one of the pioneers in the development and practice of well documented indigenous systems of medicine, particularly Ayurvedha, Siddha and Unani. The Indian population has depend on mostly plant based crude drugs for the treatment of various ailments. Medicinal plants

sector has traditionally occupied an important position in the socio-cultural, spiritual and medicine arena of rural and tribal lives of Tamil Nadu.

**Contribution by Tamil Nadu**

Siddha system is one of the ancient traditional systems of medicine in India. Siddhars, the spiritual scientists of Tamil Nadu are the founders of the scientific system. The basic concept of siddha of medicine is

**"Food is medicine; Medicine is food"**.

Saint Thiruvalluvar says,

***"Miginum kuraiyinum noiseyyum noolar***

***Vali mudhala enniya moondru".***

"Wind, bile, phlegm three causes diseases

So doctors deem it more or less"

And regarding diagnosis,

***"Noi naadi noimudhal naadi athuthanikum***

***Vaai naadi vayppa seyal"***

"Test disease, it's causes and cure

And apply remedy that is sure"[7].

**Modern Medicine – Demerits**

Inspite of tremendous advances made in the modern system of medicine there are still a large number of conditions for which suitable drugs are not available in the modern system of medicine and thus, we

should denote, time, efforts, and found in those areas where new drugs are urgently needed and such areas include tropical diseases, rheumatic diseases, viral infection, liver disease, bronchial asthma, diabetes, immunomodulators as adjuvant for chemotherapy and adaptogens[8].

**Natural products for modern medicine**

Plants have been used for medicine from the time immemorial because they have fitted the immediate personal need, are easily accessible and inexpensive. Researchers have no doubt that nature is still the preeminent synthetic chemist and that in plants particularly, there are almost infinite reserves of fascinating chemical constituents with actual and potential effects on the human body.

Plant products can also be useful as starting material for the semi synthetic preparation of other drugs. The main examples are plant steroids hormones. Natural products continue to form a significant proportion of drugs in current use and of those under investigation. It has been estimated that 56% of the lead compounds for medicinal in the British National Formulary are natural products or are derived from natural products.

Natural products will continue to be important in three areas of drug discovery.

1. As targets for production by biotechnology.
2. As a source of new lead compounds of novel chemical structure.
3. As the active ingredients of useful treatments derived from traditional systems of medicine[9].

**Priorities in Medicinal plant research**

The plant kingdom represents as enormous reservoir of biologically active molecules and so far only a small fraction of plants with medicinal activity has been assayed. Nearly 50% drugs used in medicine are of plant origin.

There is therefore much current research devoted to the phytochemical investigation of higher plants which have ethnobotanical information associated with them.

The phytochemical isolated are then screened for different type of biological activity.

The crude plants extracts can be first assayed for particular activities and the active fraction then assayed phytochemically[10].

**Phytonutrients – The Natural Drugs of the Future**

**Degenerative Diseases**

Many advances in the diagnosing of diseases and the discovery of a myriad of new names to diseases has occurred, the battle to increase

quality of life and the overcoming, curing and proper treatment of **degenerative diseases** has been all but lost. Allopathic medicine has not changed its treatment of cancer in over 40 years. Though new drugs keep popping up, the results are still the same and the most effective treatment for cancer is still surgery. Heart disease, diabetes, arthritis, leukemia, **Alzheimer's,** Parkinson's, Hodgkin's and dozens of other disease names strike fear into our minds and as we age, many of us contemplate... ". Which one will take me from this life and how will I fight it?" Today with the current standard of allopathic medicine it is only a matter of time before you are beaten by one of the many known or even unknown degenerative diseases. There are no known cures to any of these degenerative diseases.

Currently your only option is a long list of prescription drugs that may alleviate symptoms but slowly eat away your body's immunity and quality of life.

There is hope however. For more than 20 years now scientists have been researching plants and discoveries made within the last 15 years may hold some of the answers to combating many of the degenerative diseases[11,12].

**Alzheimer's disease**

Alzheimer's disease (AD), also called Alzheimer disease or simply Alzheimer's, is the most common type of dementia. Alzheimer's is a degenerative and terminal disease for which there is no known cure. In its

most common form, it afflicts individuals over 65 years old, although a less prevalent early onset form also exists. It is estimated that 26.6 million people worldwide were afflicted by AD in 2006, which could quadruple by 2050, although estimate very greatly. The disease was first described by Alois Alzheimer.

**Symptoms of AD**

In the early stages, the most commonly recognized symptom is memory loss, such as the difficulty to remember recently learned facts. Earlier occurring symptoms are often mistaken as being noncritical age – related complaints, or forms of stress. As the disease advances symptoms include confusion, anger, mood swings, language breakdown, long – term memory loss, and the general withdrawal of the sufferer as his or her senses decline. Gradually, minor and major bodily functions are lost, leading ultimately to death.

The cause and progression of Alzheimer's disease are not well understood.

**Characteristics of AD**

The disease course is divided into four stages, with a progressive pattern of cognitive and functional impairment expressed from one stage to the next.

1. Pre dementia
2. Early dementia

3. Moderate dementia
4. Advanced

Three major competing hypothesis exist to explain the cause of the disease are

1. Cholinergic hypothesis (Selective neuronal loss)
2. Amyloid hypothesis (Senile plaques)
3. Tau protein hypothesis (Neurofibrillary tangles)

**Current Treatment**

Four medications, to treat the cognitive manifestations of AD, are currently approved by regulatory agencies, including the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMEA). Three are acetylcholinesterase inhibitors and the other is memantine, an NMDA receptor antagonist. No drug has an indication for delaying or halting the progression of the disease.

The recent development of new AChE inhibitors as drugs capable of reducing the symptoms of AD. Examples – tacrine, donepezil, galanthamine, rivastigmine through inhibition of the enzyme acetylcholinestetase, responsible for breakdown of acetyl choline in the neural synapse[13].

Although very few drugs are currently approved by regulatory authorities for treating multi – factorial ailments and disorders of cognition such as Alzheimer's disease, certain plant – derived agents including, for

example, galantamine (from Galanthus nivalis L.) and rivastigmine (a semi – synthetic derivative of physostigmine) are finding an application in modern medicine. However, in Ayurveda, the Indian traditional system of medicine, which is more than 5000 years old, selected plants have long been classified as ‘medhya rasayanas’ from the Sanskrit words “medhya”, meaning intellect or cognition and rasayana”, meaning “rejuvenation”. These plants are used both in herbal and conventional medicine and offer benefits that pharmaceutical drugs lack[14].

However, these drugs are known to have limitations for clinical use due to their short half lives and /or unfavorable side effects (elevated transaminase, nausea, vomiting, diarrhoea, dyspepsia and etc)[15].

So, it is necessary to search for AChE inhibitors with lower side effects from natural resources.

**List of medicinal plants having AChE inhibition**

| Plant Name | | Phytoconstituent |
|---|---|---|
| *Galanthus nivalis* | - | Galanthamine[14] |
| *Corydalis ternata* | - | Protopine[16] |
| *Origanum majorana* | - | Ursolic acid[17] |
| *Salvia (sage) species*<br>*S. Fruticosa,*<br>*S. lavandulafolia,*<br>*S. Officinalis*<br>*Salria santolifolia* | - | Essential oil[18] |
| *Melissa officinalis* | - | Essential oil[18] |
| *Rosemarinus officinalis* | - | Essential oil[18] |

**Ursolic acid and AChE inhibition**

Ursolic acid, also known as urson, prunol, micromerol, and malol, is a pentacyclic triterpenoid compound which naturally occurs in a large number of vegetarian foods, medicinal herbs, and other plants.

Ursolic acid can be used for several pharmacological effects, such as, anti-tumor (skin cancer), hepato protective, anti inflammatory (oral and topical), anti ulcer, anti microbial, anti hyperlipidemic and anti viral. Ursolic acid and its isomer, oleanolic acid both are similar in pharmacological activity.

Ursolic acid inhibits acetylcholinesterase. This the enzyme catalyses the breakdown of the neurotransmitter chemical acetylcholine, which is important for effective cognition and reasoning. The degeneration of acetylcholine – producing neurons in the brains of people with Alzheimer's disease (AD) has been blamed for the symptom of memory loss associated with AD. Thus, sustaining acetylcholine levels may help delay this memory loss[19].

**Reason for selection of plant *P. granatum***

In order to find a natural source which has the ursolic acid its one of the phytoconstitutents, preliminary literature survey revealed that the leaves of ***Punica granatum*** contains ursolic acid, one of its constituents.

So we planned to investigate the widely available leaves of *P.granatum* belongs to the family punicaceae for its AChE inhibitory activity.

# REVIEW OF LITERATURE

## ENTIRE PLANT

### Ethnomedical Information

Siang S.T (1983) stated that dried parts of the plant is used for burns in traditional Chinese and Western Medicine as local application[20].

Jain S.P et al (1984) reported that fresh paste of the plant was used for snakebite as local application[21].

### Pharmacognosy

Anonymous (2005) geographical distribution, varieties, cultivation, including soil, propagation, manuring etc have been described in detail[22].

Shastri V.M et al (1996) determined ashes and mineral content quantitatively by gravimetric and colorimetric methods[23].

### Pharmacology

Prakash A.O et al (1986) reported that acetone, methanol and hydro alcoholic extracts showed no embryotoxic effect (200 mg/kg) in pregnant rat by gastric intubation method[24].

### Microbiology

Anesini C et al (1993) reported that hot aqueous extract showed antimicrobial activity (62.5 mg/ml) against *E.coli* and *A. niger*[25].

## AERIAL PARTS

### Pharmacological Activity

Dhawan B.N et al (1977) reported that hydro alcoholic extract parts of the plant was used as diuretic (0.063 mg/kg), hypothermic (0.125 mg/kg) in rat[26].

### Microbiology

Aynehchi Y et al (1982) reported the antibacterial activity of the ethanolic extact (100 mg/ml) against *P. valgaris, S. paratyphi, B. anthracis* by agar plate method[27].

## FLOWERS

### Ethnomedical Information

Bellakhar J et al (1991) reported that flowers of the plant was used for diarrhoea in Moroccan pharmacopoeia[28].

Jafri M.A et al (2000) reported that powdered flower buds was used for bronchitis and diabetes[29].

Li Y. et al (2005) reported that flowering part was used for diabetes in Unani system of medicine[30].

Wang R et al (2006) stated that flower was used locally for grey hair of young man in traditional Chinese medicine and also as an astringent, haemostatic[31].

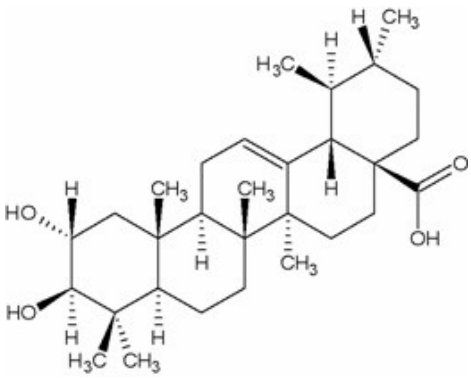
Bagri P et al (2008) stated that decoction was used as mouthwash in oral and throat inflammation[32].

Celik I et al (2008) reported that flower is consumed worldwide as beverage[33].

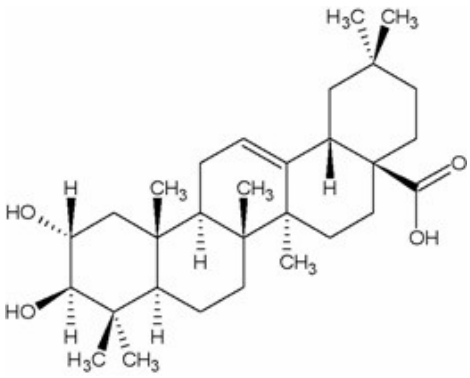
**Phytochemistry**

Batta A.K et al (1973) isolated crystalline chemical components like maslinic acid, asiatic acid [34].

**ASIATIC ACID**



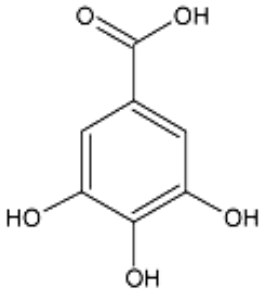
**MASLINIC ACID**



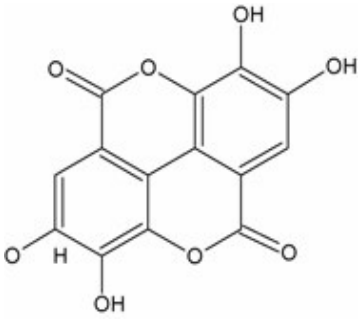
Kirillova W et al (1988) reported the presence of cyanidine, delphinidine[35].

Huang T.H et al (2005) identified the presence of **Ursolic acid,** oleanolic acid, gallic acid[36,37].

**GALLIC ACID**



**ELLAGIC ACID**



Anonymous (2005) mentioned that the presence of pelargonidine 3,5-di glucoside (a light red dye) used for dying cloth[22].

Wang R et al 2006 reported a new compound pomegranate and six known compounds namely ellagic acid, 3, $3^1$, $4^1$ – tri – O – methyl ellagic acid, ethyl brerifolin carboxylate, **Ursolic acid,** masilinic acid and daucosterol[31].

Jurenka M.T.J et al (2008) reported the presence of **Ursolic acid,** maslinic acid, styptic acid, gallic acid[38].

**Pharmacology**

Naqvi A.H et al (1992) stated that the aqueous extract showed antiamoebic activity (25 mg / 10 gm, oral)[39].

Jafri M.A et al (2000) stated that hydro alcoholic extract showed antidiabetic activity (400 mg/kg, oral) in rats[29].

Li Y. et al (2005) reported that methanolic extract was active in postprandial hyperglycemia ($IC_{50}$ 1.8 mg/ml, oral) in rats[30].

Wang R et al (2006) reported that conjugate diene inhibition of LDL (0.5 mg/ml, oral)[31].

Kaur G et al (2006) stated that ethanolic extract of showed antioxidant and hepatoprotective effect (50 – 150 mg/kg, oral) in mice[40].

Bagri P et al (2008) reported that aqueous extract showed antidiabetic activity, regulation of abnormalities in lipid profiles, oxidative stress (250, 500 mg/kg)[32].

Celik I et al (2008) reported that flower beverage showed significant improvement in degenerative liver disorder[33].

## FRUIT

### Ethnomedical Information

John D et al (1984) stated that tender fruit paste in boiled milk was used in dysentery[41].

Goh S.H et al (1984) stated that dried fruit was used in child birth disorders in Malaysia[42].

Arseculeratne S.N et al (1985) stated that fresh fruit aqueous extract was used in dysentery and as a cooling agent[43].

Singh Y.N et al (1986) reported that fresh fruit was used in cold and running nose[44].

Caceres A et al (1987) stated that dried fruit aqueous extract was used locally in conjunctivitis, wounds, ulcers, sores, bruises, mouth lesions, stomatitis, leucorrhea, vaginitis[45].

Nagaraju N et al (1990) stated that immature fruit powder was used in peptic ulcers[46].

Anonymous (1992) stated that whole fruit powder was used in diarrhoea and as diet[47].

Ghazanfar S.A et al (1993) stated that fruit was used in anthelmintic and diarrhoea[48].

Desta B et al (1994) stated that shade dried fruit hydro alcoholic extract was used in fertility control[49].

Fujita T et al (1995) reported that fruit paste was used locally in hemorrhoids[50].

Navarro F.M et al (1996) stated that shade dried fruit hulls infusion was used in infectious diseases[51].

Vazquer F.M et al (1997) stated that dried fruit was used as an astringent, diuretic, digestive[52].

Lamar A.Z et al (2008) reported that fruit was used in acidosis, dysentery, diarrhoea, helminthiasis, microbial infections, hemorrhage, respiratory pathologies in Cuban traditional Medicine[53].

**Pharmacological Activity**

Prakash A.O et al (1976) reported that ethanolic extract showed no abortifacient and embryotoxic effect (200 mg/kg, oral)[54].

Goh S.H et al (1984) reported uterine stimulant activity (0.3 ml) but not hypotensive effect (0.1 ml)[42].

Kamboj V.P et al (1988) reported anti implantation effect[55].

Igea J.M et al (1991) stated allergenic effect[56].

Desta B (1994) reported that ethanolic extract showed anti implantation effect (1.82 gm/kg, i.g)[49].

Alkofahi A et al (1999) stated that ethanolic extract showed antiulcer activity (400 mg/kg, i.g) in rats[57].

Jung S.H et al (2003) reported that methanolic extract showed aldose reductase inhibition effect ($IC_{50}$, 0.27 mg/ml)[58].

Vidal A et al (2003) reported that hydroalcoholic extract showed antiviral activity (0.4 – 1.2 mg/kg, intranasal)[59].

Okamotto J.M et al (2004) reported antidepressant activity in mice[60].

Sudeesh S et al (2005) reported that the flavonoid fraction, acetone extract showed antioxidant activity (10 mg/kg, i.g)[61].

Vinutha B et al (2007) stated that methanolic extract showed no acetylcholinesterase inhibitory (AChE) activity[62].

Patel C et al (2008) reported that aqueous extract showed no acute and subchronic toxicity (i.p or oral)[63].

Lamar A.S et al (2008) stated that hydroalcoholic extract showed no genotoxicity[53].

**Microbiological Review**

Konowalchuk J et al (1976) reported that aqueous extract showed antiviral activity[64].

Caceres A et al (1987) reported that ethanolic extract showed no antiyeast and antibacterial activity (30 ml/disc)[45].

Desta B (1994) reported that acid ethanolic extract showed antiyeast and antibacterial activity (0.2 ml/disc)[49].

Menezer S et al (2006) reported that hydroalcoholic extract showed antiyeast activity[65].

Jurenka M.T.J et al (2008) reported that hydroalcoholic extract showed antibacterial (dental plaque) activity[38].

## FRUIT JUICE

### Ethnomedical Information

Singh Y.N (1986) stated that fresh fruit juice was used in diarrhoea and jaundice[44].

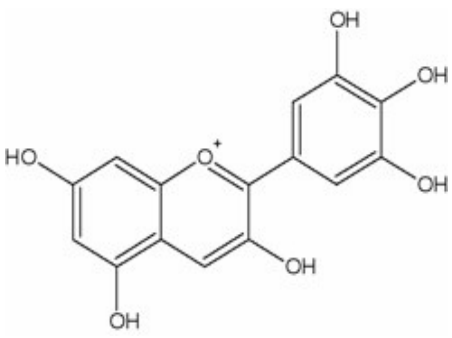
Singh V.K et al (1994) stated that fresh fruit juice was used in high fever, with loss of senses[66].

Khan M.A et al (2006) reported that fresh fruit juice used in anthelminitic, anaemia, diarrhoea, dyspepsia, tonic[67].

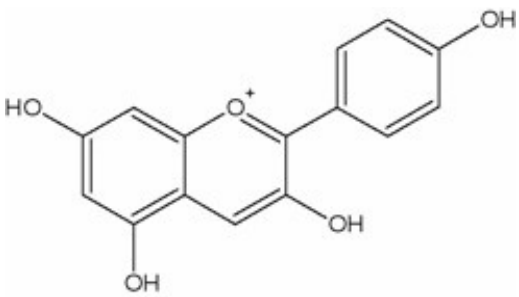
### Phytochemistry

Mavlyanov S.M et al (1997) reported the presence of delphinidine, pelargonidine[68].

**DELPHINIDINE**

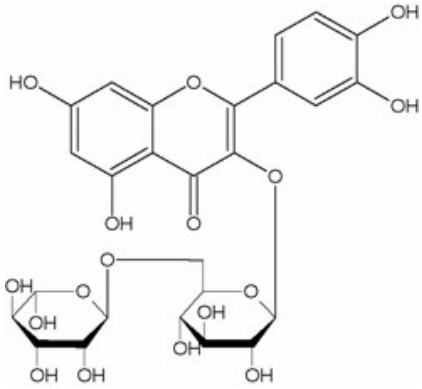



**PELARGONODINE**



Artik N et al (1998) determined the presence of caffeic acid, chlorogenic acid, p – coumaric acid, quinic acid, quercetin, rutin by HPLC[69].

**RUTIN**



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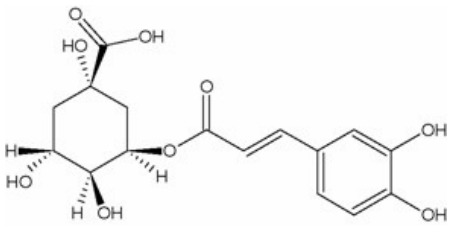

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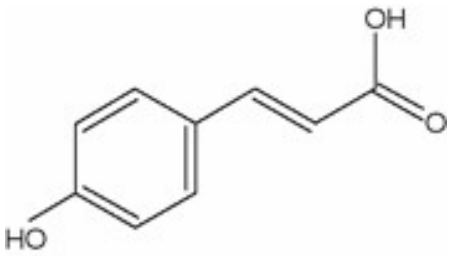

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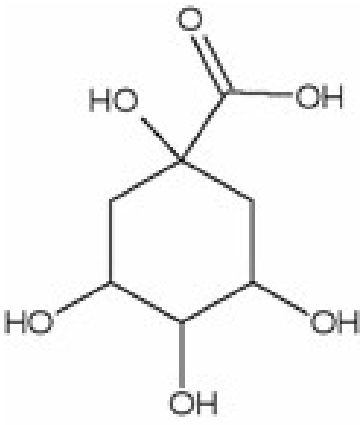
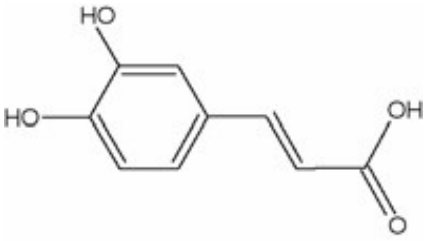
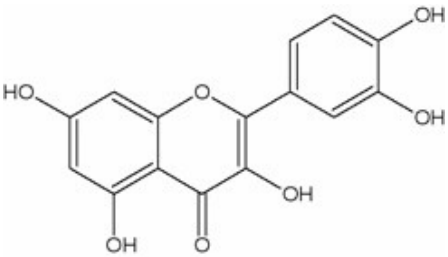


**CAFFEIC ACID**

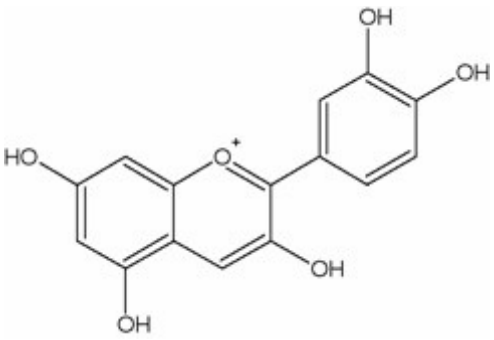


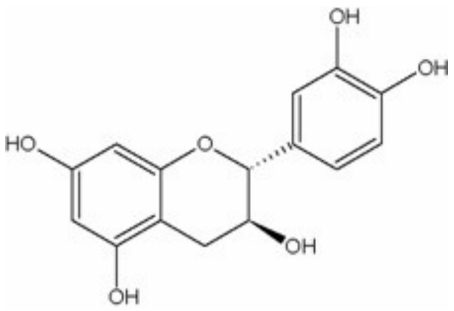
**QUERCETIN**



Hernadez F et al (1999) stated the presence of delphinidine, pelargonidine, cyanidine[70].

**CYANIDINE** **CATECHIN**




Gil M.I et al (2000) stated the presence of punicalagin[71].

Teresa S.D.P et al (2000) reported catechin, epicatechin, epigallocatechin 3 – gallate[72].

Poyrazoglku E et al (2002)[73], Lansky E.P et al (2007)[74], Jurenka M.T.J (2008)[38], were reported aliphatic organic acids (citric acid) phenolic compounds (chlorogenic acid) flavonols (quercetin, catechin).

**Pharmacology**

Gil M.I et al (2000) reported antioxidant activity[71].

Aviram M et al (2000) stated antioxidant, antiatherogenic, and platlet aggregation inhibition activity[75].

Kalpan M et al (2000) reported antioxidant, antiatherogenic, and cholesterol inhibition activity in mice[76].

Noda Y et al (2002) stated that the ethanolic extract showed superoxide radical scavenging activity[77].

Kim N.D et al (2002) reported chemopreventive and adjuvant therapy in human breast cancer[78].

Toi M et al (2003) stated antiangiogenic activity[79].

Kawaii S et al (2004) reported differentiation promotion effect[80].

Van Elswijk D.A. et al (2004) reported that crude fermented and unfermented juice showed antiproliferative activity[81].

Herber D et al (2007) stated antioxidant activity, dietary supplement[82].

Jurenka M.T.J et al (2008) reported for the treatment of hyperlipidemia, hypertension, prostate cancer, diabetes, atherosclerosis, carotid artery stenosis, **Alzheimer's disease**[38]**.**

**FRUIT (PEEL, RIND, PERICARP)**

**Ethromedical Information**

Latorre D.L et al (1977) stated that aqueous extract used in menstrual disorder[83].

Singh Y.N et al (1986) reported that aqueous extract used in diabetes[44].

Mascolo N et al (1987) reported that aqueous extract showed anti inflammatory activity[84].

Bellakhdar J et al (1991) stated that pericarp used as an antiulcer, hypoglycemiant, G.I disorder and also as vaginal antiseptic as local application[28].

Heinrich M et al, reported its use in G.I disorders[85].

Pillai N.R. et al (1992) was reported antidiarrhoeal activity[86].

Defeo V et al (1992) stated that decoction used as an astringent, anthelmintic, febrifuge[87].

Bhattarai N.K et al (1993) stated that decoction used in diarrhoea, dysentery[88].

Perez C et al (1994) stated that decoction used in urinary and respiratory tract infections[89].

Alkofalhi A et al (1996), Ahmed I et al (2001) were reported that infusion used in diarrohea, dysentery[90].

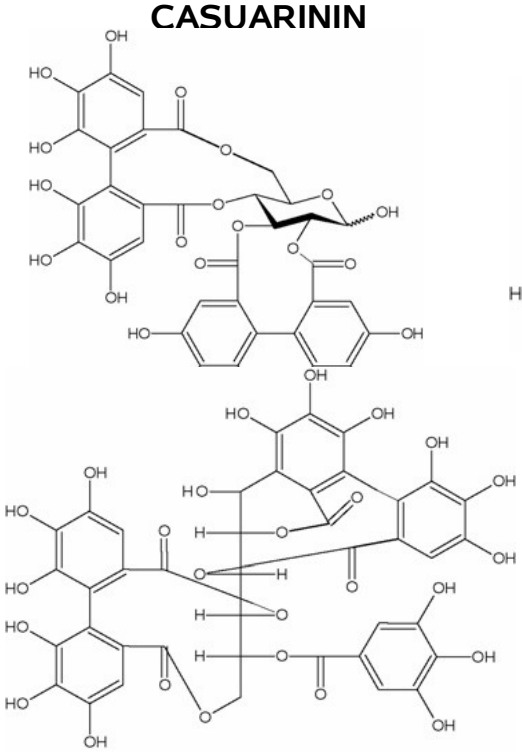
Sudeesh S et al (2003) reported that pericarp used for diarrhoea, dysentery, stomachic, colitis, piles, uterine disorders, cardiotonic, astringent[61].

**Phytochemistry**

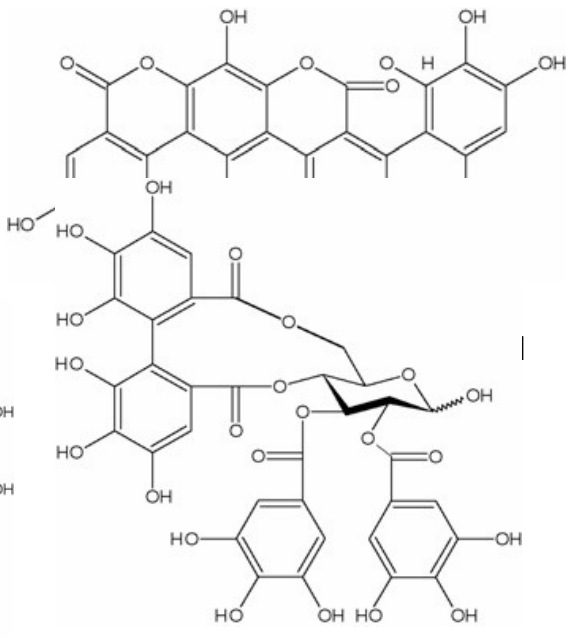
Tanaka T et al (1990) determined the structure of granatin – A, B[91].

Satomi H et al (1993) reported the presence of casuarinin, gallaglydilactone, pedunculagin, tellimagrandin[92].

**CASUARININ**



**GALLAGLYDILACTONE**



Poyrazoglku E et al (2002) stated the presence of gallic acid[73].

Noda Y et al (2002) reported the presence of delphinidine, pelargonidine, cyanidine[77].

Van Elswijk D.A. et al (2004) reported the presence of luteolin, quercetin, kaempferol[81].

**Pharmacology**

Dhawan B.N et al (1958) reported that aqueous extract showed uterine stimulant activity in rats[93].

Gujral M.L et al (1960) stated antifertility activity[94].

Kim N.D et al (1974) stated that methanolic extract showed anthelmintic effect (12 mg/kg)[95].

Mascolo N et al (1987) reported that ethanolic extract showed weak antiinflammatory activity (100 mg/kg)[84].

Sato A et al (1990) stated that hot aqueous extract showed antitumor effect (120 mg/kg)[96].

Pillai N.R et al (1992) reported hydroalcoholic extract showed antidiarrhoeal activity (500 mg/kg, i.g)[86].

Hukkeri V.I et al (1993) stated that aqueous extract showed anthelmintic activity[97].

Khennouf S et al (1999) reported antiulcer activity in rats[98].

Roos R.G et al (2001) stated that powder of the plant showed immune stimulant effect (100 mg/kg, i.g) in rabbits[99].

Noda Y et al (2002) stated that ethanolic extract showed antioxidant activity ($IC_{50}$ 10 mg/ml)[77].

Murthy K.N.C et al (2002) reported that methanolic extract showed hepatoprotective activity (50 mg/kg)[100].

Aslant M et al (2003) stated antidiabetic activity (500 mg/kg) in rats[101].

Bafna P et al (2003) reported that methanolic extract showed antimotility, antiulcer, anti diarrhoeal activity in animals[102].

Ajaykumar K.B et al (2005) stated that methanolic extract showed gastro protective effect (500 mg/kg)[103].

Qnais P et al (2007) reported that aqueous extract showed antidiarrhoeal activity[104].

Sestili, P et al (2007) stated cytoprotective effect[105].

**Microbiological Review**

Naovi S.A.H et al (1991, 1992) reported that aqueous and ethanolic extract showed anti bacterial activity (10 mg/ml) and antiamoebic activity (10mg/10gm)[106].

Miranda D et al (1993) stated that petroleum ether extract showed antibacterial activity[107].

Perez C et al (1994) reported that hot aqueous extract showed antibacterial activity[89].

Goto W et al (1996) stated that aqueous extract showed antiviral activity (1mg /ml)[108].

Prasanth D et al (2001), Ahmed I et al (2001) were reported equivocal effect in antibacterial activity[109].

Ahmed I et al (2003) stated that acetone or methanol extract was showed anti – MRSA activity[110].

**Leaves**

**Ethnomedical information**

Arnason T et al (1980) stated that hot aqueous extract used in women's disorder as local application[111].

Ilham M et al (1995) reported that infusion used as tonic[112].

Singh V.K et al (1996) stated leaf extract used in menstruration disorders[66].

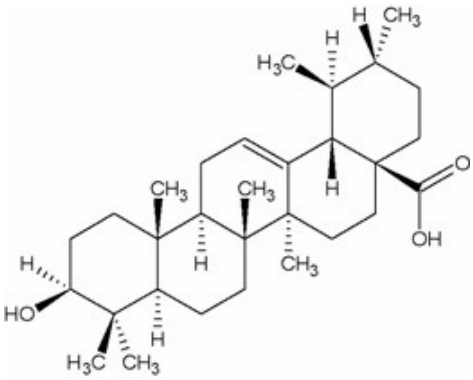
Nadkarni K.M et al (1954)[113], Jafri M.A et al (2000)[29], Ross R.G et al (2001)[99] were reported that paste used in conjunctivitis as local application.

Nadkarni K.M et al (1954) stated that juice used as an astringent, styptic[113].

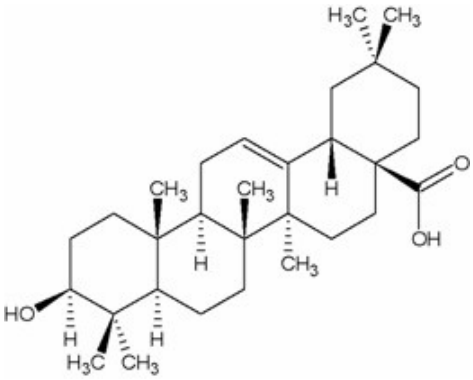
**Phytochemistry**

Elgamal M.H.A et al (1965) reported the presence of **ursolic acid**, oleanolic acid, betulic acid[114].

**URSOLIC ACID**



**OLEANOLIC ACID**



Fayez M.B.E et al (1967) reported the presence of **ursolic acid,** betulic acid, β-sitosterol[115].

Robert M.F et al (1967) stated the presence of unstable alkaloid [2-(2-propenyl)-Δ' – Piperideine][116].

Gawienoski A.Metal (1969) reported the presence of pregnenolone[117].

Pavanasasivam G et al (1974) reported the presence of betulinic acid[118].

Tawfik N.I et al (1978) stated the presence of alkaloids, carotene, coumarins[119].

Tanaka T et al (1985) reported the presence of corilagin, granaitin – A, B, strictinin, punicafolin[120].

Kirillova V.V et al (1988) stated the presence of delphinidine[35].

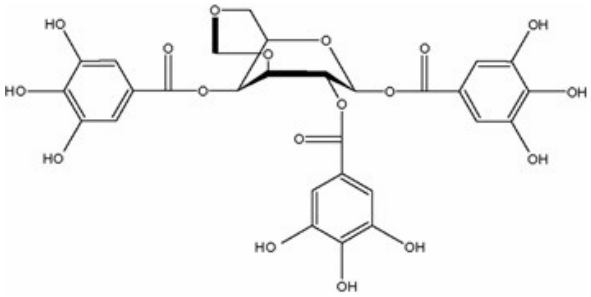
Nawwar M.A.M et al (1994) reported the presence of brevifolin, punicafolin, granaitin-B, xanthoxylin, ellagic acid[121].

Nawwar M.A.M et al (1994) reported the NMR structure analysis of apigenin, luteolin, piperidine alkaloids[122].

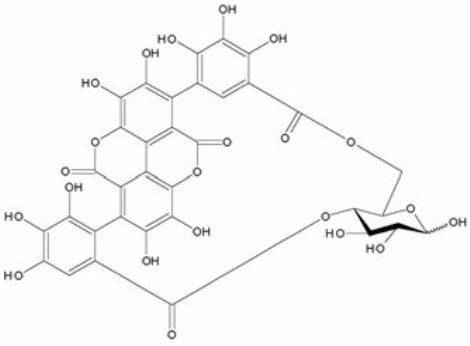
Anonymous (2005) mentioned reported the presence of **ursolic acid,** and betulic acid[22].

Lansky E.P. et al (2007) mentioned the presence of apigenin, brevifolin carboxylic acid 10-mono phosphate sulphate, apigenin – 4'-O-β - glucopyranoside, luteolin – 4'-O-β-glucopyranoside, luteolin – 3'-O-β - glucopyranoside, luteolin – 3'-O-β - xylopyranoside, puniclain, punicalagin, corilagin, punicafolin, piperidine alkaloids (N-(2',5' – Dihydroxy phenyl pyridium chloride)[74].

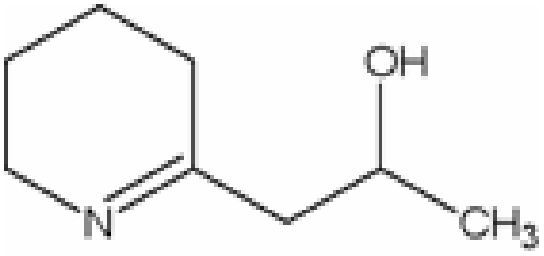
**PUNICAFOLIN**



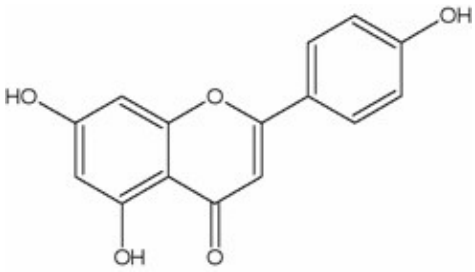
**PUNICALIN**



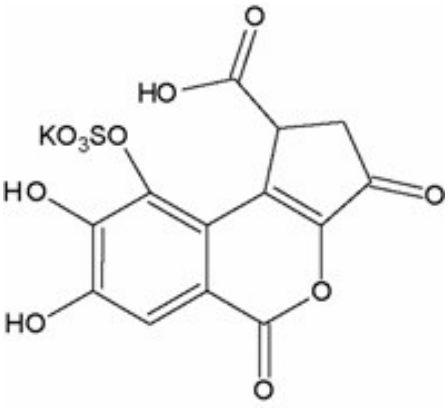
**PIPERIDINE ALKALOID**



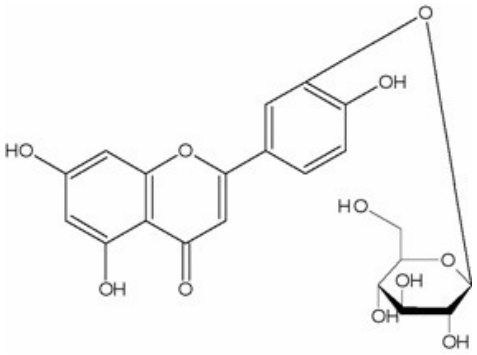

**APIGENIN**




**BREVIFOLIN CARBOXYLIC ACID**




**LUTEOLIN 3- PYRANOSIDE**




## Pharmacological

Lei F.X.N et al (2007) reported antiobesity action[123].

## Microbiology

Collier W.A. et al (1949) stated that the saline extract showed antibacterial activity in agar plate method[124].

Misas C.A.J et al (1979) stated that aqueous, ethanol, acetone extracts showed antibacterial activity[125].

Ayudhaya T.D et al (1987) stated that methanolic extract was inactive against *P.falciparum*[126].

Ilham M et al (1995) reported that ether extract was inactive in tumor promotion effect in cell culture method in mouse[112].

Alkofahi A et al (1997) reported that ethanolic extract of shade dried leaf and stem showed antibacterial, anti yeast activity in agar plate method[127].

**Seed**

John D (1984) reported that mature seeds used in hemorrhoids[41].

Okamoto J.M et al (2004) stated that seed oil used as an antioxidant[60].

Anonymous (2005) mentioned that seed oil used in stomachic[22].

**Phytochemistry**

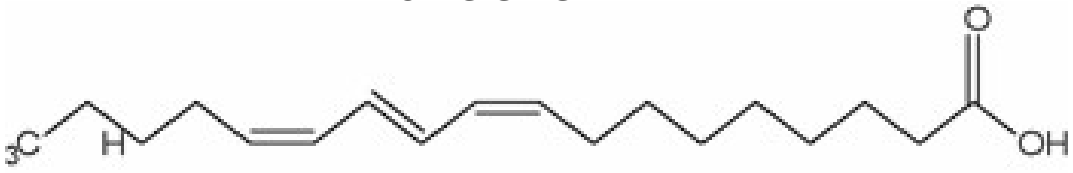
Heftmann E et al (1966) identified the presence of esterone[128].

Ahmed R et al (1995) isolated and identified the presence of **Ursolic acid**[129].

Schubert S.Y et al (1999) reported punicic acid, linoleic acid, stearic acid, palmitic acid and Oleic acid[130].

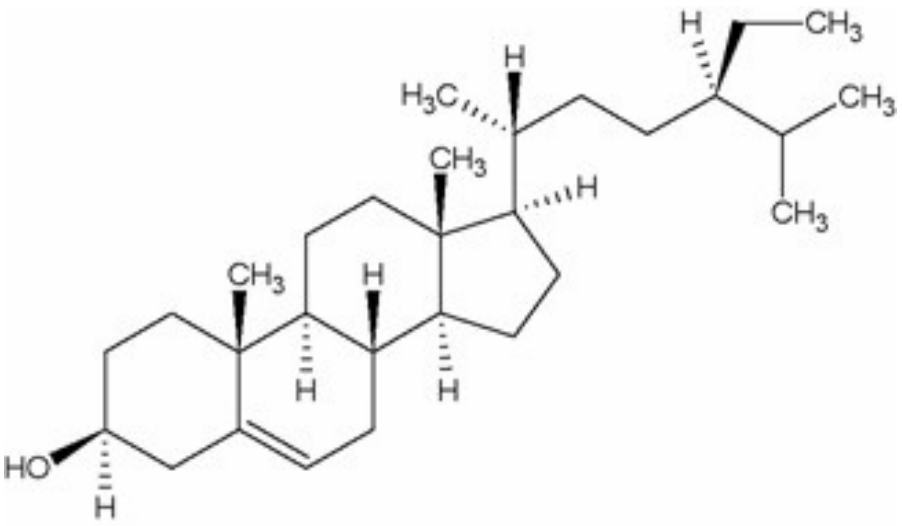
Kim N.D et al (2002) reported the presence of 17 – α – estradiol, γ – tocopherol[78].

**PUNICIC ACID**



Lansky EP et al (2007) mentioned that the presence β-Sitosterol testosterone, 17α estradiol, tocopherol, cereboside[74].

**β - SITOSTEROL**




**Pharmacology**

Dean P.D.G. et. Al (1971) stated uterine relaxation, esterogenic effect (0.2 ml, 0.4 ml)[131].

Lee J.W et al (1992) reported that aqueous extract showed weak hepatoprotective effect (1mg/ml)[132].

Abd El Wahab et al (1998) stated that seed oil showed esterogenic, androgenic effect (15 mg / Kg., 40 mg/kg; s.c)[133].

Das A.K et al (1999) reported that methanolic extract showed antidiarrhoeal activity (200 mg/kg, i.g.)[134].

Schubert S.Y et al (1999) stated that seed oil showed antioxidant, eicosanoid enzyme inhibition effect[125].

Van Elswijk D.A. et al (2004) reported that seed oil showed antiproliferative effect in human breast cancer[81].

Selvasubramanian S. et al (2000) stated that seed powder showed anti nematodal effect (0.75gm/kg)[135].

Kwak H.M et al (2005) reported that seed husk showed β-secretase inhibitory activity[136].

Jurenka M.T.J (2008) stated anti-inflammatory, antioxidant, prostate cancer activities[38].

**Bark**

**Ethnomedical Information**

Singh V.P et al (1980) stated that aqueous extract used in leucorrhea, leprosy, menorrhagia[137].

Jafri M.A et al (2000) reported that decoction used in dysentery[29].

Bharadwaj S. et al (2003) stated the bark used for dysentery in Mizoram tribal medicine[138].

Anonymous (2005) mentioned that decoction used as taenicide[22].

## Phytochemistry

Tanaka T et al (1985)[120], Neuhofer H et al (1993)[139] Lansky E.P et al (2007)[24] were reported the presence of punicalin, punicalagin, punigluconin, pelletierine, punicacortein – A, B, C, D[120].

Chauhan D et al (2001) stated the presence of quercetin, pelargonidine, ellagic acid[140].

Anonymous (2005) mentioned the presence of friedlin, betulic acid, pelletierine[22].

## Pharmacology

Tripathi S.M et al (2004) reported the bark showed enzyme inhibition AChE by molluscicidal agent[141].

## Root

## Ethnomedical Information

Caius J.F et al (1923) Darivas V et al (1989) stated that hot aqueous extract used anthelmintic[142].

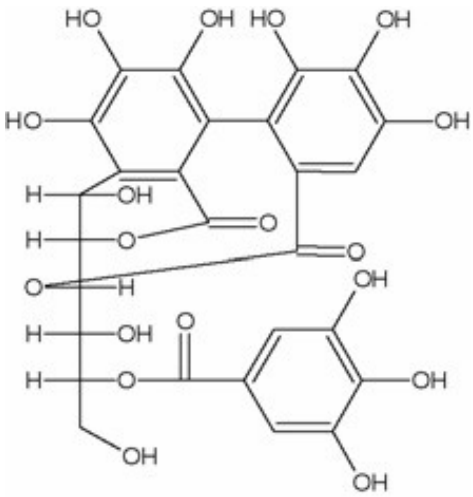
Yazicioglu A et al 1996 reported that decoction used for removal of kidney stones[143].

Bharadwaj et al (2003) stated its use in dysentery[133].

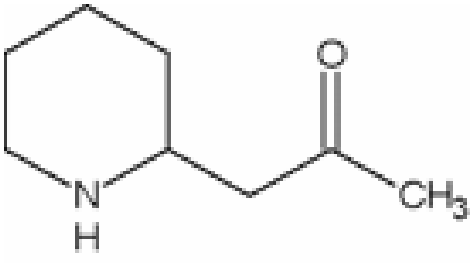
## Phytochemistry

Tanaka T et al (1985)[120], Neuhofer H et al (1993)[134], Lansky E : P et al (2007)[74], Jurenka M.T.J et al (2008)[38] reported the presence of punicalin, punicalagin, punicacortein – A, B, C, D, pelletierine alkaloids.

**PUNICACORTEIN- A**




**PELLETIERINE ALKALOIDS**




## Pharmacology

Sousa P.D et al (1974) reported that aqueous, ethanolic extract showed molluscicidal activity (1000 ppm)[144].

Singhal K.C et al (1984) stated that chloroform extract showed anthelmintic effect in mice[145].

Desta A.B et al (1995) reported that powder showed taenicide effect[146].

## Microbiological Review

Kurokawa M et al (1993) reported that hot aqueous extract showed antiviral activity. (0.5, 1 mg/ml) in mice[147].

## Acute toxicological studies

Michael, A.S., et al (1956) mentioned Artemia Salina as a test Organism for a bioassay[148].

Vanhaecke P., et al (1981) mentioned that a short – term toxicity test with Artemia nauplil[149].

Sleet R.B., et al (1983) Improved Methods for harvesting and counting synchronous populations of Artemia nauplii for use in developmental toxicology[150].

Sadhana Sathaye et al (2001) mentioned Brine shrimp lethality test a bench top bioassay was carried out to screen the toxic potential of herbal preparations. Number of deaths of larvae was taken as bioactivity or toxicity[151].

## Screening Methods of Ache Inhibitory Activity

Chung Y.K et al (2001) reported that the AChE assay was performed according to the colorimetric method of Ellman et al using acetyl thiocholine iodide as a substrate[17].

Sung S.H et al (2002) reported that the AChE assay was performed according to the colorimetric method of Ellman et al using neostigmine as positive control[152].

Gupta R.K (2004) mentioned that AChE inhibitory activity done by Ellman method[153].

Nino J. et al (2006) reported that the AChE assay was performed according to the colorimetric method of Ellman et al using esering as positive control[14].

Adsersen A et al (2007) reported that the AChE and BChE inhibitory activity was detected by a TLC bioautographic assay and the concentration of the isolated compounds that inhibited the enzyme activity by 50% ($IC_{50}$) was determined by a microtitre plate assay based on Ellman method, Galanthamine HBr was used as a positive control[154].

Vinutha B et al (2007) reported that the AChE inhibition assay was carried out as per the method of Atta-ur-Rahman et al (2001), and TLC bioautography for AChE inhibition was performed as per Martson et al (2002), eserine was used as positive control[62].

# AIM AND OBJECTIVE

**Aim**

To study the pharmacognostic, phytochemical and invitro study on acetylcholinesterase inhibitory activity of leaves of ***Punica granatum L.*** **Var: Ganesh**. Family **Punicaceae.**

**Objective**

The objective of the study was divided into three parts.

**Part – I**

**Pharmacognostic Studies**

- Identification, collection and authentification of leaves of *P. granatum*.
- Detailed pharmacognositc study includes morphological study, microscopical study and quantitative microscopy

**Part – II**

**Preliminary Phytochemical Studies**

- Preliminary phytochemical analysis of the leaf powder and different extracts.
- Identification and estimation of ursolic acid by TLC and HPLC for different extracts.

**Part III**

- **Acute toxicological study**

  To perform **BSLB** (Brine Shrimp Lethality Bioassay) as a preliminary assessment of the toxicity of for different extracts of ***P. granatum L.***

- **Acetylcholinesterase Inhibitory Activity**

**Part – A**

Standardization & Validation of Ellman method which includes

- Determination of Acetylcholinesterase concentration.
- Determination of substrate (ATCI) concentration.
- Determination of Ellman reagent (DTNB) concentration.
- Determination of pH maxima.
- Determination of Temperature maxima.

**Part – B**

- To evaluate the effect of the extracts having **ursolic acid** for invitro acetylcholinesterase inhibitory activity.

**Note:** The following text *P. granatum* means **Punica granatum L. Var: Ganesh**

# PHARMACOGNOSTIC STUDIES

## SECTION - A

## MACROSCOPICAL STUDIES OF THE LEAVES OF *Punica granatum Linn*[22,155,156,157,158,159].

***Punica granatum Linn*** is a dicotyledonous plant belonging to the family **Punicaceae.**

On the basis of the structure of the ovary and the typical fruit (the balusta), Punica has been classified as the solitary genus under a separate family *Punicaceae* distinct from *Lythraceae*.

***Taxonomy***

| | | |
|---|---|---|
| Kingdom | : | Plantae |
| Subkingdom | : | Tracheobionta |
| Division | : | Magno liophyta |
| Class | : | Magno liopsida |
| Sub Class | : | Rosidae |
| Order | : | Myrtales |
| Family | : | Punicaceae |
| Genus | : | ***Punica*** |
| Species | : | ***granatum*** |

***Synonym***[22]

Pomegranate

***Vernacular Names***[22,155,156,160,161,162]

| | | |
|---|---|---|
| English | : | Pomegranate |

| | | |
|---|---|---|
| Hindi | : | Anar, Dhalim |
| Kannada | : | Dalimba |
| Malayalam | : | Matalam |
| Sanskrit | : | Dadimah |
| Tamil | : | Madulai, Madalai Uôçû[, UôRû[ |
| Telugu | : | Danimma, Dadima |
| Bengali | : | Dalimb |
| Marathi | : | Dalimba |
| Gujarathi | : | Dadam |
| Panjabi | : | Daru |
| Arabic | : | Shajratur - rumman |
| French | : | Greanadier |
| German | : | Granatbaum |

***Geographical Distribution***[22,155]

**Native**

Considered to be Iran, Afghanistan, Baluchistan wild in the warm valleys of Himalayas 900 – 1800m and cultivated throughout India.

**Subspecies**[22]

1. Chlorocarpa
2. Porphyro carpa

**Habit and Habitat**[22]

*P.granatum* is a shrub or small tree, 5 – 10m high, dicotyledonous plant belonging to the family punicaceae. It is a subtropical fruit tree, growing best in semi – acrid climate where cool winters and hot summer prevail. It grows under a wide range of climatic conditions and is planted upto $35^0$N in USA. It stands considerable frost and is injured only when the temperature goes below – $11^0$C. It thrives even under desert

conditions. Although it is highly drought resistant, the pomegranate bears well only under irrigation (Plate-1,4) (Fig-1).

**Description of the plant**[22,155,161]

Leaves: 2-8 cm long oblong or obovate shinning above, bright green beneath, opposite, glabrous, minutely pellucid – punctuate (Plate-3,6,7).

Flowers : Scarlet red or sometimes yellow, 3.7 – 5cm long mostly solitary, some times 2 – 4 together, stamens very numerous inserted on the carlyx below the petals at various levels (Plate-2).

Fruits : Globose, crowned by the persistent calyx (Plate-5).

Rind : Coriaceous and woody, interior septate with membranous walls, containing numerous seeds.

Seeds : Angular with red, pink or whitish fleshy testa.

Bark : 5 – 10 cm long and 1-3 cm wide root bark consists of irregular, curved, flattish or recurred fragments.The outer surface is rough, earthy yellow with darker patches; the inner surface is smooth and yellow, with irregular, darker, brown blotches; the fracture is short. The Stem Bark is in straighter channeled pieces or in quills, the outer surface shows occasional shallow, longitudinal furrows and pale bands of cork[22].

**Varieties**[22]

1. Alandi or Vadki
2. Bedana
3. Dholka

4. Fabul
5. Fandahari
6. Musket Red
7. Paper shell
8. Poona
9. Spanish Ruby
10. Vellodu
11. Ganesh

**Cultivation**

**Soil**

Deep loamy soil preferably calcareous soil containing time nodules and tolerates soil alkalinity.

**Propagation and culture**

Pomegrante is propagated by seed, seedlings and hard wood cuttings.

**Manuring**

Well – rotted, old farmyard manure mixed with ashes, groundnut cake is advantageous.

# SECTION – B

# MICROSCOPICAL STUDIES OF THE LEAVES OF *Punica granatum L.*

## MATERIALS AND METHODS

### *Collection of Specimens*[163,164,165,166]

The plant specimens were collected from Department of Horticulture Tamil Nadu Agricultural University, Madurai, Tamil Nadu during August 2008. Care was taken to select healthy plants and for normal organs. The leaves were cut and removed from the plant and fixed in FAA (Formalin- 5 ml + Acetic acid – 5 ml + 70% ethyl alcohol-90 ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of tertiary-butyl alcohol. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60$^0$c), until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

### *Sectioning*

The paraffin embedded specimens were sectioned with the help of rotary microtome. The thickness of the sections was 10-12 µm. After dewaxing the sections were stained with toluidine blue. Since toluidine blue is a polychromatic stain, the staining results were remarkably good and some cyto chemical reactions were also obtained. The dye rendered pink color to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc., Where ever necessary, section were also stained with safranin and fast-green and potassium iodide (for starch).

For studying the stomatal morphology, venation pattern and trichome distribution, paradermal sections (sections taken parallel to the surface of leaf) as well as clearing of leaf with 5% sodium hydroxide and epidermal peeling by partial maceration employing

jeffrey's maceration were prepared. Glycerine mounted temporary preparations were made for macerated/cleared materials.

***Photomicrographs***

Microscopic descriptions of tissues are supplemented with micrographs where ever necessary. Photographs of different magnifications were taken with Nikon labphoto 2 microscope unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light was employed. Since these structures have birefringent property, under polarized light they appear bright against dark background.

## MICROSCOPY OF LEAF

The leaf has thick semicellular midrib and thin lamina. In cross sectional view, the lamina exhibites distinct dorsiventral symmetry.

**Midrib** (Plate-8,8A), (Fig-2,2A)

Size : 400 μm thick in vertical plane

600 μm wide in horizontal plane.

has wide dorsal concavity and abaxial side hemispherical.

**Epidermis** (Plate-9,9A,9B)

Small elliptical cells, adaxial in surface view, the cells are either rectangular or polygonal with slightly wavy anticlinal walls. In the cells stained with safranin thin cuticular striations are seen prominently, which are parallel to each other.

**Abaxial epidermis**

Stomata anomocytic (Ranunonculaeous), no distinct subsidiary cells are seen. Walls are undulate, amoeboid outlined, with deep folds in stomata. The guard are cells are oblong, eliptic and have narrow slit like aperture.

**Vascular bundle**

Simple, wide and bowel shaped with adaxial cavity.

**Xylem**

Dense parallel lines, narrow thick walled angular in out line.

**Phloem**

Thin, present in abaxial layer.

**Lamina**

80μm thick and has thick adaxial epidermis which consists of wide rectangular cells 20 μm in thick (Plate-10,10A,10B).

**Mesophyll**

Single layer adaxial palisade cells, 20-30 μm in height, spongy parenchyma about five layers of large, circular closed arranged cells.

**Idioplast**

Specially modified, dilated cavities bearing calcium oxalate crystals are frequently seen. Crystals may be prismatic (20-40 μm thick) or druses (30μm thick).

**Venation Pattern** (Plate-11,11A)

Secondary and tertiary veins are uniformly thin and wavy. Some vein islets are distinct. The vein terminations are short, straight ends or simple.

**Petiole** (Plate-12,12A,12B)

The proximal end of petiole side semi circular with wide abaxial, and concavity two short and thick lateral wings, 1.2mm in vertical axis and 1.4mm in horizontal axis.

**Distal end**

The distal end of petiole has deep 'V' shape adaxial groove, long and prominent wings, which is 1mm vertical, 1.25mm horizontal, epidermal layer of small squarish cells. Ground tissue is parenchymatous which are angular thick walls and compact. Vascular stand is single, arc shaped, having densed parallel files of long, narrow xylem elements which are thick walled with narrow lumen. Phloem prominent in the lower part of the xylem, vascular strand is 600μm wide, 250μm thick.

## POWDER MICROSCOPY OF LEAF

**Organoleptic characters**

Nature - Coarse

Colour - Green

Odour - Agreeable Odour

Taste - Tasteless

The powder microscopy of the leaf powder reveals the following charaters (Fig-3)

- Epidermal cells with thin & slighty wavy anticlinal walls.
- Anomocytic stomata (Ranunculaceous)
- Spongy parenchyma with prismatic crystals
- Xylem, phloem vessels
- Fragments of leaf

# SECTION – C

# QUANTITATIVE MICROSCOPY FOR LEAVES OF P. grantum L.

***Microscopic Schedules***[167,168,169,170,171]

The vein islet number, vein terminal number, stomatal number and stomatal index were determined on fresh leaves using standard procedures.

**A. *Vein islet number and Vein terminal number***

The term vein islet in used to denote the minute area of photosynthetic tissue encircled by the ultimate divisions of the conducting strands. The number of vein islets per sq. mm., area is called vein islet number.

Vein terminal number may be defined as the number of vein terminals present in one sq., mm. area of the photosynthetic tissue.

***Determination of Vein Islet Number and Vein Termination Number***

Leaf lets were cleared in chloral hydrate, stained and mounted on a slide.

A camera lucida is set up and by means of a stage micrometer the paper in divided into squares of $1mm^2$ using a 16mm objective. The stage micrometer is then replaced by the cleared preparation and the veins are traced in four contiguous squares, either in a square 2mm x 2mm (or) rectangle 1mm x 4 mm.

When counting, it is convenient to number each vein-islet on the tracing. Each numbered area must be completely enclosed by veins, and those which are incomplete are excluded from the count if cut by the top and left-hand sides of the square (or) rectangle but included if cut by the other two sides.

Ten readings for vein islet and vein termination number were recorded.

**Table - 1**

***VEIN ISLET NUMBER AND VEIN TERMINATION NUMBER OF***

***P. granatum L.***

| Observation number | Vein Islet Number | Vein Termination Number | |
|---|---|---|---|
| 1 | 32 | 21 | |
| 2 | 29 | 25 | |
| 3 | 31 | 28 | |
| 4 | 32 | 22 | |
| 5 | 30 | 25 | |
| 6 | 27 | 25 | |
| 7 | 24 | 23 | |
| 8 | 33 | 25 | |
| 9 | 37 | 24 | |
| 10 | 36 | 27 | |
| **Range** | **Minimum** | **Average** | **Maximum** |
| Vein islet Number | 24 | 31 | 37 |
| Vein Termination Number | 21 | 24 | 28 |

**Stomatal Number**

Stomatal number is defined as the number of stoma present in one square mm area of the photosynthetic tissues.

**Method**

Using fresh leaves, replicas of leaf surface may be made which are satisfactory for the determination of stomatal number and stomatal index. An approximate 50% gelatin and water gel is liquified on a water – bath and smeared on a hot slide. The fresh leaf is added, the slide inverted and cooled under a tap and after about 15-30 min the specimen is stripped off.

The imprint on the gelatin gives a clear outline of epidermal cells, stomata and trichomes.

**Table - 2**

**STOMATAL NUMBER**

| Observation Number | | Lower epidermis | |
|---|---|---|---|
| 1 | | 209 | |
| 2 | | 213 | |
| 3 | | 203 | |
| 4 | | 187 | |
| 5 | | 217 | |
| 6 | | 196 | |
| 7 | | 205 | |
| 8 | | 212 | |
| 9 | | 217 | |
| 10 | | 194 | |
| **Range** | **Minimum** | **Average** | **Maximum** |
| Lower epidermis | 187 | 205 | 217 |

**B. Stomatal Index**

It is the percentage, which the numbers of stomata from the total number of epidermal cells, each stoma being counted as one cell.

I, Stomatal index = S/S+E x 100

Where S = Number of stomata per unit area

E= Number of epidermal cells in the same unit area

**Determination of Stomatal Index**

The procedure adopted in the determinations of stomatal number was observed under high power (45 x).The epidermal cells and the stomata were counted. From these values the stomatal index was calculated using the above formula.

**Table – 3**

**STOMATAL INDEX**

| Observation Number | | Lower epidermis | |
|---|---|---|---|
| 1 | | 12.16 | |
| 2 | | 9.82 | |
| 3 | | 10.80 | |
| 4 | | 12.24 | |
| 5 | | 10.12 | |
| 6 | | 10.74 | |
| 7 | | 10.71 | |
| 8 | | 11.80 | |
| 9 | | 9.42 | |
| 10 | | 10.62 | |
| **Range** | **Minimum** | **Average** | **Maximum** |
| Lower epidermis | 9.42 | 10.84 | 12.24 |

## Section - C

## QUANTITATIVE SCHEDULES FOR LEAVES OF *P. granatum L.*

***Ash Value***

The ash values were determined by using air dried powder of the leaf as per the official method.

***Total ash***

Two grams of the air dried leaf powder was accurately weighed in a platinum crucible separately. The powder was scattered into a fine even layer on the bottom of the crucible and incinerated by gradually increasing the temperature not exceeding 450°c, until free from carbon. Then it was cooled and weighed for constant weight. The percentage of ash with reference to the air dried powder was calculated.

***Water soluble Ash***

The ash obtained from the total ash procedure was boiled with 25 ml of water for 5 minutes and the insoluble matter was collected on an ash less filter paper and washed with hot water. Then it was ignited for 15 minute at a temperature not exceeding 450°c. The weight of the insoluble matter was subtracted from the weight of the total ash. The difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried powder.

***Acid insoluble ash***

The ash obtained from the total ash was boiled for five minutes with 25 ml of dilute hydrochloric acid. The insoluble matter was collected in a tarred sintered glass crucible. The residue was washed with hot water, dried and weighed. The percentage of acid insoluble ash with reference to the air dried drug was calculated.

**Table - 4**

**ASH VALUES FOR THE LEAVES OF *P. granatum L.***

| Observation Number | Total Ash (%) | Acid Insoluble ash (%) | Water soluble Ash (%) |
|---|---|---|---|
| 1 | 11.45 | 1.46 | - |
| 2 | 11.26 | 1.52 | - |
| 3 | 10.90 | 1.50 | - |
| 4 | 11.12 | 1.53 | - |
| 5 | 11.49 | 1.56 | - |
| 6 | 10.80 | - | 2.96 |
| 7 | 11.20 | - | 2.60 |
| 8 | 10.91 | - | 2.52 |
| 9 | 11.12 | - | 3.02 |
| 10 | 10.78 | - | 2.90 |

| Range | | | |
|---|---|---|---|
| **Minimum** | 10.78 | 1.46 | 2.52 |
| **Average** | 11.10 | 1.51 | 2.8 |
| **Maximum** | 11.49 | 1.56 | 3.02 |

***Determination of Loss on Drying***

For the determination of loss on drying, the method described by wallis was followed.

One gram of the powdered leaf was accurately weighed in a tarred Petri dish, previously dried under the conditions specified in IP '96. The powder was distributed as evenly as practicable, by gentle sidewise shaking. The dish was dried in an oven at 100 – $105^0$ c for 1 hour. It was cooled in a desiccator and again weighed. The loss on drying was calculated with reference to the amount of the dried powder taken.

**Table - 5**

**PERCENTAGE LOSS ON DRYING FOR THE LEAVES OF *P. granatumL.***

| Observation Number | | Loss on Drying (%) W/W | |
|---|---|---|---|
| | | Leaf | |
| 1 | | 8.90 | |
| 2 | | 8.50 | |
| 3 | | 9.10 | |
| 4 | | 8.60 | |
| 5 | | 9.00 | |
| **Material** | **Minimum** | **Average** | **Maximum** |
| Leaf | 8.50 | 8.82 | 9.10 |

***Extractive Values***

***By using solvents successively with increasing order of polarity[172]***

Five grams of the coarsely powdered leaf was extracted continuously in soxhlet apparatus for six hours individually, separately with solvents of increasing order of

polarity. After six hours the solvents was removed and evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°c and weighed. The percentage of the individual solvent soluble extractive value was calculated with reference to the air dried powder.

**Table – 6**

**EXTRACTIVE VALUES (SUCCESSIVE SOLVENTS)**

| **Solvents** | **Extractive Value (%)** |
|---|---|
| | **Leaf** |
| Hexane | 1.13 |
| Chloroform | 2.66 |
| Acetone | 7.66 |
| Ethanol | 11.13 |
| Water | 16.8 |

# PHYTOCHEMICAL STUDIES

## SECTION – A
## PHYTOCHEMICAL STUDIES
## FOR THE LEAF OF *P.granatum*

The plant ***P. granatum L.*** was collected from Department of Horticulture Tamil Nadu Agricultural University, Madurai, in Tamilnadu during the first week of August and it was Authenticated by the Taxonomist and Horticulturist. The leaves portion was washed thoroughly and dried in shadow. The shadow dried leaves were powdered separately and then subjected to the following preliminary phytochemical studies.

**ORGANOLEPTIC EVALUATION**

| | | |
|---|---|---|
| Nature of the Powder | : | Coarse |
| Colour | : | Green |
| Odour | : | Distinct odour |
| Taste | : | Taste less |
| Shaken with Water | : | No Frothing occurs |
| Pressed in between two filter paper | : | No oily mark on the paper. |

# PHYTOCHEMICAL STUDIES FOR THE LEAF POWDER AND EXTRACTS OF P. granatum L.173,174,175,176

Powdered Materials and their individual extracts obtained from different increasing polarity were subjected to the following chemical test and the results were presented in the table.

***Test for Alkaloids***

***Various procedures to liberate alkaloids***

- Powdered drug was mixed thoroughly with 1 ml of 10% ammonia solution and then extracted for 10 minutes with 5 ml methanol, under reflux. The filtrate was then concentrated.
- Powdered drug was mixed thoroughly with 1 ml of 10% sodium carbonate solution and then extracted for 10 minutes with 5 ml methanol, under reflux. The filtrate was then concentrated.
- Powdered drug was ground in a mortar for about 1 minute with 2 ml of 10% ammonia solution and then thoroughly mixed with 7 gram basic aluminum oxide. The mixture was then loosely packed in to a glass column and 10 ml chloroform was added, eluted, dried and methanol was added.
- Powdered drug was shaken for 15 minute with 15 ml of 0.1 N sulphuric acid and then filtered. The filter was washed with 0.1 N sulphuric acid to a volume of 20 ml filtrate; 1 ml concentrated ammonia was then added. The mixture was then shaken with two portions of 10 ml diethyl ether. The ether was dried over anhydrous sodium sulphate, filtered and evaporated to dryness and the resulting residue was dissolved in methanol.
- Powdered drug was mixed with one gram of calcium hydroxide and 5 ml of water, made into a smooth paste and set aside for 5 minutes. It was then evaporated to

dryness in a porcelain dish on a water bath. 20 ml of 90% alcohol was added, mixed well and then refluxed for half an hour on a water bath. It was then filtered and the alcohol was evaporated. To that dilute sulphuric acid was added.

The above made extracts were tested with various alkaloid reagents and the results were as follows.

| | | | | |
|---|---|---|---|---|
| **1.** | **Mayer's reagent** | - | Cream color precipitate | Indicating the **presence** of alkaloids |
| **2.** | **Dragendorff's reagent** | - | Reddish brown precipitate | |
| **3.** | **Hager's reagent** | - | Yellow precipitate | |
| **4.** | **Wagner's reagent** | - | Reddish brown precipitate | |

***Test for purine group (Murexide test)***

The residue obtained after the evaporation of chloroform was treated with 1 ml of hydrochloric acid in a porcelain dish and 0.1g of potassium chlorate was added and evaporated to dryness on water bath. Then the residue was exposed to the vapours of dilute ammonia solution.

No purple colour was obtained indicating the **absence** of purine group of alkaloids.

***Test for Carbohydrates***

- ***Molisch's test***

The aqueous extract of the powdered material was treated with alcoholic solution of $\alpha$ - naphthol in the presence of sulphuric acid**.**

Purple colour was obtained indicating the **presence** of carbohydrates.

- ***Fehling's test***

The aqueous extract of the powdered material was treated with Fehlings I and II solution and heated on boiling water bath.

Reddish brown precipitate was obtained indicating the **presence** of free reducing sugars.

❖ ***Benedict's test***

The aqueous extract of the powdered drug was treated with Benedict's reagent and heated over a water bath.

Reddish brown precipitate was obtained indicating the **presence** of reducing sugars.

***Test for Glycosides***

***General test***

❖ ***Test A***

200 mg of the powdered drug was extracted with 5 ml of dilute sulphuric acid by warming on a water bath, filtered and neutralised with 5% sodium hydroxide solution. Then 0.1 ml of Fehlings solution A and B were added, until it becomes alkaline and heated on a water bath for 2 mts.

❖ ***Test B***

200 mg of the powdered drug was extracted with 5 ml of water instead of sulphuric acid. Boiled and equal amount of water was added instead of sodium hydroxide solution. Then 0.1 ml of Fehlings solution A and B were added, until it becomes alkaline and heated on a water bath for 2 mts.

The quantity of red precipitate formed in test A is greater than in test B indicating the **presence** of glycosides.

❊ ***Anthraquinones***

❖ ***Borntrager's test***

The powdered leaf was boiled with dilute sulphuric acid, filtered and to the filtrate benzene was added and shaken well. The inorganic layer was separated and ammonia solution was added slowly.

No color reaction was observed in ammoniacal layer indicating the **absence** of anthracene derived glycosides.

❖ ***Modified Borntrager's test***

About 0.1 gram of the powdered leaf was boiled for two minutes with dilute hydrochloric acid and few drops of ferric chloride solution was added, filtered while hot and cooled. The filtrate was then extracted with benzene and the benzene layer was separated. Equal volume of dilute ammonia solution was added to the benzene extract and shaken well.

No color was observed in ammoniacal layer indicating the **absence** of anthracene derived glycosides.

***Test for cyanogenetic glylosides***

Small quantity of the powdered leaf was placed in a stoppered conical flask with just sufficient water to cover it. A sodium picrate paper strip was inserted through the stopper so that it was suspended in the flask and it was set aside for 2 hours in a warm place.

No change in the colour of the sodium picrate paper was observed indicating the **absence** of cyanogenetic glycosides

❊ ***Test for cardiac glycosides***

❖ ***Keller Killiani test***

About 1 gram of the powdered leaf was boiled with 10 ml of 70% alcohol for two minutes, cooled and filtered. To the filtrate 10 ml of water and 5 drops of solution of lead sub acetate were added and filtered. The filtrate was then extracted with chloroform and

the chloroform layer was separated and evaporated to dryness. The residue was dissolved in 3 ml of glacial acetic acid containing a trace of ferric chloride. To this 3 ml of concentrated sulphuric acid was added to the sides of the test tube carefully.

No reddish brown layer acquiring bluish green color after standing was observed indicating the **absence** of deoxy sugars of cardiac glycosides.

- ***Raymond Test***

To the alcoholic extract of the leaf, hot methanolic alkali was added.

No Violet color was produced indicating the **absence** of cardiac glycosides.

- ***Legal's Test***

To the alcoholic extract of the powdered drug, pyridine and alkaline sodium nitro pruside solution were added.

No blood red color was formed indicating the **absence** of cardiac glycosides.

***Coumarin glycosides***

A small amount of powdered leaf was placed in test tube and covered with a filter paper moistened with dilute sodium hydroxide solution. The covered test tube was placed on water bath for several minutes. Then the paper was removed and exposed to UV light.

No green fluorescence was observed indicating the **absence** of coumarin glycosides.

***Test for Phytosterols***

The powdered leaf was first extracted with petroleum ether and evaporated. The residue obtained was dissolved in chloroform and tested for sterols.

❖ ***Salkowski Test***

Few drops of concentrated sulphuric acid were added to the above solution, shaken well and set aside.

The chloroform layer of the solution turned red in color indicating the **presence** of sterols.

❖ ***Libermann – Burchard's Test***

To the chloroform solution few drops of acetic anhydride was added and mixed well 1 ml of concentrated sulphuric acid was added through the sides of the test tube and set aside for a while.

A brown ring was formed at the junction of the two layers and the upper layer turned green indicating the **presence** of sterols.

***Test for Saponins***

About 0.5 gram of the powdered leaf was boiled gently for 2 minute with 20 ml of water and filtered while hot and allowed to cool. 5 ml of the filtrate was then diluted with water and shaken vigorously**.**

No frothing occurred indicating the **absence** of saponins.

***Test for Tannins***

To the aqueous extract of the powdered leaf, few drops of ferric chloride solution were added.

Bluish black color was produced, indicating the **presence** of tannins.

❖ ***Gold beater's skin test***

2% hydrochloric acid was added to a small piece of gold beater skin and rinsed with distilled water and placed in the solution to be tested for five minutes. Then washed with distilled water and transferred to a 1% ferrous sulphate solution.

Formation of brown color indicates the **presence** of tannins.

***Test for Proteins and Free Aminoacids***

- ***Millon's test***

The aciduous alcoholic extract of the powdered leaf was heated with Millon's reagent.

The colour was changed to red on heating indicating the **presence** of proteins.

- ***Biuret test***

To the alcoholic extract of the powdered leaf 1 ml of dilute sodium hydroxide was added. Followed by this one drop of very dilute copper sulphate solution was added.

Violet color was obtained indicating the **presence** of proteins.

- ***Ninhydrin Test***

To the extract of the powdered drug, ninhydrin solution was added, and boiled.

Formation of violet color indicating the **presence** of Aminoacids

***Test for Mucilage***

To the aqueous extract of the powdered leaf, ruthenium red solution was added.

No Reddish pink color was produced indicating the **absence** of Mucilages.

***Test for Flavonoids***

- ***Shinoda Test***

A little amount of the powdered leaf was heated with alcohol and filtered. To the alcoholic solution a few magnesium turnings and few drops of concentrated hydrochloric acid were added, and boiled for 5 minutes.

Purple color was not obtained indicating the **presence** of flavonoids.

- ***Alkaline reagent test***

To the alcoholic extract of the powdered leaf, few drop of sodium hydroxide solution was added.

Yellow color was not formed, indicating the **presence** of flavonoids

- ***Zinc Hydrochloride Test***

To the alcoholic extract, mixture of zinc dust and concentrated hydrochloric acid was added.

No formation of red color indicating the **presence** of flavonoid

***Test for Terpenoids***

The powdered leaf was shaken with petroleum ether and filtered. The filtrate was evaporated and the residue was dissolved in small amount of chloroform. To the chloroform solution tin and thionyl chloride were added.

Pink color was obtained indicating the **presence** of Terpenoids.

**Test for Volatile Oil**

About 100 gram of fresh leaves, were taken in a volatile oil estimation apparatus (Cocking Middleton apparatus) and subjected to hydro distillation for four hours.

No Volatile oil was obtained indicating the **absence** of volatile oil.

**Test for Fixed Oil**

A small amount of the powdered leaf was pressed in between in the filter paper and the paper was heated in an oven at 105°C for 10 minutes.

No translucent greasy spot occurred indicating the **absence** of fixed oil.

***Table - 7***

**RESPONSE TO THE PRELIMINARY PHYTOCHEMICAL SCREENING FOR THE CRUDE LEAF POWDER OF *P. granatum L*.**

| S.NO | TEST | OBSERVATION |
|---|---|---|
| **I.** | **ALKALOIDS** | |
| | Mayer's reagent | + |
| | Dragendorff's reagent | + |
| | Hager's reagent | + |
| | Wagner's reagent | + |
| **II** | **CARBOHYDRATES** | |
| | Molisch's test | + |
| | Fehling's test | + |
| | Benedict's test | + |
| **III** | **GLYCOSIDES** | |
| | General Test | + |
| | Anthraquinone | - |
| | Cardiac | - |
| | Cyanogenetic | - |
| | Coumarin | - |
| **IV** | **PHYTOSTEROLS** | |
| | Salkowski test | + |
| | Liberman Burchard's test | + |
| **V** | **SAPONINS** | - |
| **VI** | **TANNINS** | + |
| **VII** | **PROTEINS AND FREE AMINO ACIDS** | |
| | Millon's test | + |
| | Biurett test | + |
| **VIII** | **GUMS AND MUCILAGE** | - |
| **IX** | **FLAVONOIDS** | |
| | Shinoda test | + |
| **X** | **TERPENOIDS** | + |
| **XI** | **VOLATILE OIL** | - |
| **XII** | **FIXED OIL** | - |

The above described tests were also performed on the different extracts of leaf powder of ***P. granatum L.*** and the results were as follows,

**Table – 8**

**RESPONSE TO THE PRELIMINARY PHYTOCHEMICAL SCREENING FOR THE DIFFERENT EXTRACTS OF LEAF OF *P. granatum L.***

| Tests | Hexane extract | Chloroform extract | Acetone extract | Ethanolic extract | Aqueous extract |
|---|---|---|---|---|---|
| **ALKALOIDS** | | | | | |
| Mayers Reagent | - | + | - | - | - |
| Dragendorffs reagent | - | + | - | - | - |
| Hagers reagent | - | + | - | - | - |
| Wagners reagent | - | + | - | - | - |
| **CARBOHYDRATES** | | | | | |
| Molishch's Test | - | - | - | + | + |
| Fehlings Test | - | - | - | + | + |
| Benedicts Test | - | - | - | + | + |
| **GLYCOSIDES** | | | | | |
| General Test | - | - | - | + | + |
| Anthraquinone | - | - | - | - | - |
| Cardiac | - | - | - | - | - |
| Cyanogenetic | - | - | - | - | - |
| Coumarin | - | - | - | - | - |
| **PHYTOSTEROLS** | | | | | - |
| Salkowski Test | + | + | - | - | - |
| Libermann Burchard Test | + | + | - | - | - |
| **SAPONINS** | - | - | - | - | - |
| **TANNINS** | - | - | - | + | + |
| **PROTEINS & FREE AMINO ACID** | | | | | |
| Millons test | - | - | - | + | + |
| Biuret test | - | - | - | + | + |
| Ninhydrin test | - | - | - | + | + |
| **GUMS & MUCILAGE** | - | - | - | - | - |
| **FLAVONOIDS** | | | | | |
| Shinoda test | - | - | + | - | - |
| Alkaline Reagent test | - | - | + | - | - |
| Zinc hydrochloric acid test | - | + | + | - | - |
| **TERPENOIDS** | + | + | + | + | - |
| **FIXED OIL** | - | - | - | - | - |

"+" Indicate Positive reaction "-" Indicate Negative reaction

# SECTION – B

# TLC & HPLC ANALYSIS FOR VARIOUS EXTRACTS OF LEAVES OF P.granatum L.[173,177,178]

Among the various methods of separating and isolating plant constituents the "chromatographic Procedure" originated by Tswett is one of the most useful techniques of general application. All finely divided solids have the power to adsorb other substances are capable of being absorbed some much more readily than others. This phenomenon of selective adsorption is the fundamental principle of chromatography.

**Principle**

When a mixture of compound is spotted on a TLC plate the compound which readily soluble and not strongly absorbed moves up readily along with this solvent. Those which are not so soluble, and are more strongly absorbed moves up less readily to the separation of the compound.

The advancement of the TLC techniques has provided the organic chemists and biochemists a tool which combines in itself sensitivity and rapidity compared to the conventional paper chromatographic technique.

**Preparation of Extracts**

The dried leaf powder of ***P. granatum L.*** was extracted in a Buchi rotavapor – R - 220 extraction apparatus and the extracted solution was concentrated under reduced pressure.

The extract was dissolved in Methanol. Small quantity was taken in a capillary tube and it was spotted on TLC plate 1cm above its bottom end the start points ere equally sized as for as possible.

**Development of chromatogram**

The plates were developed in a chromatographic tank by using a range of solvents from non-polar to polar as a mobile phase. The plates were allowed to develop ¾ of the length and then removed. The solvent front was immediately marked and the plates were allowed to dry. Then the plates were examined visually or under UV (or) sprayed with different reagents.

The spots were identified and Rf values were determined.

| | | | | |
|---|---|---|---|---|
| Stationary Phase | : | TLC Aluminium sheet precoated with slicagel 60 F254. (Merck) | | |
| Mobile phase used | : | Chloroform | : | Methanol |
| | | 8 | : | 2 |
| Detecting Agent | : | Methanolic Sulphuric acid | | |

The Rf value was calculated by the following formula.

$$Rf = \frac{\text{Distance travelled by solute front}}{\text{Distance traveled by solvent front}}$$

**Table – 9**

**RESULTS OF TLC ANALYSIS OF VARIOUS EXTRACTS FROM THE LEAVES OF *P. granatum L.***

| S. No | Type of Extract | Observation | Rf value |
|---|---|---|---|
| 1. | Reference Standard (ursolic acid) | 1 spot obtained ❖ pink | 0.25 |
| 2. | Chloroform Extract | 3 spots obtained. ❖ pink ❖ yellow and ❖ green. | 0.25, 0.41, 0.53 |
| 3. | Acetone Extract | 3 spots obtained. ❖ pink ❖ yellow and ❖ green. | 0.25, 0.43, 0.55 |
| 4. | Ethanol Extract | 3 spots obtained. ❖ pink ❖ yellow and ❖ green. | 0.25, 0.47, 0.58 |

The $R_f$ value (0.25) of various extracts coincides with the standard of ursolic acid. Hence the pink colour spot may be to ursolic acid. (Plate – 13)

Further the amount of ursolic acid present in various extracts of leaves of *P.granatum* were quantified using HPLC analysis.

# "HPLC ANALYSIS OF VARIOUS EXTRACTS OF LEAVES OF P. granatum L."

**High performance liquid chromatography**[179]

HPLC was referred to as high pressure liquid chromatography, but nowadays the term high performance liquid chromatography is performed. Since it better describes the characteristics of the chromatography and avoids creating the impression that high pressures are an inevitable pre-requisite for high performance.

A typical HPLC unit consist of the following

- A solvent reservoir and mixing system
- A high pressure pump
- A sample inlet pump
- A column (stainless steel)
- A detector and recording unit

**Procedure**

The appropriate solvents (mobile liquid phase) from the reservoirs are allowed to enter the mixing chamber where a homogenous mixture is obtained. A pump capable of maintaining high pressures draws the solvent from the mixing chambers and pushes it through the column.

The sample is injected through a port into the high pressure liquid carrier stream between the pump and the column. The separation takes place on the columns which vary from 50 – 100cm in length and 2.3 m.m in i.d. Typical flow rates are 1-2ml/min with pressure up to several thousand psi. The column effluent passes through a non-destructive detector where a property such as ultraviolet absorbance, refractive index (or) molecular fluorescence is monitored, amplified, and recorded as a typical detector response vs retention time chromatogram. The effluent may be either discarded,

recycled, or saved for the further studies in a fraction collector which is synchronized with the detector.

**Practical Procedure**

There are two methods which are generally used.

First method makes use of micro syringe designed to with stand high pressure. The sample is injected through a septum in an injection port, either directly on to the column packing (or) on to a small plug of inert material immediately above the column packing. This can be done while the system is under pressure, or the pump may be turned off before injection and when the pressure has dropped to near atmospheric, the injection is made and the pump switched on again. This is termed a stop flow injection.

In the second method, the sample is introduced by use of a loop injector. This consists of a metal loop of small volume which can be filled with the sample. By means of an appropriate valve, the eluant from the pump is channeled through the loop, the outlet of which leads directly on to the column. The sample is thus flushed on to the column by the eluant, without interruption of solvent flow to the column.

Repeated application of highly impure samples such as sera, urine which have preferably been deproteinated may eventually cause the column to lose its resolving power.

To prevent this occurrence a guard column is installed between the injection and the analytical column.

**HPLC Analysis of various extracts of P. granatum L. leaves (Fig. 5,6)**

| | | |
|---|---|---|
| Instrument | : | Shimadzu BM – 101 |
| Liquid Chromatogram | : | 10 ATVP Shimadzu liquid chromatography |
| Column Used | : | C-18 ODS Column |
| Column No | : | SLL / P / HPLC / 68 |
| Detector | : | SPD – 10 AVP Shimadzu UV – Vis detector |
| Wave length | : | 210 nm |
| Mobile Phase | : | ACN : Methanol |
| Flow rate | : | 0.5ml/1min |
| Injection volume | : | 20 µl |
| Standard (Ursolic acid) | : | 30.40mg / 100ml |
| Ursolic acid Potency | : | 84.84 |

**Results**

By comparing the peaks obtained from various (chloroform, acetone, ethanol) extracts of *P.granatum* with the peak area of reference standard ursolic acid, it was found that the various extracts contain ursolic acid and the HPLC data were as follows (Fig – 4 – 8).

**Table – 10**

**RESULTS OF HPLC ANALYSIS FO VARIOUS EXTRACTS FROM THE LEAVES OF P.granatum**

| S.NO | Parameters | Standard | Type of Extract | | | |
|---|---|---|---|---|---|---|
| | | Ursolic acid (Fig-4,4A,4B) | Chloroform (Fig-5,5A) | Acetone (Fig-6,6A) | Ethanol (Fig-7,7A) | Aqueous (Fig-8,8A) |
| 1. | Weight (in mg/100ml) | 30.40 | 421 | 283.60 | 243.50 | 401.20 |
| 2. | Peak Area – 1 | 3576647 | 11744297 | 129228 | 48457 | Not detected |
| 3. | Peak Area – 2 | 3530697 | 11942826 | 125300 | 52757 | |
| 4. | Peak Area – 3 | 3593624 | - | - | - | |
| 5. | Average | 3566989.33 | 11843561.50 | 127264 | 50607 | |
| 6. | Standard Deviation | 32556.18 | 140381.20 | 2777.52 | 3040.56 | |
| 7. | Assay (%w/w) | As such | 20.34 | 0.32 | 0.1503 | |

**Calculation :**

The percentage of ursolic acid present in the Chloroform extract.

$$= \frac{\text{Test Area}}{\text{Standard Area}} \times \frac{\text{Standard dilution}}{\text{Test dilution}} \times \frac{\text{Potency}}{100} \times 100$$

$$= \frac{11843561.50}{3566989.33} \times \frac{30.40/100}{421/100} \times \frac{84.84}{100} \times 100 \qquad = 20.34\%$$

# ACUTE TOXICOLOGICAL STUDY USING BRINE SHRIMP LETHALITY ASSAY

The importance of medicinal plants and traditional health systems solving the health care problems of the world is gaining increasing attention. Most of the developing countries have adopted traditional medical practice as an integral part of their culture.

In order to study the toxicity of these medicinal plants we performed **Brine Shrimp Lethality Bioassay** which based on the ability to kill laboratory cultured brine shrimp (***Artemia nauplii***). The brine shrimp assay was proposed by Michael[148] et al latter developed by Vanhaecke et al[149] Sleet and Brendel et al[150]. The assay is a useful tool for preliminary assessment of toxicity and it has been used for the detection of fungal toxins, plant extract toxicity, heavy metals, pesticides and cytotoxicity testing of dental materials.

The method is attractive because it is very simple, inexpensive and low toxin amounts are sufficient to perform the test in the micro well scale.

***Production of Artemia Strain***

Artemia is a non-selective filter feeder of organic detritus, micro algae and bacteria. Artemia are naturally found in saltpans, hypersaline lakes and coastal lagoons as well as in the man made Saltpans. When the cysts are inoculated in seawater for 24 hrs, the free – swimming nauplii are hatched out.

***Cytotoxicity Bioassay[151]***

Ten nauplii were drawn through a glass capillary and placed in each vial containing 4.5 ml of brine solution. In each experiment, 0.5 ml of the extract was added to 4.5 ml of brine solution and maintained at room temperature for 24 hrs under the light and surviving larvae were counted. Experiments were conducted along with control (vehicle treated), different concentrations of the chloroform extract (100-7500 ppm) in a set of three tubes per dose.

***Lethality Concentration Determination***

The percentage lethality was determined by comparing the mean surviving larval of the test and control tubes. $LC_{50}$ value was obtained from the best – fit line, plotted concentration verses percentage lethality. Podophyllotoxin was used as a positive control in the bio assay (Fig – 9).

***Statistical Analysis***

The percentage lethality was calculated from the mean survival larvae of extract treated tubes and control. $LC_{50}$ values were obtained by best-fit line method.

**Table - 11**

**EFFECT OF VARIOUS CONCENTRATIONS OF CHLOROFORM EXTRACT FROM LEAVES OF *P. granatum L.* ON *ARTEMIA NAUPLII***

| Extract | Concentration in ppm | Numbers released | Numbers dead after 24 hrs | Mortality (%) | Corrected Mortality by Abbot's formula |
|---|---|---|---|---|---|
| Chloro form extract | 100 | 10 | 3 | 30 | 23.3 |
| | | 10 | 2 | 20 | |
| | | 10 | 2 | 20 | |
| | 200 | 10 | 2 | 20 | 23.3 |
| | | 10 | 2 | 20 | |
| | | 10 | 3 | 30 | |
| | 500 | 10 | 3 | 30 | 33.3 |
| | | 10 | 4 | 40 | |
| | | 10 | 3 | 30 | |
| | 1000 | 10 | 4 | 40 | 43.3 |
| | | 10 | 5 | 50 | |
| | | 10 | 4 | 40 | |
| | 2500 | 10 | 3 | 30 | 43.3 |
| | | 10 | 5 | 50 | |
| | | 10 | 5 | 50 | |
| | 5000 | 10 | 5 | 50 | 50.0 |
| | | 10 | 5 | 50 | |
| | | 10 | 5 | 50 | |
| | 6000 | 10 | 6 | 60 | 63.3 |
| | | 10 | 7 | 70 | |
| | | 10 | 6 | 60 | |
| | 7500 | 10 | 7 | 70 | 66.6 |
| | | 10 | 7 | 70 | |
| | | 10 | 6 | 60 | |
| Control | | 10 | - | - | - |
| | | 10 | - | - | - |
| | | 10 | - | - | - |

# PHARMACOLOGICAL SCREENING

**Introduction**

In Alzheimer's Disease (AD), the brain is characterized by selective neuronal loss, neurofibrillary tangles, and extracellular deposits of insoluble amyloid that form senile plaques. The nucleus basalis of Meynert, the diagonal band of Broca, and the medial setum of the basal forebrain provide cholinergic projection to the cerebral cortex, hippocampus and amygdala. The cholinergic system is responsible for the storage and retrieval of items in memory. Its degradation correlates well with the severity of cognitive and memory impairment. Moreover, the severity of dementia is closely correlated with both synapse and neuronal loss in the neocortex and hippocampus (Plate 16 – 18).

As the cognitive dysfunction and other features of AD are mediated by the loss of function at cholinergic synapses in the neocortex and hippocampus, agents replacing the lost cholinergic function should be useful in the management of this disease.

One strategy for ameliorating symptoms of AD is the restitution of a near normal acetylcholine concentration in the synaptic cleft to enhance cholinergic neurotransmission. Acetylcholinesterase inhibitors reduced the hydrolysis of acetylcholine to boost the endogenous level of acetylcholine in the brain and thereby to boost cholinergic neurotransmission. This resulted in the improvement of cognitive function in mild to moderate AD. Some patients, however, demonstrated a dramatic improvement in cognitive scores that was readily observable in their daily functions

Ursolic acid (UA), a pentacyclic triterpene acid, has been isolated from many kinds of medicinal plants, such as *Eriobotrya japonica, Rosemarinus officinalis, Glechoma hederaceae*. Ursolic acid has been reported ro produce anti-tumor activities, including the inhibition of skin tumorigenisis and inhibition of tumor promotion . It also induced tumor cell differentiation by the regulation of the expression of differentiation-

specific genes in mouse F9 teratocarcinoma cells. In addition, ursolic acid was shown to possess an anti-angiogenic effect on the chick chorioallantoic membrane, and an anti invasive activity on HT1080 human fibrosarcoma cells. It was proved that ursolic acid from *Origanum majorana* appeared to be a potent AChE inhibitor in Azhimer's disease[17].

In our study it was found that chloroform, acetone, ethanol extracts of defatted, shade dried, powdered leaves of *P.granatum* contains ursolic acid and the percentage were determined by HPLC.

The previous report of ursolic acid as potent AChE inhibitor and its presence in the leaves of *P. granatum* which is available freely at lower cost and not used for any other purpose after fruit harvest has inspired us to carryout AChE inhibitor activity.

**METHOD**

Ellman Esterase Assay procedure was performed.The method aims to determine the rate of hydrolysis of acetylthiocholine (ATCh) by acetylcholinesterase(AChE) in tissues taken from laboratory rat[17,62,152,153,154].

**PRINCIPLE**

This assay is a spectrophotometric method, which involves two linked reactions to produce a coloured compound. The production of the compound is monitored by measuring the absorbance of light by the reaction mixture over time. ATCh is hydrolyzed enzymatically to give acetate and thiocholine. Thiocholine reacts with 5,5[1]-dithiobis-2-nitrobenzoic acid(DTNB) producing the yellow coloured 5 thiobis-2-nitrobenzoic acid(TNB). TNB has absorbance maxima at a wavelength of 412 nm.

## MATERIALS AND METHODS

### 1. REQUIREMENTS

| Apparatus/Instrument used | | Name of the Company |
|---|---|---|
| Microplate Reader | : | Fluostar optima, BMG |
| Sonicator | : | Analytical lab suppliers |
| Vortex mixer | : | Serwell instruments Ltd |
| pH meter | : | Thermo |
| Analytical balance | : | Sartorius |
| Micro pipette | : | Thermo |

### 2. MATERIALS

| Apparatus/Instrument used | | Name of the Company |
|---|---|---|
| Eppendorf vials | : | Tarson |
| Pipette tips 2-200 µl | : | Tarson |
| Pipette tips 200- 1000 µl | : | Tarson |
| Beakers | : | Borosil |
| 96 well micro plate(white) | : | Becton Dickinson |
| Distilled water | : | Spectrum |
| Sptula | : | - |

### 3. REAGENTS

| Reagent used | | Name of the Company |
|---|---|---|
| Sodium phosphate(monobasic) | : | Merck |
| Sodium phosphate(Dibasic) | : | Merck |
| Acetylthiocholine iodide(ATCI) | : | Sigma |
| Acetylcholinesterase(AChE) | : | Rat brain tissue |
| 5,5[1]-dithiobis-2-nitrobenzoic acid(DTNB) | : | SRL |
| Tacrine | : | Sigma |

### 4. Reagent preparation

- **Phosphate buffer (0.1 M, pH 8)**

2.69 grams of Dibasic sodium phosphate, 0.146 grams of monobasic sodium phosphate was added in 100 ml of double distilled water(DD water) and pH was adjusted to pH 8 and the volume was made upto 200 ml by adding DD water.

❖ **Enzyme**

100 mg of rat brain tissue was homogenized with 5 ml of phosphate buffer ; it was then aliquoted into vials under sterile conditions and stored at -80°

❖ **Substrate (ATCl)**

Molecular weight of substrate = 289.18 g.

❖ **Ellman reagent (DTNB)**

Molecular weight of DTNB = 396.35 g.

❖ **Control (Tacrine)**

Molecular weight of Tacrine = 234.73 g.

❖ **Sample**

Stock solution = 500 mg of samples were weighed and dissolved in 1000 µl DMSO (Dimethyl sulphoxide)

**PROCEDURE**

Rat striata are homogenized in 10 volumes of 0.1 M phosphate buffer(pH 8.0). Five minutes after the addition of 400 µl of homogenate, 30 µl of test compound solution at various concentrations, and 100 µl of DTNB(10mM) to 2.45 ml of 0.1 M phosphate buffer(pH 8.0) are added and AChE activity is determined at $25^0$ C over 1 minute in a photocell after the addition of 20 µl of acetylthiocholine iodide(ATCl) as substrate.

A substrate blank(i.e., no tissue, only substrate and buffer and DTNB) should be run with each group of assays; this measures nonenzymatic substrate hydrolysis. A tissue blank(i.e., only tissue, buffer and DTNB but no substrate) should be run for each tissue to determine the degree of binding of DTNB to sulfhydryls in the tissue sample. If this is minimal after the preincubation period, then the tissue blank will not be needed on a regular basis for that tissue.

The activity was calculated by using the formula

$$\text{Velocity (V)} = \frac{\text{Change in Absorbance / min}}{\text{molar extinction coefficient} \cdot 13600}$$

SECTION – A

## STANDARDIZATION AND VALIDATION OF ELLMAN METHOD

**1. Standardization of assay using different concentrations of Enzyme**

1. Into the 96 well (white) microtitre plate, 150µl (0.1M Phosphate) buffer in enzyme blank (EB), substrate blank (SB) and 140-50µl buffer in respective activity wells.
2. 50µl enzyme was added in enzyme blank, except in substrate blank well.
3. **10-100µl of enzyme** was added in respective activity wells.
4. 50µl of DTNB was added in all wells.
5. 50µl of ATCI was added in all wells except enzyme blank.
6. Absorbance was read at the intervals of 10 minutes at 405 nm in microplate reader in **duplicate**.

**Table - 12**

**STANDARDIZATION OF ENZYME CONCENTRATION**

| Contents (µl) | EB | SB | A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Buffer | 150 | 150 | 140 | 130 | 120 | 110 | 100 | 90 | 80 | 70 | 60 | 50 |
| Enzyme | 50 | - | 10 | 20 | 30 | 40 | 50 | 60 | 70 | 80 | 90 | 100 |
| DTNB | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| ATCI | - | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |

**Table -13**

**EFFECT OF DIFFERENT VOLUME OF ENNZYME IN AChE ACTIVITY (Fig – 10)**

| S.No. | Volume of Enzyme | Absorbance | Activity |
|---|---|---|---|
| 1. | 10 | 0.04585 | 3.37132 |
| 2. | 20 | 0.077 | 5.66176 |
| 3. | 30 | 0.1088 | 8 |
| 4. | 40 | 0.13465 | 9.90074 |
| 5. | 50 | 0.14955 | 10.9963 |
| 6. | 60 | 0.15215 | 11.1875 |
| 7. | 70 | 0.1554 | 11.4265 |
| 8. | 80 | 0.13485 | 9.91544 |
| 9. | 90 | 0.12475 | 9.17279 |
| 10. | 100 | 0.12755 | 9.37868 |

**Result**

The volume of enzyme at 40µl, 50µl were found to be ideal concentration at which the enzyme activity will be linear. Above the enzyme volume 50µl showed saturation so we performed the same experiment to confirm the suitable concentration from 40µl, 50µl.

Finally, the enzyme at 50 µl volume was selected for the assay at which the enzyme activity will be linear.

**2. Standardization of assay using different concentration of substrate**

1. Into the 96 well microtitre plate, 150 µl buffer in enzyme blank, substrate blank and 100 µl buffer in respective activity wells.
2. 50µl enzyme was added in all wells except in substrate blank
3. 50µl of DTNB was added in all wells.

4. 50µl of ATCI different concentrations from 4mM – 0.125mM was added in respective activity wells (A to Q).
5. Absorbance was read at the intervals of 10 minutes at 405nm in microplate reader.

**Table -14**

**STANDARDIZATION OF SUBSTRATE CONCENTRATION**

| Contents (µl) | EB | SB | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Buffer | 150 | 150 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Enzyme | 50 | - | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| DTNB | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| ATCI | - | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |

**Table - 15**

**EFFECT OF VARIOUS CONCENTRATON OF SUBSTRATE IN AChE ACTIVITY (Fig – 11)**

| S.NO. | Concentration mM [S] | Absorbance. V | Activity | 1/[S] | 1/V |
|---|---|---|---|---|---|
| 1. | 4 | 0.092155 | 6.776103 | - | - |
| 2. | 3.75 | 0.1154 | 8.485294 | - | - |
| 3. | 3.5 | 0.1073 | 7.889706 | 0.285714 | 9.319664 |
| 4. | 3.25 | 0.1047 | 7.698529 | 0.307692 | 9.551098 |
| 5. | 3 | 0.10395 | 7.643382 | 0.333333 | 9.62001 |
| 6. | 2.75 | 0.10825 | 7.959559 | 0.363636 | 9.237875 |
| 7. | 2.5 | 0.1082 | 7.955882 | 0.4 | 9.242144 |
| 8. | 2.25 | 0.10575 | 7.775735 | 0.444444 | 9.456265 |
| 9. | 2 | 0.10935 | 8.040441 | 0.5 | 9.144947 |
| 10. | 1.75 | 0.1121 | 8.242647 | 0.571429 | 8.920607 |
| 11. | 1.5 | 0.11725 | 8.621324 | 0.666667 | 8.528785 |
| 12. | 1.25 | 0.1153 | 8.477941 | 0.8 | 8.673027 |
| 13. | 1 | 0.1083 | 7.963235 | 1 | 9.23361 |
| 14. | 0.75 | 0.11205 | 8.238971 | 1.333333 | 8.924587 |
| 15. | 0.5 | 0.1056 | 7.764706 | 2 | 9.469697 |
| 16. | 0.25 | 0.08975 | 6.599265 | 4 | 11.14206 |
| 17. | 0.125 | 0.0434 | 3.191176 | 8 | 23.04147 |

**Determination of $K_m$ value**

1. Michalis - Menten graph can be plotted by taking [S] in mM concentration on X - axis and V in mM / min on Y - axis.

Michalis - Menten equation :

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

Where, V = Initial rate of production of product

$V_{max}$ – Maximum initial rate of production of product

[S] – substrate concentration

$K_m$ – Michalis - Menten rate constant (The substrate concentration that produces half the maximum velocity)

Lineweaver Burk plot is sensitive to data error and is strongly biased in fitting data in low concentration range. The reciprocal of Michalis - Menten equation gives Lineweaver Burk plot. It was plotted by taking 1/[S] on X – axis and 1 /V on Y – axis (Fig – 12).

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}$$

Y = mx + c

Where, m = slope ($K_m/V_{max}$)

C = Intercept ($1/V_{max}$)

From the graph we got

Y = 1.6114 x + 7.977

$V_{max}$ = 1/c = 0.1253

$K_m = V_{max}$ x m

$K_m$ = 0.2019

**Result**

From $K_m$ value, the substrate at **0.5 mM** was found to be the ideal concentration at which the enzyme activity will be linear.

**3. Standardization of assay using different concentrations of Ellman reagent (DTNB)**

1. Into the 96 well microtitre plate, 150 µl buffer in enzyme blanks, substrate blank and 100 µl buffer in respective activity wells.
2. 50µl enzyme was added in all wells except in substrate blank.
3. 50 µl of DTNB different concentrations from 5.28 mM – 0.0412 mM was added in respective activity wells (A to H).
4. 50 µl of 0.5mM ATCI was added in all wells except enzyme blank well.
5. Absorbance was read at the intervals of 10 minutes at 405nm in microplate reader.

**Table – 16**

**STANDARDIZATION OF ELLMAN REAGENT**

| CONTENTS (µl) | EB | SB | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|---|---|
| Buffer | 150 | 150 | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Enzyme | 50 | - | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| DTNB | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| ATCI | - | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |

**Table - 17**

**EFFECT OF DIFFERENT CONCENTRATON OF ELLMAN REAGENT IN AChE ACTIVITY (Fig – 13)**

| S.No. | Concentration (mM) | Absorbance | Activity |
|---|---|---|---|
| 1. | 5.28 | 0.0975 | 0.0975 |
| 2. | 2.64 | 0.11135 | 0.11135 |
| 3. | 1.32 | 0.11335 | 0.11335 |
| 4. | 0.66 | 0.11945 | 0.11945 |
| 5. | 0.33 | 0.1161 | 0.1161 |
| 6. | 0.165 | 0.11055 | 0.11055 |
| 7. | 0.0825 | 0.10365 | 0.10365 |
| 8. | 0.0412 | 0.0377 | 0.0377 |

**Results**

From this experiment the ideal concentration of DTNB was found to be **0.33 mM**.

**4. Estimation of pH Maxima**

1. Into the 96 well microtitre plate, 150 µl buffer in enzyme blank, substrate blank and 100 µl buffer of different **pH 7 – 9** was added in respective activity wells (A to E)
2. 50 µl enzyme was added in all wells except in substrate blank.
3. 50µl of 0.33mM DTNB was added in all wells.
4. 50µl of 0.5mM ATCI was added in all wells except enzyme blank.
5. Absorbance was read at the intervals of 10 minutes at 405nm in microplate reader.

**Table - 18**

**ESTIMATION OF pH MAXIMA**

| Contents (µl) | EB | SB | ACTIVITY | | | | |
|---|---|---|---|---|---|---|---|
| | | | A | B | C | D | E |
| Buffer | 150 | 150 | 100 | 100 | 100 | 100 | 100 |
| Enzyme | 50 | - | 50 | 50 | 50 | 50 | 50 |
| DTNB | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| ATCI | - | 50 | 50 | 50 | 50 | 50 | 50 |

**Table - 19**

**EFFECT OF pH IN AChE ACTIVITY (Fig – 14)**

| S.No. | pH | Activity (Units) |
|---|---|---|
| 1. | 7 | 9.29 |
| 2. | 7.5 | 11.64 |
| 3. | 8 | 12.92 |
| 4. | 8.5 | 12.045 |
| 5. | 9 | 10.54 |

**Result**

It was proved from the experiment that the buffer in pH 8, showed good enzyme activity, so we have selected **pH 8** for the assay.

**5. Estimation of Temperature Maxima**

1. Into the 96 well microtitre plate, 150 µl buffer in enzyme blank, substrate blank and 100 µl buffer of pH 8 was added in respective activity wells.
2. 50µl enzyme was added in all wells except in substrate blank.
3. 50µl of 0.33 mM DTNB was added in all wells.

4. 50µl of 0.5mM ATCI was added in all wells except enzyme blank.
5. Absorbance was read at the intervals of 10 minutes at 405nm in microplate reader at different temperature from **28 – 42$^0$C**.

**Table - 20**

**ESTIMATION OF TEMPERATURE MAXIMA**

| **Contents (µl)** | **EB** | **SB** | **ACTIVITY** | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | **A** | **B** | **C** | **D** | **E** | **F** | **G** |
| Buffer | 150 | 150 | 100 | 100 | 100 | 100 | 100 | 100 | 100 |
| Enzyme | 50 | - | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| DTNB | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |
| ATCI | - | 50 | 50 | 50 | 50 | 50 | 50 | 50 | 50 |

**Table - 21**

**EFFECT OF TEMPERATURE IN AChE ACTIVITY (Fig – 15)**

| **S.No.** | **Temp** | **Activity (Units)** |
|---|---|---|
| 1. | 28$^0$C | 13.365 |
| 2. | 30 | 12.85 |
| 3. | 33 | 13.49 |
| 4. | 35 | 13.425 |
| 5. | 37 | 13.51 |
| 6. | 39 | 13.245 |
| 7. | 42 | 13.51 |

**Result**

It was proved from the experiment that the temperature at 37$^0$C showed good enzyme activity, so we selected the temperature at **37$^0$C** for the assay.

## Section B

## SCREENING OF SAMPLES FOR AChE INHIBITORY ACTIVITY

### 1. Estimation of AChE inhibitors Tacrine, Ursolic acid and extracts

1. Into the 96 well microtitre plate, 150µl buffer in enzyme blank, substrate blank, 100 µl buffer in activity well and 50µl buffer of **pH 8** was added in activity inhibition well.
2. **50µl enzyme** was added in all wells except in substrate blank.
3. 50µl of 0.**33mM DTNB** was added in all wells.
4. 50µl of **reference standard** ursolic acid, tacrine, were added in activity inhibition well.
5. 50µl of **0.5mM ATCI** was added in all wells except enzyme blank.
6. Absorbance was read at the intervals of 10 minutes at 405nm in microplate reader at **37°C**.

**Table - 22**

**ESTIMATION OF AChE INHIBITORY ACTIVITY FOR TACRINE AND URSOLICACID**

| CONTENTS (µl) | EB | SB | Activity | Inhibition |
|---|---|---|---|---|
| Buffer | 150 | 150 | 100 | 50 |
| Enzyme | 50 | - | 50 | 50 |
| DTNB | 50 | 50 | 50 | 50 |
| Inhibitor | - | - | - | 50 |
| ATCI | - | 50 | 50 | 50 |

**Table - 23**

**EFFECT OF DIFFERENT CONCENTRATION OF URSOLIC**

**ACID IN AChE INHIBITION (Fig – 16)**

| S.No. | Concentration (μM) | % Inhibition | % Inhibition |
|---|---|---|---|
| 1. | 500 | 96.533330 | 95.338870 |
| 2. | 250 | 86.285710 | 86.700770 |
| 3. | 125 | 67.174610 | 71.483380 |
| 4. | 62.5 | 45.968250 | 50.831200 |
| 5. | 31.125 | 28.761910 | 36.636830 |

**Table - 24**

**EFFECT OF DIFFERENT CONCENTRATION OF TACRINE**

**IN AChE INHIBITION (Fig – 17)**

| S.No. | Concentration (nM) | % Inhibition | % Inhibition |
|---|---|---|---|
| 1. | 1600.0 | 89.926620 | 92.524590 |
| 2. | 800.0 | 85.523680 | 88.983600 |
| 3. | 400.0 | 71.914610 | 79.868850 |
| 4. | 200.0 | 65.510340 | 65.311480 |
| 5. | 100.0 | 48.298870 | 49.114750 |
| 6. | 50.0 | 29.686460 | 32.721310 |
| 7. | 25.0 | 18.212140 | 19.213120 |
| 8. | 12.5 | 7.938626 | 7.344262 |

**Table - 25**

**SIGMOIDAL DOSE – RESPONSE (VARIABLE SLOPE)**

| Best fit values | Ursolic Acid | Tacrine |
|---|---|---|
| Bottom | 0.0 | 0.0 |
| Top | 100.0 | 100.0 |
| Log $EC_{50}$ | 1.787 | 2.049 |
| Hillslope | 1.244 | 0.9806 |
| **$EC_{50}$** | **61.19** | **111.8** |
| Std. Error | | |
| Log $EC_{50}$ | 0.02194 | 0.01580 |
| Hill slope | 0.08550 | 0.03376 |
| 95% Confidence Intervals | | |
| Log $EC_{50}$ | 1.736 to 1.837 | 2.015 to 2.082 |
| Hill slope | 1.046 to 1.441 | 0.9082 to 1.053 |
| $EC_{50}$ | 54.46 to 68.75 | 103.4 to 120.9 |
| Goodness of Fit | | |
| Degrees of Freedom | 8 | 14 |
| $R^2$ | 0.9820 | 0.9940 |

**2. SCREENING OF AChE INHIBITORY ACTIVITY FOR VARIOUS EXTRACTS OF LEAF *P. granatum***

**Preparation of the test solution :**

Test solution for the evaluation of AChE inhibitory activity were prepared by dissolving the dried extracts in DMSO (Dimethyl Sulphoxide).

The percentage inhibition and $IC_{50}$ data for all the extracts were presented in table No 25.

**Table - 26**

**EFFECT OF DIFFERENT CONCENTRATION OF VARIOUS EXTRACTS IN AChE INHIBITION (Fig – 18, 19, 20)**

| S.NO. | Concentration (mg/ml) | % Inhibition of Extracts | | | |
|---|---|---|---|---|---|
| | | Chloroform | Acetone | Ethanol | Aqueous |
| 1. | 4 | 94 | 76 | 90 | Nil |
| 2. | 2 | 51 | 48 | 54 | Nil |
| 3. | 1 | 26 | 27 | 29 | Nil |
| 4. | 0.5 | 12 | 11 | 14 | Nil |
| 5. | 0.25 | 1.3 | 6.4 | 2 | Nil |

**Table - 27**

**SIGMOIDAL DOSE – RESPONSE**

| Best fit values | Type of Extract | | |
|---|---|---|---|
| | Chloroform | Acetone | Ethanol |
| Bottom | 0.0 | 0.0 | 0.0 |
| Top | 100.0 | 100.0 | 100.0 |
| Log $EC_{50}$ | 0.2563 | 0.3329 | 0.2507 |
| **$EC_{50}$** | **1.804** | **2.152** | **1.781** |
| Std. Error | | | |
| Log $EC_{50}$ | 0.1469 | 0.06484 | 0.1121 |
| 95% Confidence Intervals | | | |
| Log $EC_{50}$ | 0.1514 to 0.6640 | 0.1662 to 0.4996 | -0.03752 to 0.5389 |
| $EC_{50}$ | 0.7057 to 4.613 | 1.466 to 3.160 | 0.9172 to 3.458 |
| Goodness of Fit | | | |
| Degrees of Freedom | 4 | 5 | 5 |
| $R^2$ | 0.8307 | 0.9451 | 0.8871 |

The data were represented by sigmoidal dose response graph (Fig. 16 – 20).

**Result**

The percentage inhibition and $IC_{50}$ data for tacrine, ursolic acid, and various extracts were presented in Table No 27.

**Table – 28**

**COMPARISON OF $IC_{50}$ VALUE AND PERCENTAGE INHIBITION OF STANDARD AND EXTRACTS IN AChE INHIBITION**

| S.NO. | Standard and Extracts | Concentration | % Inhibition | $IC_{50}$ |
|---|---|---|---|---|
| 1. | Tacrine | 1600 nM | 93% | 111.8nM |
| 2. | Ursolic acid | 500 μM | 95% | 61.19μM |
| 3. | Chloroform extract | 4 mg / ml | 94% | 1.8mM |
| 4. | Acetone extract | 4 mg/ml | 76% | 2.1mM |
| 5. | Ethanol extract | 4 mg/ml | 90% | 1.8mM |

# RESULTS AND DISCUSSION

This dissertation covers the investigations on pharmacognostic, phytochemicals and invitro AChE inhibition of the various extracts of defatted, shade dried, powdered leaves of ***P.granatum***, var.: Ganesh, Family:***Punicaceae***.

**In Chapter – 1 : Introduction,** We discussed medicinal plants global view → WHO views on medicinal plants – Status of India → Contribution by Tamilnadu → Modern medicine demerits → Natural products for modern medicine → Priorities in medicinal plant research → phytonutrients – The natural drugs of the future → Degenerative diseases → Alzheimer's disease → Symptoms of AD → Characteristics of AD → Current treatment → List of medicinal plants having AChE inhibition → Ursolic acid and AChE inhibition → reason for selection of plant ***P.granatum***.

**In Chapter 2** : **Review of Literature,** the various literature available were categorized under ethnomedical, pharmacognostical, phytochemical, pharmacological and biological screening of entire plant, aerial parts, flower, fruit, fruit juice, peel, rind, pericarp, leaves, bark root and acute toxicological study methods and AChE inhibitory activity screening methods were reviewed.

**In Chapter 3** : **Aim and Objective** were set to obtain a potential benefit to treat Alzheimer's disease by FDA approved method of AChE inhibition by considering the following reasons.

- A new effective plant based, easily available AChE inhibitor to provide a new dimension in the therapy of Alzheimer's disease.
- Hence to provide significant contribution to the chemotherapy of this disease by natural product derived compound in future in an affordable way to reduce the incidence of Alzheimer's disease.

**Chapter : 4 Pharmacognositc studies**

**Section : A**

Taxonomy, synonym, vernacular name, subspecies, geographical distribution, habit & habitat, subspecies which includes morphological description of all parts, of the plant and further climate, soil condition, manuring of the cultivation varieties of ***P.granatum*** were discussed in detail to identify the plant with support of photographs, and line drawings as an establishment of authenticity (Plate – 1 to 7, Fig.: 1)

**Morphology of the Leaf**

Size : 2 - 8 cm

Shape : Oblong or Obovate

Colour : Green

Margin : Entire

Base : Narrowed into a very short petiole

Odour : Agreeable Odour

Taste : Tasteless

**Section - B** deals with the microscopic studies of leaves of ***P.granatum*** to ascertain the arrangement of tissues (Plate No - 8 - 12). The T.S. of leaf through midrib of mature, young leaf and the powder analysis showed the following features (Fig - 3).

The leaf has thick semicellular midrib and thin lamina. In cross sectional view, the lamina exhibits distinct dorsiventral symmetry.

**Midrib**

Has wide dorsal concavity and thick hemispherical abaxial part.

**Epidermal Cells**

Are either rectangular or polygonal with slightly wavy anticlinal walls and cells are thin cuticular striations.

**Abaxial epidermis**

Has stomata anomocytic (Ranunonculaeous), no distinct subsidiary cells are seen. Walls are undulate, amoeboid out lines and guards cells are oblong, elliptic.

**Vascular bundle**

Simple, wide and bowel shaped with adaxial cavity.

## Xylem

Dense parallel lines, narrow thick walled angular in out line.

## Pholem

Thin, present in abaxial layer.

## Lamina

80 μm thick and has thick adaxial epidermis which consists of wide rectangular cells 20 μm in thick.

## Mesophyll

Single layer adaxial palisade cells, 20-30 μm in height, spongy parenchyma about five layers of large circular closed arranged cells.

### Idioplast

Specially modified, dilated cavities bearing calcium oxalate crystals are frequently seen. Crystals may be prismatic (20-40 μm thick) or druses (30μm thick).

### Venation Pattern

Secondary and tertiary veins are uniformly thin and wavy. Some vein islets are distinct. The vein terminations are short, straight ends or simple.

### Petiole

The proximal end of petiole side semi circular with wide abaxial, and concavity two short and thick lateral wings, 1.2mm in vertical axis and 1.4mm in horizontal axis.

**Distal end**

The distal end of petiole has deep 'V' shape adaxial groove, long and prominent wings, which is 1mm vertical, 1.25mm horizontal, epidermal layer of small squarish cells. Phloem prominent in the lower part of the xylem, vascular strand is 600µm wide, 250µm thick.

**Section - C** deals with the quantitative microscopy in terms of microscopical and physical parameters and the results were tabulated from table No. 1 to 6.

The following evaluations were carried out for leaf

| S.No. | Parameters | Minimum | Average | Maximum |
|---|---|---|---|---|
| 1. | Vein islet Number | 24 | 31 | 37 |
| 2. | Vein termination number | 21 | 24 | 28 |
| 3. | Stomatal Number (lower epidermis) | 187 | 205 | 217 |
| 4. | Stomatal Index (lower epidermis) | 9.42 | 10.84 | 12.24 |
| 5. | Total Ash (% W/W) | 10.78 | 11.10 | 11.49 |
| 6. | Acid insoluble ash (% W/W) | 1.46 | 1.51 | 1.56 |
| 7. | Water soluble ash (% W/W) | 2.52 | 2.8 | 3.02 |
| 8. | Percentage of Loss on Drying | 8.50 | 8.82 | 9.10 |

## EXTRACTIVE VALUES (SUCCESSIVE SOLVENTS EXTRACTION)

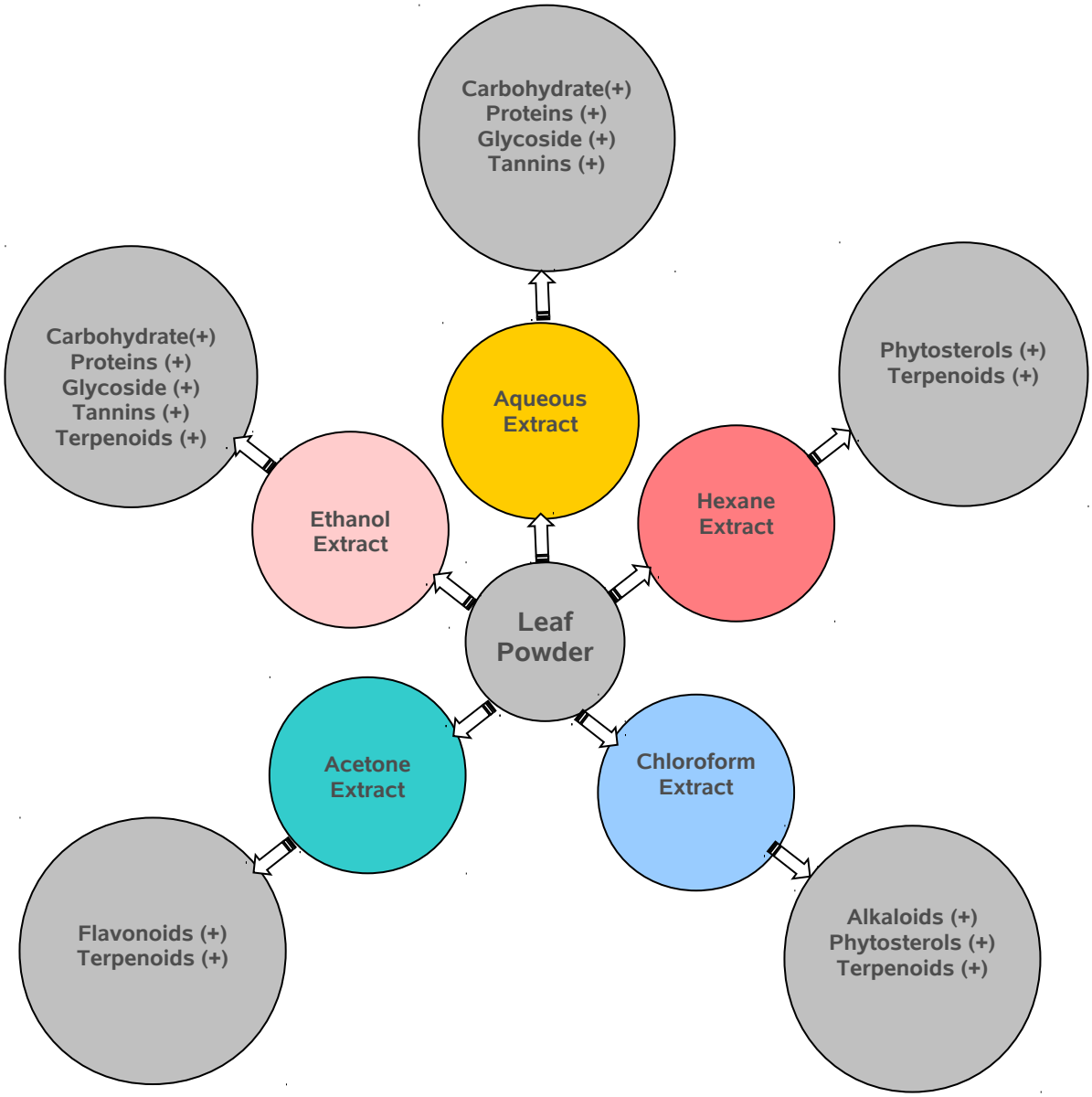
| S.NO. | Solvents | Successive Solvents Extractive Value (%) |
|---|---|---|
| 1. | Hexane | 1.13 |
| 2. | Chloroform | 2.66 |
| 3. | Acetone | 7.66 |
| 4. | Ethanol | 11.13 |
| 5. | Water | 16.8 |

## Chapter – 5 – Preliminary Phytochemical Studies

**Section – A** deals with the preliminary qualitative phytochemical examination of the powdered leaves of ***P.granatum*** and results were tabulated from Table No. : 7.

| Part | Constituents Present | Constituents Absent |
|---|---|---|
| Leaf Powder | Alkaloids, carbohydrates, proteins, phytosterol tannins, flavonoids, terpenoids, glycosides. | Volatile Oil, Fixed oil, mucillage |

The preliminary qualitative phytochemical examination of the extract obtained by individual extraction of powdered drug using various solvents of increasing polarity were carried out and the results were tabulated from Table No. 8.




Schematic representation of various constituents present in the various extracts of leaves ***P. granatum L.***

**Section – B** deals with the preparation of the Chloroform, acetone, ethanol, and aqueous extracts to determine the percentage of ursolic acid present and to screen them for AChE inhibitory activity depending upon the presence of ursolic acid.

The presence of ursolic acid in the chloroform, acetone, ethanol and aqueous extracts were identified by Co-TLC and the $R_f$ value of ursolic acid was found to be 0.25 (Table – 9, Plate - 13).

The estimation of ursolic acid content in the chloroform, acetone, ethanol and aqueous extracts were done by using HPLC analysis (Table – 10, Fig – 4 - 8)

| S.No. | Type of Extract | Description | Percentage of Ursolic Acid (% w/w) |
|---|---|---|---|
| 1. | Chloroform | Black Powder | 20.34 |
| 2. | Acetone | Dark Brown paste | 0.32 |
| 3. | Ethanol | Greenish Brown paste | 0.15 |
| 4. | Water | Brown Lumps | Nil |

**In Chapter - 6** The Acute toxicological study was carried out for the chloroform extract of ***P.grantum*** by Brine Shrimp Lethality Bioassay (Plate – 14,15).

This method is attractive because it is very simple, inexpensive and low concentration is sufficient to perform the test in micro well scale.

The methodology for cytotoxicity bioassay was discussed in this chapter. The percentage lethality was determined by comparing the mean surviving larvae of the test and control tubes.

The mortality of larvae was calculated after 24 hrs, by using Abbot's formula, it was corrected and tabulated in Table 11. The percentage of mortality recorded in the chloroform extract were 23.3, 23.3, 33.3, 43.3, 43.3, 50, 63.3, 66.6 at 100, 250, 500, 1000, 2500, 5000, 6000 and 7500 ppm respectively (Fig – 9).

The $LC_{50}$ values were obtained from the regression plot by plotting concentration verses % lethality. Podophyllotoxin was used as a positive control – 100% mortality at 3 ppm.

**The $LC_{50}$ values suggested that chloroform extract is non toxic, The $LC_{50}$ values for chloroform extract by BSLB was 2100 ppm and it need further evaluation in animal model by acute and chronic toxicological study**.

**Chapter – 7**

**Section : A** deals with description about the Alzheimer's disease, its manifestation, current therapy and its limitations, need for the research were dealt in the introduction (Plate – 16, 17, 18).

- The AChE assay was performed according to the colorimetric method of Ellman et al using ATCI as a substrate[153].
- Standardization & Validation of AChE inhibitory activity.

- Initially a test trial was conducted to standardize various parameters like pH, temperature and the suitable concentrations for AChE enzyme, substrate, reagent (DTNB). The results were tabulated in Table 12 - 20 and represented graphically. From the result it was clear that the pH maxima = 8, and temperature maxima = $37^0$C. The optimum concentrations of AChE enzyme, substrate and reagent were found to be 50µl, 0.5mM and 0.33 mM respectively (Fig 10– 15).

**Section : B** deals with the Screening of AChE inhibitory activity of various extracts of leaves of *P.granatum*:

From the results of HPLC the percentage of ursolic acid in the various extracts prepared successively using defatted, shade dried, powdered leaves of ***p.granatum*** for chloroform, acetone, ethanol, were 20.34, 0.32, 0.15 respectively. Since all these extracts containing ursolic acid, we planned to carry out AChE inhibitory activity of these extracts along with aqueous, prepared from the above marc, since it is economic, if it is effective.

Chloroform, acetone, ethanol, aqueous extracts obtained from defatted, shade dried, powdered leaves of ***P.granatum*** by successive extraction were screened by using 0.25, 0.5, 1, 2, 4 mg/ml

The percentage inhibition for reference standard and various extracts were presented in (Table No- 22,23,25).

**EFFECT OF DIFFERENT CONCENTRATION OF URSOLIC**

**ACID IN AChE INHIBITION**

| S.No. | Concentration (μM) | % Inhibition | % Inhibition |
|---|---|---|---|
| 1. | 500 | 96.533330 | 95.338870 |
| 2. | 250 | 86.285710 | 86.700770 |
| 3. | 125 | 67.174610 | 71.483380 |
| 4. | 62.5 | 45.968250 | 50.831200 |
| 5. | 31.125 | 28.761910 | 36.636830 |

**EFFECT OF DIFFERENT CONCENTRATION OF TACRINE**

**IN AChE INHIBITION**

| S.No. | Concentration (nM) | % Inhibition | % Inhibition |
|---|---|---|---|
| 1. | 1600.0 | 89.926620 | 92.524590 |
| 2. | 800.0 | 85.523680 | 88.983600 |
| 3. | 400.0 | 71.914610 | 79.868850 |
| 4. | 200.0 | 65.510340 | 65.311480 |
| 5. | 100.0 | 48.298870 | 49.114750 |
| 6. | 50.0 | 29.686460 | 32.721310 |
| 7. | 25.0 | 18.212140 | 19.213120 |
| 8. | 12.5 | 7.938626 | 7.344262 |

**EFFECT OF DIFFERENT CONCENTRATION OF VARIOUS EXTRACTS IN AChE INHIBITION**

| S.NO. | Concentration (mg/ml) | % Inhibition of Extracts | | | |
|---|---|---|---|---|---|
| | | Chloroform | Acetone | Ethanol | Aqueous |
| 1. | 4 | 94 | 76 | 90 | Nil |
| 2. | 2 | 51 | 48 | 54 | Nil |
| 3. | 1 | 26 | 27 | 29 | Nil |
| 4. | 0.5 | 12 | 11 | 14 | Nil |
| 5. | 0.25 | 1.3 | 6.4 | 2 | Nil |

The data were represented by sigmoidal dose response graph. (Figure 16 - 20).

**COMPARISON OF $IC_{50}$ VALUE AND PERCENTAGE INHIBITION OF STANDARD AND EXTRACTS IN AChE INHIBITION**

| S.No. | Standard and Extracts | Concentration | % Inhibition | $IC_{50}$ |
|---|---|---|---|---|
| 1. | Tacrine | 1600 Nm | 93% | 111.8nM |
| 2. | Ursolic acid | 500 μM | 95% | 61.19μM |
| 3. | Chloroform extract | 4 mg / ml | 94% | 1.8mM |
| 4. | Acetone extract | 4 mg/ml | 76% | 2.1mM |
| 5. | Ethanol extract | 4 mg/ml | 90% | 1.8mM |

From the study the percentage inhibition and $IC_{50}$ value for chloroform & ethanol extracts were found there 94, 1.8 mM and 90, 1.8mM respectively. **The % inhibition and $IC_{50}$ value for chloroform, ethanol extracts were promising at 4 mg/ml concentration level in a dose dependent manner.**

**The result shows that the Chloroform and ethanol extracts were active at 4mg/ml concentration but less comparable to the standard drugs tacrine and the triterpenoid ursolic acid which are active at 1600 nM and 500μM concentration respectively.**

**It was also observed that acetone extract was comparable with that of chloroform, ethanol extract in the same concentration level in dose dependent manner (76% inhibition, $IC_{50}$ 2.1mM).**

**But aqueous extract did not inhibit the AChE enzyme activity in the same concentration level. All other extracts showed greater than 75% inhibition at 4 mg/ml concentration. In the light of the present results we can expect the leaves of *P.grantum* to be helpful in neurodegenerative disorders like AD by reducing the enzymatic degradation of acetylcholine which is only currently approved treatment of AD by USFDA and EMEA.**

**The component responsible for AChE inhibition activity in the chloroform extract may be due to the higher concentration of ursolic acid (20.34%). Since it was already reported that ursolic acid act as AChE inhibitor in a dose dependent and competitive / non-competitive manner with a $K_i$ value of 6 pM representing the affinity of the enzyme and inhibitor.**

**The alcohol, acetone activity in these investigation may act on different mechanism though it contain low concentration of ursolic acid 0.32%, 0.15% respectively.**

# CONCLUSION

This dissertation covers the pharmacognostic parameters of the leaves of ***Punica granatum***, **Var. Ganesh** belonging to the family***:*** ***Punicaceae***, such as macroscopical, microscopical including powder analysis and physical standards like ash values, extractive values, etc have been studied and presented.

- Microscopical studies reveals the presence of calcium oxalate prismatic crystals, druses in the mesophyll region and uniform parallel striations in the upper epidermis along with usual leaf tissues.
- The preliminary phytochemical studies reveals the presence of alkaloid, carbohydrate, glycoside, phytosterol, tannins, proteins, flavonoids, terpenoids in leaf powder.
- TLC of the chloroform, acetone and ethanol extracts of leaves of *P.granatum* shows the presence of ursolic acid.
- The percentage of ursolic acid in chloroform, acetone, ethanol extracts were determined by **HPLC** as 20.34, 0.32, 0.15 respectively.
- The commercial exploitation of ursolic acid is recommended.

**Acute toxicological study** In the present study it was confirmed that the chloroform extract was nontoxic and the $LC_{50}$ value was 2100 ppm and it need further evaluation in animal model by acute & chronic toxicological studies.

**Preliminary invitro AChE inhibitory activity**

The Ellman method for the Acetyl cholinesterase assay was standardized and validated.

The preliminary observation of the various extracts of leaves of *P.granatum* by invitro study showed significant AChE inhibitory activity in the following order in a dose dependent manner .

Chloroform extract > ethanol extract > acetone extract.

The $IC_{50}$ value for chloroform acetone, ethanol extracts were 1.8, 2.1, 1.8mM respectively. It is comparable with the standard drug tacrine.

It is concluded that it can be optimistic that the present investigation present a herbal drug of a safe and potential chemotherapeutic target in the treatment of Alzheimer's disease and commercial exploitation of ursolic acid extraction. AChE inhibitors, which enhance cholinergic transmission by reducing the enzymatic degradation of acetylcholine, are the only source of compound currently approved for the treatment of Alzheimer's Disease (AD) by U.S. Food and Drug Administration (USFDA) and European medicines Agency (EMEA).

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