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The potential acetylcholine esterase inhibitory activity (*InVitro*)(AChE) of *Citrus limetta* Risso.. Leaf essential oil targeting Alzheimer's disease treatment

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ABSTRACT

The major role of the central nervous cholinergic pathway in learning and memory and the generation of cholinergic deficits with several neuro degenerative disorders and cognitive impairment contribute to the development of symptomatic cholinergic therapy. Inhibition of brain cholinesterase (ChE) serves as a strategy for the treatment of several cognitive diseases such as Alzheimer disease (AD), senile dementia, ataxia, myasthenia gravis, glaucoma, and Parkinson disease. Inhibition of Acetyl cholinesterase (AChE) is considered as the important therapeutic strategy against Alzheimer's disease (AD). Many plant derived phytochemicals have shown AChE inhibitory activity. In the present study, essential oil isolated from the leaves of *Citrus limetta*(EOPNL) used in Indian Ayurvedic system of medicine for improving cognitive function, was screened for acetylcholine sterase inhibitory activity by Ellman's microplate colorimetric method. The percentage AChEinhibition for 0.5,1,1.5 µl/ml of EOCLL were 59,76,92 respectively and for the standard drug tacrine was 65, 80, 89, 93 at 200, 400,800,1600Nmrespectively.GC-MS Profile of the EOCLL showed the presence of nerolidol, β-caryophyllene, cubein, globulol, α -pinene, α - terpinene, elemol, α–bisbolol, β- bisabolene, β-elemene. Acute toxicity assessment using BSLA showed safety of the EOCLL without any symptoms of toxicity .Conclusively it was optimistic that the volatile oil isolated from the leaves of *C.limetta*. is a safe and potential chemotherapeutic target in the treatment of Alzheimer's disease and commercial exploitation.

Keywords: *Citrus limetta, Rutaceace, AChE inhibitor, Neuroprotection.*

1. INTRODUCTION

Bioactive compounds of plant origin especially essential oils have received great scientific attention in the recent decades as novel therapeutic agents that could aid the healing process owing to their antioxidant, anti inflammatory and anti microbial effects. [1] *Citrus limetta* (Rutaceae) is popularly known, kolumichai in Tamil, It was reported that the GC-MS analysis of the isolated E.O from the leaves indicated the presence of following constituents D.Limonene, α –pinene, α-terpineol, citronelal, α– bisbolol, camphene, β- bisabolene, nonanal, borneol, geranial, linalool, bergamol.

The survey of literature on *C.limetta* also reveals that leaves contain various phytoconstituents like alkaloid especially flavonoid compounds. Leaves of *C.limetta* is traditionally known to be useful for the treatment of wide panel of diseases like epileptic seizures, hypertension, prostate disorder, giddiness, rheumatism, wound infection, cough, sore throat, fever, wounds, tooth decay, gastric ulceration, reproductive problems, etc. Various scientific investigation of the leaves showed and as antioxidant, anti- platelet aggregation, antimicrobial, sedative, digestive, hemostatics, diuretics, analgesics, anti-inflammatory and cardiovascular diseases. anti-diabetic activity, antiulcer, hepato- protective, antihypertensive and wound healing activity. Root traditionally used for liver diseases, tuberculosis, asthma, diabetes, hypertension, toothaches, anaemia, etc. Fruit is used for malaria, stomach pain, liver and spleen enlargement, anaemia, to induce lactation, to increase lactation, to lower hypertension and as haemapoitic, anti- microbial, sedative, diuretic and digestive, expectorant, vermifuge, etc. Researches proved its anticancer, immunomodulatory, erythropoitic,diuretic, antifungal, anti-bacterial hypertensive effect, anti-diabetic, hepatopro- tective, cardiac activities, anti-inflammatory effect etc. [2-5]

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 .As the cognitive dysfunction dementia is closely correlated with both synapse and neuronal loss in the neocortex and hippocampus .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. Acetylcholi- nesterase 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. 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 acetylcholine stetase, responsible for breakdown of acetyl choline in the neural synapse. 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 [6] So, it is necessary to search for AChE inhibitors with lower side effects from natural resources.Medicinal plants are used in the treatment of neurological disorders quite longer time, for convulsion, stroke and epilepsy, that is, *Poria cocos*, *Polygala tenuifolia*, *Uncaria rhynchophylla*, *Ginkgo biloba*, and *Lycium barbarum.*Recent pharmacological studies revealed that *Ginkgo biloba* possessed neuroprotective effects towards D- galactose, beta amyloid and ischemia-induced neuronal death. *Uncaria rhynchophylla* also prevented D-galactose, beta-amyloid, 6-hydroxydopamine and kainic acid-induced neurotoxicity. [7-16]

The economic aspect of this crop evidently proved it as commercial crop. In fact the revenue generated by this crop can be further magnified by many folds, if its medicinal applications are scientifically explored well. Its traditional use in nervous disorders and the presence of considerable quantity of EO prompted us to investigate its protective effect on neurotoxicity for the development of drug for neurodegeneration like Alzheimer‟s disease.

1.1. Collection and Authentication

The leaves of the healthy plant *C.limetta* Risso. selected for our study was collected from **Annavasal**, Pudhukkottai Tamil Nadu, India, during the month of Jun 2017 and was authenticated by Dr. Stephen, Department of Botany, American college, Madurai.

1.2. Isolation of essential oil from the leaves

The leaves were dried at room temperature under shade, powdered, sieved (60mesh) and stored in a well closed container. From the dried plant EO is isolated (EOCLL) by hydrodistillation using Clevenger apparatus and analyzed by GC-MS.

1.3. Identification of compounds present in the essential oil of the leaves by

GC-MS

JEOL GC MATE 11 model used, Column HP 5ms, carrier gas high pure helium gas with flow rate

1ml/mt, oven temp 50-250 deg/min, Mass analyser quadrupole with double focusing, with photon multiplier tube.

1.4. Acute toxicity study using BRINE SHRIMP (*Artemia Nauplii)* LETHALITY BIOASSAY (BSLA)

In order to study the toxicity of the EOCLL we performed Brine Shrimp Lethality Bioassay which based on the ability to kill laboratory cultured brine shrimp (*Artemia nauplii*). The brine shrimp 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. [17,18]

1.5. Toxicity assessment of EOCLL of the leaf

Ten free swimming hatched out *nauplli* were drawn through a glass capillary and placed in each vial containing 4.5 ml of brine solution. In each experiment, 0.5ml brine solution containing various concentration of EO (ppm) was added to 4.5 ml of brine solution and maintained at room temperature for 24 hrs under the light then surviving larvae were counted. Experiment was conducted along with control (vehicle treated), different concentrations of the EO (100-1000 ppm) in a set of three tubes per dose. The percentage lethality was determined by comparing the mean surviving larval of the test and control tubes. LC50 value was obtained from the best – fit line, plotted concentration verses percentage lethality. Podophyllotoxin was used as a positive control in the bio assay.

1.6. Acetylcholinesterase Inhibitory Activity

Ellman Esterase Assay procedure was performed.The method aims to determine the rate of hydrolysis of acetylthiocholine (ATCh) by acetylcholinesterase(AChE). 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. [19-23]

Eppendorf vials, 96 well micro plate(white). Reagents: Sodium Phosphate(monobasic) (Merck), Sodium phosphate(Dibasic) (Merck), Acetylthiocholine iodide(ATCI) (Sigma), Acetylcholinesterase (AChE) (Rat striata-sigma), 5,5[1]-dithiobis-2-nitrobenzoic acid(DTNB) (SRL), Tacrine (Sigma).

2. MATERIALS

Microplate Reader,(Fluostar optima, BMG) Sonicator, Vortex mixer, Micro pipette

2.1. METHOD

Homogenized rat striata 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, (0.5,1,2µl/ml), tacrine 1600nm 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(ATCI) 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.

Velocity(V) = Change in Absorbance / min molarextinctioncoefficient13600

3. RESULTS

Percentage of essential oil was found to be 0.2 to 0.35 % of dry leaf powder. Greenish yellow in colour, aromatic odour, Bitter Aromatic taste, Slightly viscous, Soluble in petroleum ether, toluene, chloroform and ethanol, immiscible in water, refractive index 1.4745, specific gravity 0.6798 at 20ºC, optical rotation 1.364 at 20ºC. GC-MS Profile of the EOCLL showed the presence of d-limonene, β-pinene, α-pinene, α-terpineol, Citronellal, α-Bisabolol, Camphene, β-Bisabolene, Nonanal, Borneol, Geranial, Linalool, Bergamol. In continuation of our efforts to verify the safety of EO, we performed Brine shrimp lethality assay (BSLA) using free swimming hatched out *Artemia nauplii* which based on the ability to kill laboratory cultured brine shrimp. It was observed that 100% of mortality above 800 ppm for EOCLL. LC50 for EOCLL was about 419ppm in 24hrs.100% mortality was observed at 3ppm for podophyllotoxin positive control. This prescreen showed safety of the EOCLL without any symptoms of toxicity.

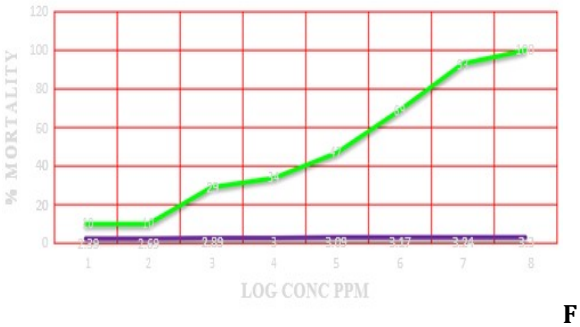

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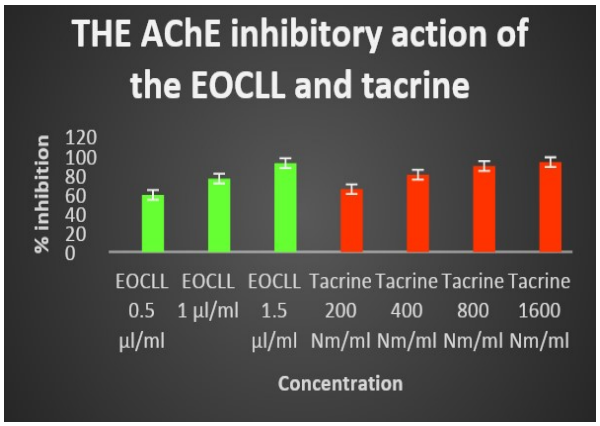
Figure -1: Acute toxicity study of eocll

The Ellman method for the Acetyl cholinesterase inhibitory assay was perfomed . The preliminary observation of the EOCLL by *in vitro* study showed significant AChE inhibitory activity dose dependent manner .The percentage AChE inhibition for 0.5,1,1.5 µl/ml of EOCLL were 59,76,92% respectively. (Figure,2)The inhibition for the standard drug tacrine was 65,80,89,93% at 200,400,800,1600Nm respectively. IC50 for EOCLL and tacrine were 0.5 µl/ml,111.8nm respectively.



4. DISCUSSION

Essential oils (E.O) are valuable natural products find applications in much area, including pharmaceuticals, cosmetics, perfumes, aromatherapy, phytotheraphy, spices etc. Attention of many scientists was attracted towards the screening of plants to study the biological activities of their oils from phytochemical and pharmacological to therapeutic aspects. This may be hopefully lead to new directions on plant applications and new perspectives on the potential therapeutic use of these natural products. E.O' are complex mixtures comprising many single compounds. The knowledge of its composition permits for a better and specially directed application. Esssential oils consist of monoterpenes and sesquiterpenes which are the lipophilic secondary metabolites of plants.Neurodegenerative diseases represent a group of chronic disorders characterized by progressive and selective decline in neuronal and cognitive functions, found in about 5% of reported cases of brain diseases . The unique pattern by which each neurodegenerative disease causes progressive neuronal damage and their ability to produce disease-specific cellular biomarkers have been of importance in their classification . Alzheimer's disease (AD) is a neurodegenerative disorder characterized by decline in acetylcholine (ACh) neurotransmitter, deposition of senile plaques, neurofibrillary tangles and progressive loss of cognitive function . Several studies have reported the potential neuroprotective properties of essential oil from a number of plants used either alone or in combination with other plants in many traditional medical practices. In one study, essential oil from clove bud (*Syzygium aromaticum* (L.) Merr. & Perry) and Ethiopian pepper (*Xylopia aethiopica* Dun. A. Rich, Annonaceae) showed neuroprotective properties in vitro by exhibiting anticholinesterase and antioxidant activities . These essential oils also exhibited membrane-stabilizing properties by inhibiting quinolinic acid induced lipid peroxidation in rat brain homogenate . In another study, essential oils from peels of sweet orange (*Citrus sinensis* (L.) Osbeck) and lemon (*Citrus limon* (L.) Osbeck) were investigated for their in vitro antioxidant and membrane stabilizing properties, and inhibitory activity of acetylcholinesterase(AChE)and butyrylcholinesterase (BChE) enzymes. The authors observed that, based on these biological activities, essential oils could be used in the treatment of neurodegenerative disorders, especially Alzheimer's disease.[24] The present study has been designed to investigate the nootropic potential of EOCLL using the Ellman AChE inhibitory assay using ATCI as a substrate. From the study the percentage inhibition and IC50 value were promising at 2µl/ml in dose dependent manner comparable to the standard drug tacrine(1600nm).The preliminary acute toxicity study showed the nontoxicity o the EOCLL.

It is concluded that it can be optimistic that the present investigation present essential oil isolated from the leaves of *C.limetta* is a safe and potential chemotherapeutic target in the treatment of Alzheimer's disease and commercial exploitation. AChE inhibitors, which enhance cholinergic transmission by reducing the enzymatic degradation of acetylcholine, are the source of compound currently approved for the treatment of Alzheimer's Disease (AD). The above finding require the detail investigation for its exact mechanism of action which can result in complementary to those of existing AChE inhibitor agents to deduce a definite conclusion. This plant may provide inexpensive, safe drug template.

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