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VOLATILE OIL OF THE LEAVES OF *P.nigrum* L. ALLEVIATES BETA AMYLOID PATHOLOGY IN *Drosophila melanogaster*

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ABSTRACT

Objective: To prescreen the *in vivo* neuro protective activity of the VO of the leaves of *P.nigrum* family Piperaceae commonly called as pepper, using the model organism Aβ42-amyloid neuro toxicity induced *Drosophila melanogaster*. **Method:** Isolation of leaf VO and its analysis by GC-MS were carried out. Acute toxicity assessment were also performed. The neuro protective effect of the VO of the leaves of *P.nigrum* (VOPNL) *in vivo* was evaluated on the transgenic Aβ42 model of *Drosophila melanogaster*, a novel model system for screening drugs for Alzheimer disease by Longivity Assay, Climbing Assay, Pseudopupil assay and nail polish imprint technique and SEM. **Results:** GC-MS Profile of the VO showed the presence of nerolidol, β-caryophyllene, cubein, globulol, α -pinene, α -terpinene, elemol, α–bisbolol, β- bisabolene, β-elemene. Toxicity assessment using brine shrimp lethality bioassay (BSLA) of the VOPNL showed nontoxic upto LC_{50} 500ppm. The VOPNL possesses potential *in vivo* neuro protective activity on *Drosophila melanogaster* against beta amyloid induced neuronal toxicity. **Conclusion:** We investigated the *in vivo* neuroprotection of the VOPNL on the representative of Alzheimer disease model of transgenic *Drosophila* by overexpression of beta amyloid protein, Aβ42. In the present study we have presented the first evidence that the VO of the leaves could significantly ameliorate the adverse morphological changes from Aβ42 protein in *Drosophila*, as indicated by prolonging the lifespan, by improving locomotor abilities and rescuing neurodegeneration in ommatidia of Aβ expressing *Drosophila* which is comparable with donepezil. So it demonstrated the novel use of VOPNL against Aβ induced neurodegeneration which may be mediated through the multiple components of the VO. Based on this findings we suggest developing VO of the leaves of *P.nigrum* as a potential therapeutic intervention for neurodegenerative diseases like Alzheimer's disease. VOPNL effectively protect, rescue and most importantly restore the impaired movement activity (i.e climbing capability) in *Drosophila melanogaster*, a valid model of AD. It provides a framework for future studies to assess the suppression of oxidative stress and to restore and or maintain locomotor activity in AD patients. Elucidation of their mechanism of action will provide new insight for new targets.

KEYWORDS: *Piper nigrum*, Piperacae, Neurooprotective, *Drosophila melanogaster*, Voaltile oil, Alzheimer disease.

INTRODUCTION

Beta-amyloid (Aβ) protein plays a vital role in Alzheimer's disease (AD). Although the mechanism of the disease is unknown, the deleterious effect of beta-amyloid is quite clear. The protein self-aggregate into a plaque[1], which lead to the generation of reactive oxygen species, membrane potential disruption and increased vulnerability to excite toxicity and cause eventually neuronal death[2] and related cognitive defects.[3] Recent report revealed an increasing prevalence of dementia all over the world, from 36 million in 2010 to 66 million by 2030, with majority of Alzheimer disease (AD).[4] Recently AD threatens our aged people with the possible loss of memory and cognitive functions and leads to increasingly health care burden to our future economy. In spite of advances in medical interventions, AD is fatal and at present, there is no cure. Complex pathology of AD, it is not very responsive to current modern medicines.[5, 6] Increasing researches have turned to the traditional medicinal herbs, which are multitargeting, to search for a novel way of AD treatment.[7, 8] *Drosophila melanogaster* developed as a model organism of drug screening for neurodegenerative diseases. It possesses several unique features such as stable and fully known genetics, highly conserved disease pathways, high-throughput, and cost effective.[9] It was reported that

many of the genes implicated in human AD pathogenesis have *Drosophila* homologs, including amyloid precursor protein (APP),γ-secretase, and tau.[10] But there are some dissimilarities, such as the absence of β-secretase, which cause a defect in endogenous production of Aβ42.[11] In this investigation, the *Drosophila* models that overexpress human Aβ42 would be used. This model neurodegeneration would result in reduced lifespan, reduced locomotor activity, eye degeneration and histological change to the neuronal structure.[10, 12] These pathological phenotypes could be observed within a few weeks, much faster than that of the counterpart phenotypes in transgenic mice.[13] Therefore, *Drosophila* as model of AD provides excellent tools for performing drug screens to identify drug candidate that can suppress the toxicity associated with Aβ accumulation. 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.*[8, 14] Recent pharmacological studies revealed that *Ginkgo biloba* possessed neuroprotective effects towards D-galactose[15], beta amyloid[16] and ischemia-induced neuronal death.[17] *Uncaria rhynchophylla* also prevented D-galactose[18], beta-amyloid[19], 6-hydroxydopamine[20] and kainic acid-induced neurotoxicity.[21] In this study we selected a widely available plant *P.nigrum* (Piperacae). It is popularly known, black pepper in English, melagu in Tamil, seetalchini or kababchini in Hindi. It was reported that the GC-MS analysis of the isolated V.O from the leaves indicated the presence of following constituents Nerolidol, β- caryophyllene, Cubein, Globulol, α -pinene, α -terpinene, elemol, α–bisbolol, β- bisabolene, β-elemene.[22] Leaves of *P.nigrum* of this family, is traditionally known to be useful for the treatment of wide panel of diseases like epileptic seizures,[23] hypertension, prostate disorder, giddiness, rheumatism, wound infection, cough, sore throat, fever, wounds, tooth decay, gastric ulceration, reproductive problems, etc. and as antioxidant, anti-platelet aggregation, antimicrobial, sedative, digestive, hemostatics, diuretics, analgesics, anti-inflammatory and cardiovascular diseases. Various scientific investigation of the leaves showed anti-diabetic activity,[24] anti-microbial activity, antiulcer, analgesic, anti-inflammatory, anti-microbial, anti-oxidant, hepatoprotective,[25] antihypertensive and wound healing activity.

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, hepatoprotective, cardiac activities, anti-inflammatory effect etc.

Root traditionally used for liver diseases, tuberculosis, asthma, diabetes, hypertension, toothaches, anaemia[26], etc. The survey of literature on *P.nigrum* also reveals that leaves contain various phytoconstituents like alkaloid especially flavonoid compounds. 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. By a well-coordinated effort, we can properly exploit this plant. Therefore research on development of herbal products from this plant is required to be initiated immediately for exploring the unique potential of this crop which would also minimize the menacing wastage especially the leaves. It may be further envisaged that the revenue generated by this plant would easily exceed that generated by any major crop of the country even with a present level of traditional agro economic practices. Therefore a well-coordinated effort by the farmers, traders, scientist, technologists, extension workers, physician, administrators, and policy makers is required to be initiated to boost up the national economy as well as the proper exploitation of this for proper therapeutic purpose. The review of literature showed some lacuna exists in the phytochemical and pharmacological studies in the leaves of *P. nigrum.* Its traditional use in nervous disorders and the presence of considerable quantity of VO prompted us to investigate its protective effect on neurotoxicity in *Drosophila* model organism for the development of drug for neurodegeneration like Alzheimer's diseseae.

MATERIALS AND METHODS

CHEMICALS

Donepezil, Drosophila food.

COLLECTION AND AUTHENTICATION

The leaves of the healthy plant *P.nigrum* selected for our study was collected from Hailey Buriar estate, Idduki Dist, Kerala, India, during the month of July 2016 and was authenticated by **Dr. Stephen,** Department of Botany, American college, Madurai and **Dr. Sasikala** Director (Retd) of Siddha Central Research Institute, Arumbakkam.

SEM SAMPLE PREPARATION

Sample for SEM analysis were mounted on the specimen stub using carbon adhesive sheet. Small sample were mounted with I sq. cm glass slide and kept in carbon adhesive sheet. Samples were coated with gold to a thickness of 100 AO using Hitachi vacuum evaporator. Coated sample were analysed in a Hitachi Scanning electron Microscope 3000 H model. Laboscope model Microscope with Photomicrograph & CCTV was used. Aβ42 transgenic *Drosophila melanogaster* model was kindly provided by Prof. Dr. S.C. Lakhotia, Department of Zoology, Banarus Hindu University, Varanasi, India.

ISOLATION OF VOLATILE 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 VO is isolated (VOPNL) by Clevenger apparatus and analyzed by GC-MS.

IDENTIFICATION OF COMPOUNDS PRESENT IN THE VOLATILE OIL OF THE LEAVES BY GC-MS ANALYSIS

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.

ACUTE TOXICITY STUDY USING BRINE SHRIMP (*Artemia nauplii)* LETHALITY BIOASSAY (BSLA)

In order to study the toxicity of the extract 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.[28,29]

TOXICITY ASSESSMENT OF VOPNL 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 VO (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 VO (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. 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.

CULTURE OF *Drosophila melanogaster*

Flies were raised at 25°C on standard corn meal medium supplemented with dry yeast.

***IN VIVO* NEURO PROTECTIVE ACTIVITIES OF THE VOPNL ON *Drosophila melanogaster*[30,31]**

Longivity assay

Genetic crosses were performed in the vials containing the diet with treatments. The normal control, which did not express Aβ42, The Aβ42 expressing control (the positive control) were maintained on the normal diet. Standard drug treated group fed with diet containing 10mmol donepezil/g of *Drosophila* media whereas the three VOPNL groups were fed with diets containing 1, 2 and4 µl/g of *Drosophila* media, respectively. Newly hatched *Drosophila* in each group was transferred to a new vial (30 *Drosophila* per vial), continued with their respective treatments and incubated at 25∘C. Dead *Drosophila* were counted on day 1 and 5 in a 7-day cycle, and the remaining live *Drosophila* were transferred to a new vial containing the same diet. The feeding lasted for 65 days. 100 *Drosophila* were tested for each group.

Climbing Assay

Locomotor function of *Drosophila* was measured by climbing assay. In brief, 30 male *Drosophila* were placed at the bottom of a 15mL falcon tube and were given 10 s to climb up the tube. At the end of each trial, the number of *Drosophila* that climbed up to a vertical distance of 8 cm or above was recorded. *Drosophila* were tested on day 1 and 5 in a 7-day cycle. Each trial was performed three times. 100 *Drosophila* were tested for each group.

Pseudopupil Assay

The control and Aβ42 *Drosophila* were treated with the same treatments as described above. *Drosophila* heads were examined under a light microscope. Briefly, the compound eye of 5 days old *Drosophila* was viewed under microscope in a dark field. There were eight photoreceptors in each ommatidium and seven of them were visible. Each photoreceptor projected a darkly staining rod, the rhabdomere, into the center of the ommatidium.The dissected heads with the neck was put on a slide. Shine light from the top through the compound eye. The rhabdomeres efficiently absorb light. Due to the precise architecture of the retina, the superposition of the pictures of several ommatidia appears as one single pseudopupil in the postero dorsal quadrant of the eye. Under the microscope, the rhabdomeres appeared as bright spots and rhabdomeres in each ommatidium were counted. In the control group, 7 rhabdomeres could be observed in each ommatidium. One hundred ommatidia were observed from 5 to 10 eyes, and the average rhabdomeres count per ommatidium was calculated.30 drosophila were tested for each group in triplicate.

Nail Polish Imprint Technique[33]

In this technique, a nail polish (transparent) was used to create an exact replica of the external surface of the eye, which was then examined using a light microscope. An imprint of adult eye of the fly was obtained by a small drop of transparent nail polish placed on the surface of a clean glass slide. The fly under examination was anaesthetized, placed on a dry area of the slide and decapitated with a sharp blade or needle.

The head was held with forceps or needles and is dipped in the still fluid drop of nail polish. The head is then placed in a clean and dry area of the same slide and the nail polish layer on the eyes is allowed to dry at room temperature (preferably 24°C) for 5– 10 min. The dried layer of nail polish was easily peeled-off from the eye with the help of fine dissecting needles. The isolated

peel, being an exact replica of the eye surface, assumes a goblet-shaped appearance. It was carefully placed on another clean glass slide with the imprint side facing upright. This imprint can be directly examined and photographed under a stereo binocular microscope to provide a low magnification image of the eye surface. For higher magnification and for better image of the eye surface, the peel is carefully flattened by gently placing a cover slip over it and carefully applying a slight pressure. The eye imprint is then examined under a microscope using 10X, or 45X differential interference contrast objective. The eye surface was observed under scanning electron microscope(SEM).

RESULTS

Percentage of volatile oil was found to be 1.2% v/w of dry leaf powder. Yellow in colour, aromatic odour, pungent taste, greasy, soluble in organic solvents, immiscible in water, refractive index 1.505, specific gravity 0.9462 at 25°C, optical rotation +1.9. GC-MS Profile of the VOPNL showed the presence of nerolidol, β- caryophyllene, cubein, globulol, α -pinene, α -terpinene, elemol, α–bisbolol, β- bisabolene, β-elemene. In continuation of our efforts to verify the safety of VO, 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 VOPNL. LC_{50} for VOPNL was about 500ppm respectively in 24hrs.100% mortality was observed at 3ppm for podophyllotoxin positive control. This prescreen showed safety of the VOPNL without any symptoms of toxicity "Fig.1".

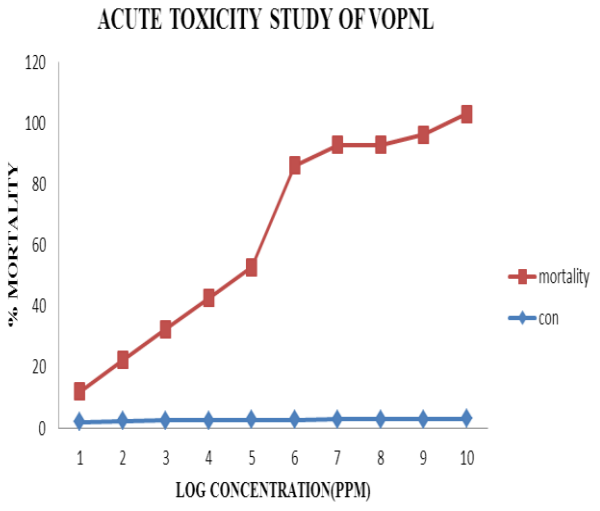
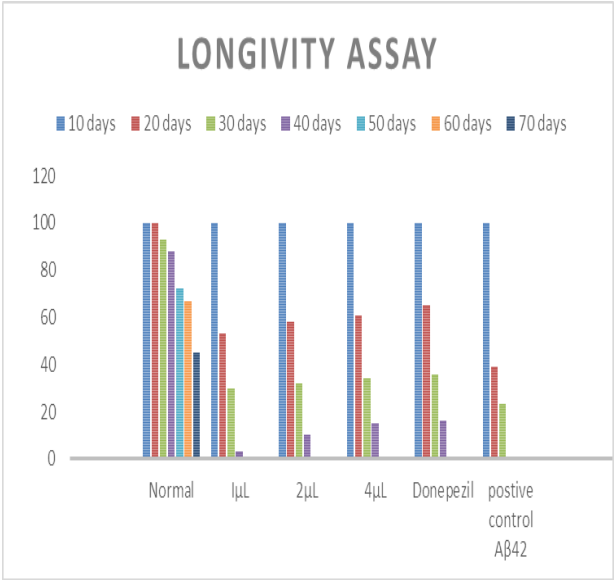


Fig-1

In the present study neuroprotective effect was evaluated using *Drosophila* AD model. In the lifespan experiments Aβ42 *Drosophila* showed a complete reduction in lifespan between 30 -40 days when compared to control. VOPNL treatment significantly improved the survival of *Drosophila* dose dependently in the tested concentrations ($p<0.001$). All treated groups showed significant improvement of survival. 4µl/mg showed maximum lifespan increase equivalent to the standard drug donepezil "Fig.2".



'Fig-2'

For locomotor abilities determination Aβ42 expressing *Drosophila* showed significant impaired locomotion from the age of day 9 onwards. VO treated flies showed an improvement in locomotor activity dose dependently. 4µl/g conc showed improvement in the locomotion equivalent to the std drug donepezil "Fig. 3".

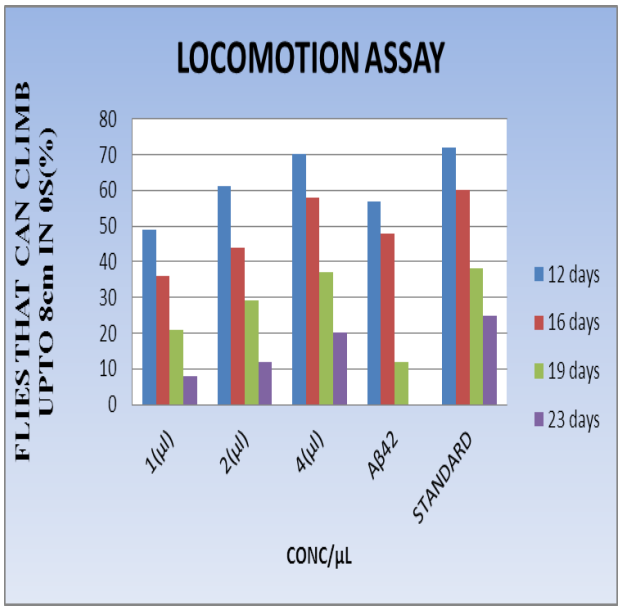


Fig-3

In this study the effect of Aβ42 expressing *Drosophila* on degeneration of retinal tissue which were mainly neurons. Aβ42 expressing *Drosophila* contained significantly more degenerating rhabdomeres compared with the normal fly. The number of degenerated

rhabdomeres was 2±0.258. The treated group has significantly rescued rhabdomeres in each ommatidium with increase in number of count which reflected a preventive effect of the VOPNL on neurodegeneration. This effect was comparable to the standard drug donepezil "Fig.4,5".

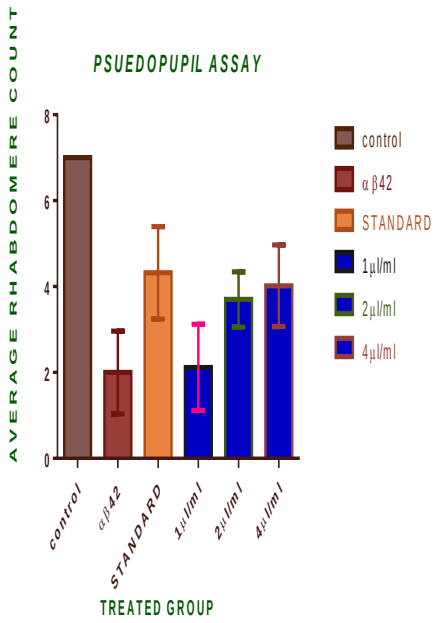
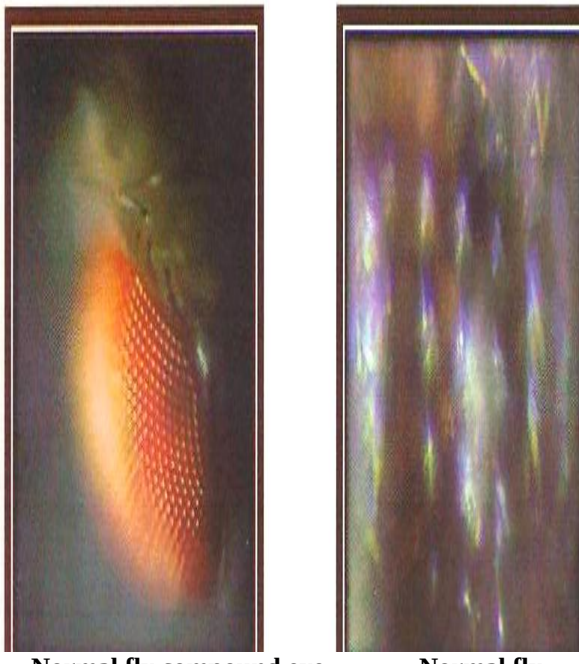
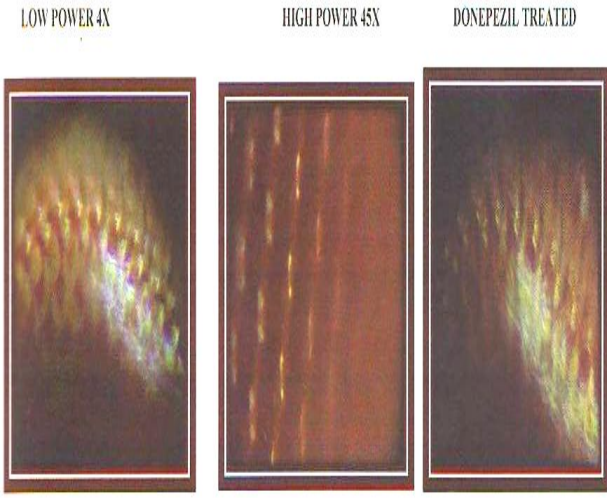


Fig-4

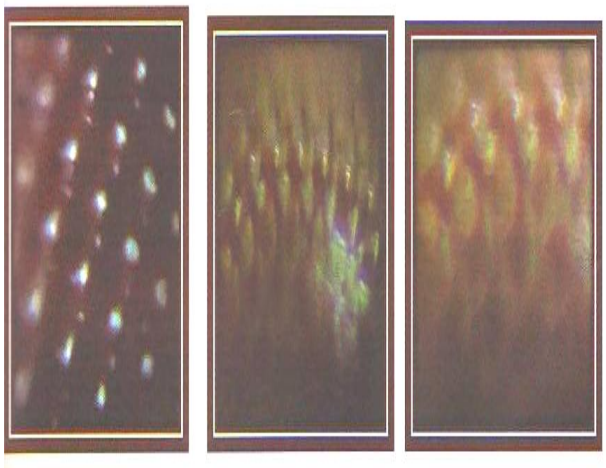


Normal fly compound eye **Normal fly**

Fig.5 EVALUATION OF RHABDOMERES COUNT IN OMMATIDIA



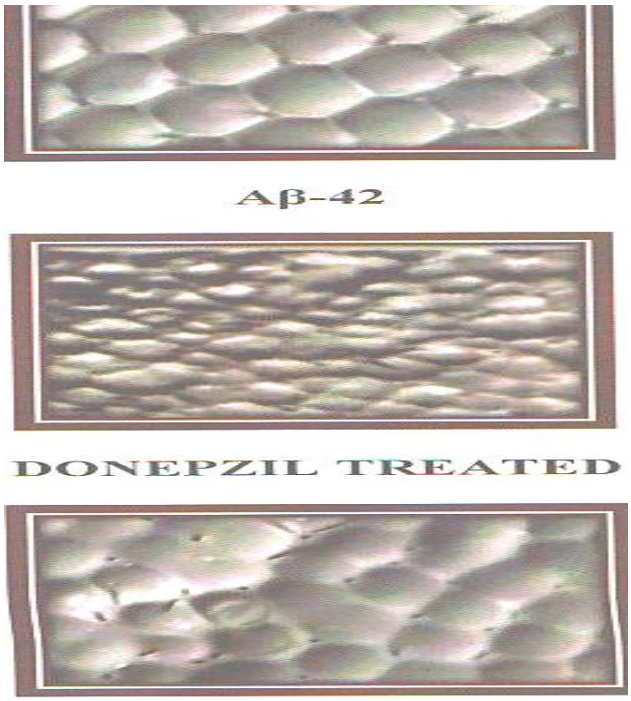
VOPNL TREATED FLY



Evaluation of surface organization of ommatidia in adult eyes by nail – polish imprints and Scanning Electron Microscope (SEM)

Transgene expression in the eye cells severely disrupts the regular arrays of ommatidia due to neurodegeneration and its pattern was highly disorganized compared to the regular arrays of Ommatidia in the eyes of control flies. Interestingly a majority of treated groups the ommatidial organization and overall structure of the eye surface was significantly better showing restoration of ommatidial integrity and axons projecting from rhabdomeres to the optic lobe in the brain. VOPNL(4µl) treated Aβ-42 transgenic fly ommatidia showed good surface improvement equivalent to donepezil treated by nail polish imprint method. The substantial improvements in the neurodegeneration phenotypes viz. eye morphology, formation and organization of rhabdomeres clearly showed that VOPNL suppresses neurodegeneration in fly models of AD toxicity. Surface morphology was observed under scanning electron microscope also to confirm the above findings "Fig. 6,7". It can be concluded that VOPNL provides a balanced defense to neuronal cells against the toxic protein aggregates and its

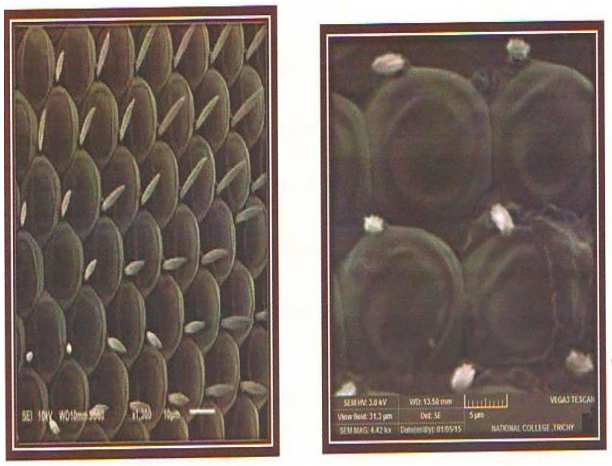
beneficial effects in suppressing inherited neurodegenerative disorders. Further investigation will be useful in developing it as convenient therapeutic formulations for combating the increasing burden of neurodegenerative disorders.



Normal Ordered Array

VO TREATED

Fig.6 NAIL POLISH IMPRESSION TECHNIQUE OMMATIDIA



VOPNL TREATED DONZEPEZIL TREATED

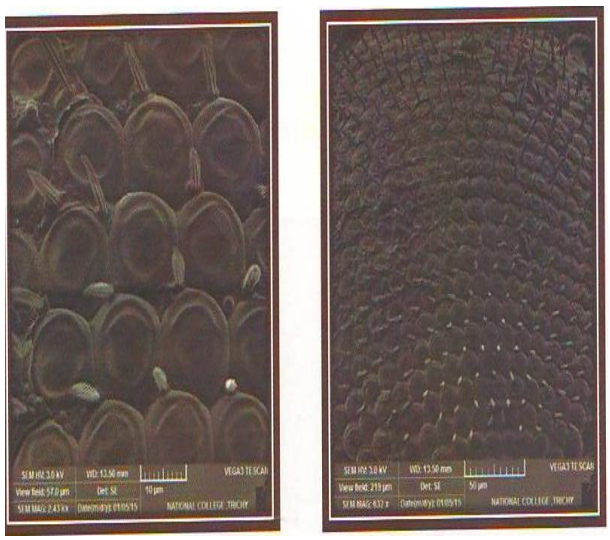


Fig .7 EVALUATION OF OMMATIDIA UNDER SEM

CONFLICT OF INTEREST STATEMENT

We do not have any conflict of interest.

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