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ORIGINAL RESEARCH ARTICLE

Pomegranate (*Punicagranatum. Linn. var. Ganesh*) Leaf Extracts Ameliorates Neurotoxicity In Transgenic *Drosophila* Expressing Human Amyloid Pathology

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ABSTRACT

To prescreen the *in vivo* neuroprotective activity of the chloroform, ethanol extracts of the leaves of Punicagranatum L.var. Ganesh family Punicaceae, commonly called pomegranate, using the model organism $A\beta_{42}$-amyloid neurotoxicity included *Drosophila melanogaster*. Chloroform (CEPGL), ethanol, (EEPGL) extracts were prepared, and its analysis by HPLC were carried out. Acute toxicity assessments were also performed. The neuroprotective effect of CEPGL, EEPGL *in vivo* was evaluated on the transgenic $A\beta_{42}$ model of *Drosophila melanogaster*, a novel model system for screening drugs for Alzheimer's disease by longevity assay, Climbing assay, Pseudopupil assay and nail polish imprint technique, and scanning electron microscope (SEM). HPLC profile of the CEPGL, EEPGL showed the presence of Ursolic acid. Toxicity assessment using brine shrimp lethality bioassay (BSLA) of the CEPGL, EEPGL showed nontoxic up to 2500, 2000 ppm, respectively. The extracts possess potential *in vivo* neuroprotective activity on *Drosophila melanogaster* against beta-amyloid included neuronal toxicity.

Conclusion: In the present study, we have presented the first evidence of the extracts of the leaves could significantly ameliorate the adverse morphological changes from $A\beta_{42}$ protein in Drosophila, as indicated by prolonging the lifespan, by improving locomotor abilities and rescuing neuro-degeneration in ommatidia of $A\beta_{42}$ expressing Drosophila which is comparable with donepezil. So it demonstrated the novel use of Ursolic acid of the extracts CEPGL, EEPGL effectively protect, rescue and most importantly, restore the impaired movement activity (i.e., climbing capability) in *Drosophila melanogaster.*

Keywords: Alzheimer's disease, *Drosophila melanogaster, Punicagranatum,* Punicaceae, Ursolic acid.

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is associated with global mental dysfunction and cognitive deterioration. Common pathological features of AD are the accumulation of intraneuronal tau and extracellular amyloid β (Aβ) peptide.[1] Accumulation of Aβ leads to the deposition of insoluble neuritic or senile plaques, thereby initiating a pathological cascade, which results in synaptic dysfunction, synaptic loss, neuronal death, and cognitive impairments.[2] Moreover, oxidative stress and inflammation are involved in Aβ-induced neuronal death and neurotransmitter deficits and the progression of AD.[3] 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-aggregates into a plaque,[4] which leads to the generation of reactive oxygen species, membrane potential disruption and increased vulnerability to excite toxicity and cause eventually neuronal death[5] and related cognitive defects.[6] Recent report revealed an increasing prevalence of dementia worldwide, from 36 million in 2010

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to 66 million by 2030, with the majority of AD.[7] Complex pathology of AD, it is not very responsive to current modern medicines.[8,9] Increasing research has turned to the traditional medicinal herbs, which are multitargeting, to search for a novel way of AD treatment.[10,11] Drosophila melanogaster developed as a model organism of drug screening neurodegenerative diseases. It possesses several unique features such as stable and fully known genetics, highly conserved disease pathways, high- throughput, and cost-effectiveness.[12] 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.[13] But there are some dissimilarities, such as the absence of β-secretase, which causes a defect in endogenous production of $A\beta_{42}$.[14] In this investigation, the *Drosophila* models that overexpress human $A\beta_{42}$ would be used. This model neurodegeneration would result in reduced lifespan, reduced locomotor activity, eye degeneration and histological change to the neuronal structure.[13,15] These pathological phenotypes could be observed within a few weeks, much faster than that of the counterpart phenotypes in transgenic mice.[16] Therefore, *Drosophila* as model of AD provides excellent tools for performing drug screens to identify drug candidates that can suppress the toxicity associated with Aβ accumulation. Medicinal plants are used to treat neurological disorders quite longer, for convulsion, stroke, and epilepsy, that is, *Poriacocos*, *Polygala tenuifolia*, *Uncariarhynchophylla*, *Ginkgo biloba*, and *Lycium barbarum.*[11,17] Recent pharmacological studies revealed that *Ginkgo biloba* possessed neuroprotective effects towards D- galactose,[18] beta amyloid,[19] and ischemia-induced neuronal death.[20] *Uncariarhynchophylla*also prevented D-galactose,[21] beta-amyloid,[22] 6-hydroxydopamine,[23] and kainic acid-induced neurotoxicity.[24] In this study we selected a widely available plant *Punicagranatum. L. var. Ganesh* (Punicaceae), popularly known as pomegranate, madulai in Tamil, anar, dhalim in Hindi. *P. granatum*is a fruit-bearing deciduous shrub or small tree is widely cultivated throughout Asia and tropical Africa.[25] Some studies reported the bark, roots and leaves of the tree have antioxidant, anti-inflammatory, anti-carcinogenic medicinal benefits as well.[26-29] Unique tannins occur in pomegranate leaves, as well as in peel. Leaves of *P. granatum* also contain glycosides of apigenin, a flavone with progestinic[30] and anxiolytic[31] properties. The fresh leaves extract of *P. granatum* has been found to be associated with free-radical scavenging activity.[32] Ellagic acid, another major ellagitannin present in the leaves, showed antioxidant and anti-inflammatory properties.[33,34] Apigenin, a flavone, and Punicalgin, anellagitanin, also present in the leaves of *P. granatum* have been known to exhibit anti-inflammatory actions.[25]

The bioactive products derived from plants are being considered to be an unparalleled source to design novel and effective therapeutic agents for the treatment of dreadful diseases, including cancer, cardiovascular and neural disorders.[35] Among the categories of natural products, triterpenoids represent a large family of compounds and comprise more than 20,000 identified terpenoids, including ursolic acid (UA) with promising remedial value.[36-38] UA is a pentacyclic triterpenoid compound that exists either as free acid or as aglycone of saponins. There has been extensive research to elucidate the UA-mediated pharmacological potential. It has been reported that UA can modulate a variety of signaling pathways associated with cancer survival and progression, cardiovascular and neural injuries.[36]

The survey of literature on *P. granatum*also reveals that leaves contain various phytoconstituents like alkaloids, especially terpenoid compounds. The economic aspect of this crop evidently proved it as a commercial crop. Therefore research on the 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. Therefore a well-coordinated effort by the farmers, traders, scientists, technologists, extension workers, physicians, administrators, and policymakers is required to be initiated to boost up the national economy as well as the proper exploitation of this for proper therapeutic purpose. Its traditional use in nervous disorders and the presence of a considerable quantity of extracts prompted us to investigate its protective effect on neurotoxicity in Drosophila model organism to develop the drug for neurodegeneration like AD.

MATERIALS AND METHODS

Donepezil, *Drosophila* food, analytical grade chloroform, ethanol. $A\beta_{42}$ transgenic *Drosophila melanogaster* model was kindly provided by Prof. Dr. S.C. Lakhotia, Department of Zoology, Banarus Hindu University, Varanasi, India.

Collection And Authentication

The leaves of the healthy plant *P.granatum*selected for our study was collected from the Department of Horticulture, AC & RI, Tamilnadu Agricultural University, Madurai, Tamilnadu, India, during July 2016 and was authenticated by Dr. Stephen, Department of Botany, American college, Madurai.

SEM Sample Preparation

Sample for SEM analysis were mounted on the specimen stub using carbon adhesive sheet. Small samples were mounted with I sq. cm glass slide and kept in carbon adhesive sheets. Samples were coated with gold to a thickness of 100 AO using Hitachi vacuum evaporator. The coated sample was analyzed in a Hitachi scanning electron microscope 3000 H model. Laboscope model Microscope with Photomicrograph and CCTV was used.

Preparation of the Extracts From The Leaves

The leaves were dried at room temperature under a shade, powdered, sieved (60 mesh) and stored in a well-closed container. The dried leaf, CEPGL and ethanol (EEPGL) extracts were prepared using the rotary vacuum evaporator and analyzed by HPLC.

Identification of Compound Present in the Extracts of the Leaves by HPLC Analysis

Shimadzu BM-101 model used, Column: C-18 ODS, Mobile phase: ACN: Methanol 1:1, Flow rate: 0.5 mL/1 min, Injection volume: 20 µL, wavelength: 210 nm, standard ursolic acid 30.40 mg/100 mL.

Acute Toxicity Study Using Brine Shrimp *(Artemianauplii)* Lethality Bioassay (BSLA)

To study the toxicity of the extracts, we performed BSLA, which is based on the ability to kill laboratory cultured brine shrimp (*Artemianauplii*). 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 dentalmaterials.[39,40]

Toxicity Assessment of CEPGL, EEPGL of the Leaf

Ten free-swimming hatched out *Artemianauplli* were drawn through a glass capillary and placed in each vial containing 4.5 mL of brine solution. In each experiment, 0.5 mL brine solution containing various concentrations of extracts (ppm) were added to 4.5 mL of brine solution and maintained at room temperature for 24 hours under the light, then surviving larvae were counted. An experiment was conducted along with control (vehicle-treated), different concentrations of the extracts (100–2500 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 using Abbott's formula. The graph plotted concentration verses percentage lethality. Podophyllotoxin was used as a positive control in the bioassay.

Culture of *Drosophila Melanogaster*

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

***In vivo* Neuro Protective Activities of the CEPGL, EEPGL on Aβ_{42} Expressing *Drosophila melanogaster.*[40,41]**

Longivity Assay

Genetic crosses were performed in the vials containing the diet with treatments. The normal control, which did not express Aβ_{42}. The Aβ_{42} expressing control (the positive control) was maintained on the normal diet. Standard drug treated group fed with diet containing 10 mmol donepezil/g of *Drosophila* media whereas the three CEPGL, EEPGL groups were fed with diets containing 1, 2, and 4 mg/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℃. 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 eachgroup.

Climbing Assay

Locomotor function of *Drosophila* was measured by climbing assay. In brief, 30 *Drosophila* were placed at the bottom of a 15 mL falcon tube and were given 10 seconds 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* was tested on days 1 and 5 in a 7-day cycle. Each trial was performed three times. 100 *Drosophila* were tested for eachgroup.

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, 5 days old Drosophila's compound eye was viewed under a 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 were put on a slide. Shine light from the top through the compound eye. The rhabdomeres efficiently absorb light. Due to the retina's precise architecture, the pictures of several ommatidia's superposition appear as one pseudo pupil in the eye's posterodorsal quadrant. Under the microscope, the rhabdomeres appeared as bright spots and rhabdomeres

in each ommatidium were counted. In the control group, seven 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. A total of 30 *Drosophila* were tested for each group in triplicate.

Nail Polish Imprint Technique[41]

In this technique, a nail polish (transparent) was used to create an exact replica of the eye's external surface, which was then examined using a light microscope. An imprint of the fly's adult eye was obtained by a small drop of transparent nail polish placed on the surface of a clean glass slide. The fly under examination was anesthetized, 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 to 10 minutes. The dried layer of nail polish was easily peeled-off from the eye with the help of fine dissecting needles. The isolated peel, being a 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 a better image of the eye surface, the peel is carefully flattened by gently placing a coverslip over it and carefully applying slight pressure. The eye imprint is then examined under a microscope using a 10X or 45X differential interference contrast objective. The eye surface was observed under the SEM.

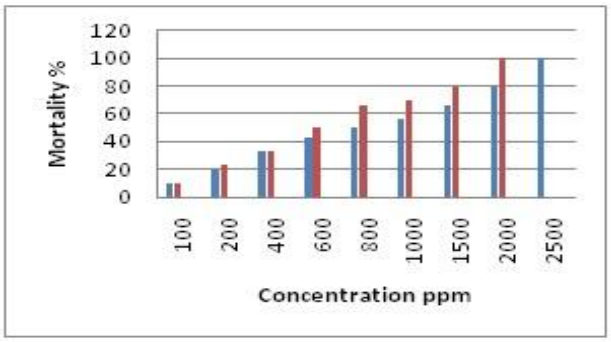
RESULTS

HPLC Profile of the CEPGL, EEPGL showed the presence of ursolic acid 20.34,0.1503%w/w respectively. In continuation of our efforts to verify the safety of extracts, we performed BSLA. It was observed that 100% of mortality for CEPGL, EEPGL was about 2500, 2000 ppm respectively in 24 hours.100% mortality was observed at 3ppm for podophyllotoxin positive control. These pre-screens showed safety of the CEPGL, EEPGL without any symptoms of toxicity (Graph.1).

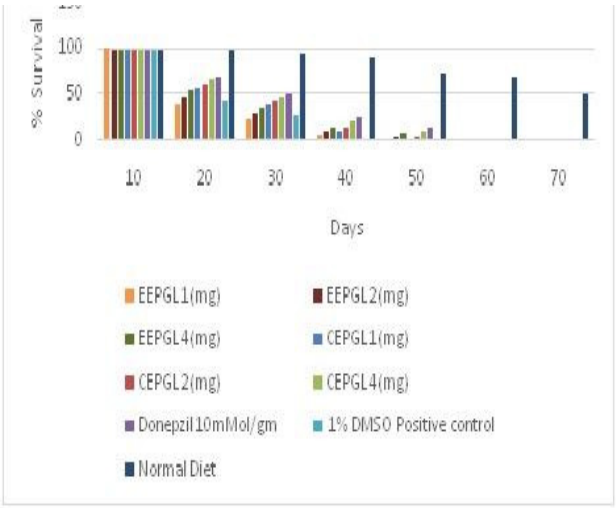
In the present study neuroprotective effect was evaluated using *Drosophila* AD model. In the lifespan experiments, Aβ42 *Drosophila* showed a complete reduction in lifespan between 30-40 days when compared to control. CEPGL, EEPGL treatment significantly improved Drosophila dose's survival dependently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

For locomotor abilities determination Aβ42 expressing *Drosophila* showed significantly impaired locomotion from the age of day 9 onwards. Extracts treated flies showed an improvement in locomotor activity dose-dependently. A 4 mg/g concentration showed improvement in the locomotion equivalent to the standard drug donepezil (Graph 3).

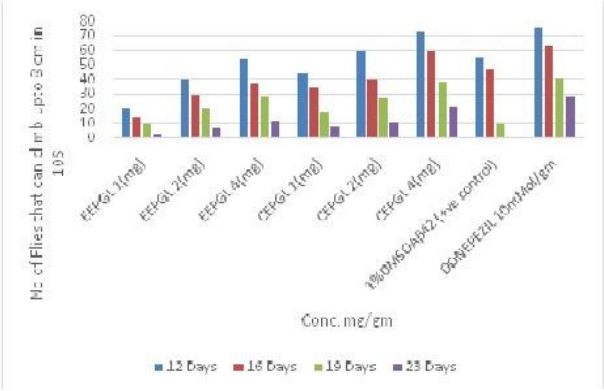
In this study, the effect of $A\beta_{42}$ expressing *Drosophila* on the degeneration of retinal tissue which was mainly neurons. $A\beta_{42}$ expressing *Drosophila* contained significantly more degenerating rhabdomeres compared with the normal fly. The number of degenerated rhabdomeres was 2 ± 0.258. The treated group has significantly rescued rhabdomeres in each ommatidium with an increased count of count, reflecting a preventive effect of the CEPGL, EEPGL on neurodegeneration (4.30 ± 0.223, 3.8 ± 0.20



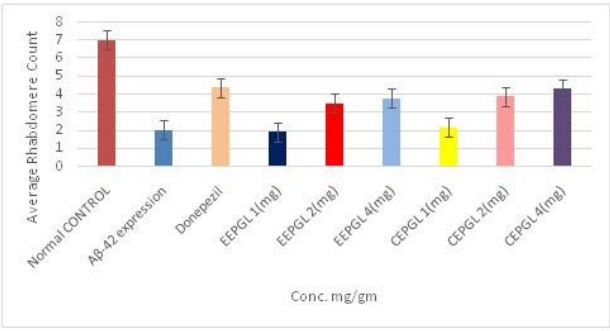
Graph.1: Acute Toxicity Study of CEPGL, EEPGL



Graph 2: Longivity Assay of CEPGL, EEPGL



Graph.3: Locomotion Assay of CEPGL, EEPGL



Graph.4: Pseudopupil Assay of CEPGL, EEPGL

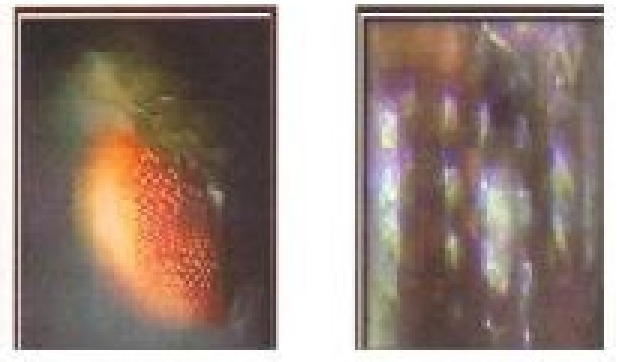


Plate 1: Compound eyes of normal fly

for 4 mg/g concentration respectively). This effect was comparable to the standard drug donepezil (Graph 4, Plate. 1-3).

Evaluation of Rhabdomeres Count

Evaluation of Surface Organization of OmmatidiaIn Adult Eyes By Nail – Polish Imprints and 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

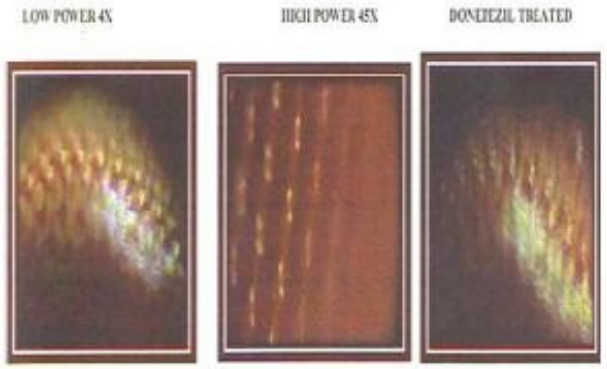


Plate 2: Over expressed Eyesof $A\beta_{42}$

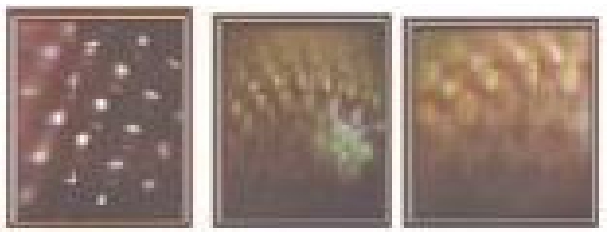


Plate 3: Extracts Treated Eyes

regular arrays of Ommatidia in the eyes of control flies. Interestingly, in most treated groups, the ommatidial organization and overall structure of the eye surface were significantly better, showing the restoration of ommatidial integrity and axons projecting from rhabdomeres to the optic lobe in the brain. CEPGL, EEPGL (4mg) treated $A\beta_{42}$ 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 rhabdomeres organization clearly showed that CEPGL, EEPGL suppresses neurodegeneration in fly models of AD toxicity. Surface morphology was observed under scanning electron microscope also to confirm the above findings. It can be concluded that CEPGL, EEPGL provides a balanced defense to neuronal cells against the toxic protein aggregates and their beneficial effects in suppressing inherited neurodegenerative disorders. Further investigation will help develop it as convenient therapeutic formulations for combating the increasing burden of neurodegenerative disorders.

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