TO STUDY THE EFFECT OF CERTAIN AYURVEDIC FORMULATIONS USING ALZHEIMER'S AND HUNTINGTON'S MODEL OF *DROSOPHILA*

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BY

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DECLARATION BY THE CANDIDATE

I declare that this thesis "**To study the effect of certain Ayurvedic formulations using Alzheimer's and Huntington's model of *Drosophila***" submitted for the award of Master of Science to THE UNIVERSITY OF TRANS-DISCIPLINARY HEALTH SCIENCES AND TECHNOLOGY, Bengaluru, is my original work, conducted under the supervision of Prof S.C Lakhotia and Kishor Patwardhan and co-supervision of Dr Megha. I confirm that no part of the work reported herein has been submitted for a degree or examination at any other university. References, funding and material obtained from other sources have been duly acknowledged, and no part of this dissertation has been plagiarized.

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CERTIFICATE FROM THESIS SUPERVISORS

This is to certify that the work incorporated in this thesis **"TO STUDY THE EFFECT OF CERTAIN AYURVEDIC FORMULATIONS USING ALZHEIMER'S AND HUNTINGTON'S MODEL OF *DROSOPHILA*"** submitted by **HARIKRISHNAN M** was carried out under our supervision. No part of this thesis has been submitted for a degree or examination at any other university. References, help and material obtained from other sources have been duly acknowledged. I confirm the originality of the work and that there is no plagiarism in any part of the thesis.

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Personal Reflection

This project provided me with valuable learning experiences. It provided me with a deep understanding of fly biology, initially from our small lab at TDU and later as part of the Cytogenetics Laboratory of the Department of Zoology at BHU. I feel honored to have received guidance and advice from eminent Distinguished Prof. Subhash Chandra Lakhotia, who has more than 50 years of experience in the field of fly biology. Additionally, I am proud to have had exposure to Prof. Kishore Patwardhan, who is open to diverse schools of knowledge. Working on my dissertation at BHU, one of the top academic institutions in India, was a stroke of luck.

During this project, I had the opportunity to handle several research equipment that are not commonly found in standard labs. Notably, the confocal microscope stands out due to its high cost and complex mechanism. Having knowledge and experience in working with various microscopes such as binocular, stereo binocular, GFP microscope, fluorescence microscope, and the confocal microscope makes me proud. Among the techniques I performed, immunostaining was one of the most challenging and memorable experiences, requiring days of patience and meticulous procedures.

Initially, planning and executing the research was a challenging task. However, continuous exposure to the research work conducted by PhD scholars in seminars provided me with insights into how experiments are planned and completed. It also helped me understand the importance of control groups and provided a glimpse into the statistical aspects of research.

Reviewing reports and literature from our lab and relevant sources, as well as going through the process of submitting reports, significantly expanded my knowledge in the field of my research. This experience also improved my communication skills, both through emails and direct interactions.

Moreover, I was introduced to various software tools like Mendeley, ImageJ, and graphing software, as well as various writing tools which enhanced the ease and quality of my work.

Throughout this project, I learned the importance of patience, practice, continuous learning, and seeking advice from experienced researchers in order to successfully complete any research endeavor. I also realized the need to foster strong bonds and coordination with colleagues for smooth research progress.

An additional achievement that I consider beneficial is the exposure to an unfamiliar environment. This encompassed aspects such as language, culture, food, climate, friends, and surroundings, which strengthened me mentally and physically.

Furthermore, this dissertation project helped me expand my professional network and personal contacts, which I consider highly valuable. I had the opportunity to participate in an all-India cell biology conference, which provided me with a broader perspective on the current trends in research.

Overall, this project was a transformative experience that enriched my knowledge, skills, and personal growth in various aspects of research and beyond.

Summary

Even though ayurvedic formulations are used in treatments for ailments like dementia, Alzheimer's, etc., their mechanism of action is not fully understood. Presently, we have tried feeding two Ayurvedic formulations, Kalyanaka gritha (KG) and Saraswat gritha (SG), at two different concentrations (0.05 g/100 ml and 0.25 g/100 ml), to fly models of Alzheimer's and Huntington's diseases. These diseases, known as proteopathies, progress with age and lead to the destruction of neurons.

In Ayurveda practice, ghee-based medicine (Gritha preparations) is found to be quite effective in the clinical treatment of neurological problems. But such treatment methods are entirely new to modern science and are to be further explored. In this seven-month dissertation, I have tried to understand the effects of these formulations in the larval as well as adult stages of *Drosophila melanogaster*. The Gal4-UAS system was used to genetically create the desired diseased model fly. Immunostaining of the eye discs of late 3rd instar larvae revealed a lesser accumulation of Aβ plagues in diseased larvae fed KG and SG. In the adult stage, formulation feeding did not improve locomotory or phototaxis behavior. Instead, diseased females fed with higher concentration of formulation showed greater locomotory deficits. However, this could be because the formulations were continuously fed to the flies, even at the adult stage. Lethality studies were also carried out, leading to the conclusion that the lower concentration of the formulation had more pronounced beneficial effects on flies expressing the neurodegenerative transgenes. These results were similar to those we concluded from immunostaining. These preliminary studies suggest that the fly model can be used to study Ayurveda formulations but dosage requires standardization.

Abbreviations & Symbols

DAPI: 4, 6-diamidino-2-phenylindole dichloride

GFP: Green Fluorescent Protein

H: Hour

min: minute

ml: milliliter

PFA: Paraformaldehyde

RT: Room Temperature

s: Second

UAS: Upstream Activating Sequence

KG: Kalyanaka Gritham

SG: Saraswatha Gritham

AB: Anti Body

Fig.: Figure

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INTRODUCTION

Ayurveda and Its branches

Ayurveda is a traditional medical system that has been used for hundreds of years in India and is becoming more and more well-known around the world. However, to ensure its safety, efficacy, and quality, there is a need for research and standardization in Ayurveda.

The word Ayurveda comes by two words Ayu and Veda. Word Ayu refers to life span and Veda denotes knowledge. Ayurveda has a science focus on improving Quality of life by preventing as well as treating the diseases. Ayurveda focus on basic principles of Tridosha which states vitiation of dosha balance in the body can lead to disease. Maintenance of the balance of these dosha is always looked for in health as well as in treatments. Vata, pitha and kapha are the three doshas. Ayurveda advices diet regiments and medicine to maintain the harmony between them.

Ayurveda has Ashta-anga or eight branches or components that deal with different aspects of healthcare and healing. These eight branches are:

1. Kayachikitsa (Internal Medicine)
2. Shalya Tantra (Surgery)
3. Shalakya Tantra (Ophthalmology and Otolaryngology)
4. Kaumara Bhritya (Pediatrics and Obstetrics):
5. Bhuta Vidya (Psychiatry):
6. Agada Tantra (Toxicology):
7. Rasayana Tantra (Rejuvenation Therapy)
8. Vajikarana Tantra (Aphrodisiacs and Fertility)

Since the present study deals with neurodegeneration diseases, Rasayana tantra, which focuses on the prevention of diseases and the promotion of longevity through the use of special herbs and therapies, is discussed below.

Rasayana and pattern of their usage

The word "Rasayana" is made up of two Sanskrit words: "Rasa" which means essence, and "Ayana" which means path or journey. Rasa can be corelated to dhatu (tissues) and the proper transport and nourishment to its excellence is the key role of Rasayana.(Deshpande et al., 2018). Treatment in Ayurveda involves modulation of diet, lifestyle, and/or administration of medicines which target Dhatu Samyata (achieving the state of homeostasis) and not merely alleviating the disease. As a result, deep analysis of health parameters, its assessment, and its maintenance is the first approach of Ayurveda. (Goyal, 2018)

Various methods of Rasayana administration are explained in classical text. One among them is Kuti Praveshika Rasayana where Rasayana drugs are continuously given to a person keeping him inside a kuti (small room devoid of sunlight). The other method is Vatatapika where medicine is given at a given dosage at some fixed time of day. The classical method of administering Rasayana drugs is only after proper shodhana followed by deepana(Chulet & Pradhan, 2009).

Medhya Rasayana is group of drugs that improve memory. Acharya Charaka has explained "Medhya ganas" (a group of 10 drugs with neurotropic property). These medicines are believed to be good for neurological disorders and are being studied using various approaches and models in the resent years(Dubey S.D., 2020).

Need for research in principles and practices of Ayurveda

One of the primary reasons for the need for research in Ayurveda is to establish evidence-based practices. While Ayurvedic treatments have been used for centuries, there is a lack of scientific research to validate their effectiveness. By conducting research, we can gather empirical evidence to support or modify the traditional knowledge of Ayurveda and determine the best treatment protocols for various ailments. Research can also help us understand the mechanisms of action of Ayurvedic treatments. By identifying the active compounds in Ayurvedic herbs and formulations, we can better understand how they work and develop more effective treatments(Patwardhan B, 2014) (S. C. Lakhotia, 2014).

It is understood that traditional health care systems like Ayurveda have been neglected due to a lack of studies that bridge the gap between ancient descriptions and modern understanding. By incorporating larger and more diverse population samples and considering physiological and genomic factors, future studies have the potential to offer a more comprehensive and scientific understanding of traditional wisdom(Lakhotia S C, 2014).

There are a few instances of noteworthy contributions made mostly by experts outside of the area of AYUSH that have significantly improved it. Contemporary scientific rigour must be used to validate Ayurvedic concepts and practices. To advance the authenticity of AYUSH, it is crucial to create academically sound methods based on solid scientific data(Lakhotia et al., 2019).

Standardization is another crucial aspect of Ayurveda that requires attention. Standardization involves establishing uniform standards for Ayurvedic medicines, including quality control measures, good manufacturing practices, and labeling requirements. Without standardization, the quality and efficacy of Ayurvedic products may vary significantly, which can lead to safety concerns and inconsistent treatment outcomes.(Vaidya et al., 2018). Moreover, standardization can aid in the regulation of Ayurvedic products and facilitate their global acceptance. Many countries have different regulations for herbal medicines, which can make it challenging for Ayurvedic products to gain acceptance in international markets. By standardizing Ayurvedic medicines, we can ensure that they meet international standards and regulations, making them more widely accepted(Chaturvedi et al., 2021; Chauhan et al., 2015).

During the past and the current century, modern medicine has made significant progress in managing various diseases. However, many conditions still cannot be fully cured, as the complex factors contributing to their development pose challenges. To address this gap, there is a growing recognition for integrating holistic principles of disease management, as explained in Ayurvedic science, with molecular understandings from modern medicine. This approach aims to provide a more comprehensive and effective strategy for managing ailments(Lakhotia SC, 2019; Thottapillil et al., 2021).

In India, there is a need to foster collaboration between modern and traditional healthcare systems to promote scientific leadership in the field of healthcare. Policy makers should ensure that their vision and strategies align with the rich legacy of Indian knowledge to facilitate this integration(Shankar & Patwardhan, 2017).

Ayurveda, an ancient Indian system of medicine, has demonstrated promising results in treating chronic ailments such as cancer, diabetes, arthritis, and asthma, which often pose challenges for modern medicine. However, the limited acceptance and recognition of Ayurveda globally can be attributed to the lack of scientific validation for some of its concepts. Therefore, it is essential to conduct evidence-based research to establish the effectiveness of Ayurveda and elevate its status in the global medical community. Improvements in research methodology are necessary to achieve this goal(Chauhan et al., 2015).

While attempting to correlate Ayurvedic science with modern science, it is important to acknowledge that there are certain aspects that remain contentious and unacceptable. The evolving nature of knowledge in modern science makes it challenging to fully comprehend some Ayurvedic concepts, requiring further research and potential revisions to provide a more comprehensive understanding. Various gaps and unanswered questions in Ayurveda need to be addressed through research to enhance its relevance and fulfill public expectations(Lakhotia S, 2023; K. Patwardhan, 2023).

LITRATURE REVIEW

Humans now live longer on average than they did a few decades ago. The poor quality of the older age life, however, is seen as a global public health concern. Neurodegenerative diseases that selectively affect various types of neurons and brain regions can develop in late adulthood or as a person enters the old age life. These diseases cause motor dysfunction, cognitive decline, and psychiatric disorders that are progressive, irreversible, incurable, and without remission periods.

The lifestyle disorders and other geriatric syndromes have seen significant increase in recent decades. In neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's disease, the loss of specific groups of neurons is characteristic. Neurodegeneration refers to the gradual and progressive deterioration of the structure and function of neurons, resulting in the loss of motor abilities and/or cognitive symptoms. This condition typically worsens with age and can significantly impact the quality of life and lifespan of affected individuals. Neurodegenerative diseases encompass a wide range of clinical conditions, with the most common ones being Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis (ALS), and various types of ataxia diseases (Dugger & Dickson, 2017; Poddar et al., 2021).

Many of the neurodegenerative diseases are marked by the presence of abnormal proteins, heightened oxidative stress, and impaired mitochondrial function. These factors contribute to the development of "protein conformational diseases. The primary molecular feature of neurodegenerative diseases involves the accumulation of modified gene products leading to disrupted redox balance and the buildup of unfolded or misfolded proteins in specific parts of brain. Reactive oxygen species (ROS) are highly reactive molecules that have been linked to the development of neurodegenerative diseases. These chemically active species are known to contribute significantly to oxidative stress, which is frequently observed at elevated levels in the brains of individuals affected by neurodegenerative disorders. While proteinopathies have long been established as the primary cause of neurodegeneration, there has been a growing interest in investigating the involvement of protein impairments in the pathogenesis of psychiatric disorders like depression and schizophrenia. Recent studies have focused on understanding the role of abnormal protein function in these mental health conditions(Ochneva et al., 2022; Oladele et al., 2021).

A common neurodegenerative condition is the Huntington's disease (HD). Since the Huntingtin (HTT) gene mutation was discovered to be the molecular cause of this brain ailment 25 years ago, a number of animal models, including the fruit fly, have been employed to investigate the cellular and molecular bases of this disorder(Brown, 2015; Loi et al., 2023).

Each neurodegenerative disease is characterized by specific symptoms, which are primarily attributed to the loss of particular populations of neurons in the adult brain. Unfortunately, most neurodegenerative diseases that lead to the progressive death of neuronal cells are currently incurable. The severity of neuronal loss directly correlates with the clinical symptoms experienced by individuals affected by these diseases.

The causes of neurodegenerative diseases are multifactorial, involving a combination of genetic and environmental factors. Some forms of neurodegeneration are caused by genetic mutations, including Polyglutamine (PolyQ) disorders, Amyotrophic lateral sclerosis (ALS), and Parkinson's disease.(Poddar et al., 2021)

PolyQ Disorders

Polyglutamine (PolyQ) disorders are inherited neurodegenerative diseases caused by the expansion of CAG repeats in specific genes. These expansions lead to the production of a chain of glutamine repeats, known as a polyglutamine tract or PolyQ tract. More than 20 neurological disorders have been linked to the CAG expansions, with nine of them being neurodegenerative diseases. Some examples include Huntington's disease, spinal and bulbar muscular atrophy, dentatorubral pallidoluysian atrophy, and several types of spinocerebellar ataxias. In these disorders, the mutant protein with the expanded PolyQ tract accumulates and forms insoluble aggregates, as well as sequesters other proteins to form inclusion bodies. These aggregates contribute to synaptic dysfunction and degeneration of specific neurons in the adult brain. The trinucleotide repeats may get expanded during DNA replication due to the formation of hairpin structures, which lead to the incorporation of additional nucleotides by DNA polymerase. This results in the expansion of the repeat length in subsequent generations.(Dugger & Dickson, 2017; Lieberman et al., 2019).

Huntington's disease

Huntington's disease, also known as Huntington’s Chorea, is a neurological disorder characterized by progressive dementia and uncontrolled limb movements, collectively referred to as ‘chorea’. It is named after Dr. George Huntington, who first identified the disease in 1872. The condition is caused by an abnormal version of the Huntington gene, which normally contains 10-35 tandem trinucleotide repeats (CAG) coding for glutamine residues in its protein coding sequence. In Huntington's disease condition, these trinucleotide tandem repeats are increased to more than 36, resulting in the production of a faulty Huntington protein with an elongated polyglutamine segment. This altered protein affects the brain's function and leads to the development of chorea symptoms. The disease is inherited in an autosomal dominant manner, with symptoms appearing at an earlier age in subsequent generations, a phenomenon known as genetic anticipation. Paternal transmission of the abnormal gene repeats is more likely, and affected fathers are often the source of inheritance. The prevalence of Huntington's disease in Asia is approximately 0.40 per 1,00,000 people, and around 10% of cases have an onset before the age of 21(Loi et al., 2023).

Alzheimer's disease

Alzheimer's disease is a neurodegenerative disease that typically begins slowly and progressively worsens over time. The most prevalent kind of dementia, accounting for 60% to 80% of all cases, is Alzheimer's disease (AD). According to estimates, this ruining neurological condition presently affects 50 million patients globally and has an indirect influence on tens of millions of others whose loved one's experience years-long cognitive deterioration(Rostagno, 2023).

The pathophysiology of Alzheimer's disease involves the injury and death of neurons, initially affecting the hippocampus region of the brain, and eventually impacting the entire brain with atrophy. Postmortem examinations reveal presence of fibrous tangles inside cells and senile plaques outside cells throughout the brain. The primary component of these plaques is a small peptide called amyloid-beta (Aβ), which is believed to be a causative factor in Alzheimer's disease. Some research studies suggest that the degradation of acetylcholine synthesis, mediated by enzymes such as acetylcholine transferase and acetylcholine esterase, may play a role in the development and progression of the disease(Dugger & Dickson, 2017; Ferreira-Vieira et al., 2016).

Research on genetic mutations that cause diseases like Huntington's, early-onset Alzheimer's, Parkinson's, and ALS, has shown that the mutated genes worsen the age-related issues in neurons. These issues include the buildup of harmful proteins, increased stress on cells, and problems with regulating ions homeostasis in cells (Mattson & Magnus, 2006). Neurons' synapse areas are particularly susceptible to ageing and neurodegenerative diseases. They are vulnerable to damage from excitotoxins as well as damage from pathogenic proteins such mutant huntingtin and amyloid-peptide(Mattson & Magnus, 2006).

Relevance of Ayurveda and Rasayana in present era

During the current wave of COVID-19, there have been numerous reports of memory decline. This cognitive impairment has been observed not only during the acute phase of COVID-19 infection but also in patients who have recovered from the illness(Ahmed et al., 2022; Bungenberg et al., 2022; Ceban et al., 2022). The traditional healthcare systems were also used by public to tackle these issues, leading to several-fold increase in demand of herbal drugs, specifically those which have proven effects in immunity and Rasayana property (Kotecha, 2021; Singh et al., 2021).

According to WHO dementia is one of the major causes of disability and dependency among older people and the sixth greatest cause of death in the world today. The most prevalent form of dementia, Alzheimer's disease, may be a factor in 60–70% of cases(World Health Organization [WHO], 2023).

The high prevalence of neurological disorders requires an integrative approach to tackle the current situation. Integration of various medical systems, especially with regard to their key potential is expected to combat the prevalent situation.

Prof. M. S. Valiathan introduced "Ayurvedic Biology" as an initiative aimed at building an evidence base for Ayurveda, including clinical trials of Rasayanas (rejuvenation) therapies(Valiathan M S, 2006). His concepts were matching with Prof S.C.Lakhota and which helped in some investigations using the fruit fly (*Drosophila melanogaster*). *Drosophila* is a good model for testing different ideas because it shares ~60% genes with humans, majority of which are involved in developmental and cell signalling pathways; significantly, 75% of the genes associated with various human diseases are conserved between human and *Drosophila* genomes(Adams et al., 2000; Pratomo et al., 2022; Reiter et al., 2001).

Prof S.C. Lakhotia has guided similar research in Ayurvedic Biology at BHU using AmlakiRasayana and Rasasindoor which has shown remarkable effects in PolyQ models of fly(Dwivedi et al., 2012a, 2015). These examples indicate the necessity of evidence for Ayurvedic treatments, which contribute to mainstreaming Ayurveda. Prof M. S. Valiathan suggests that Ayurvedic physicians can prove the effectiveness of their treatments by conducting clinical trials following WHO guidelines. They need to create national guidelines for disease treatment and integrate Ayurveda into mainstream medicine. This will require long-term cooperation between Ayurvedic physicians, government support, and evidence-based practices. It may take several decades, but it would be welcomed by the public, insurance companies, and new Ayurveda graduates (Joshi et al., 2022).

research are examples of producing evidence for Ayurvedic treatment and could contribute to mainstreaming Ayurveda. He further suggests that Ayurvedic physicians can prove the effectiveness of their treatments by conducting clinical trials following WHO guidelines. By doing so, they can create national guidelines for disease treatment and integrate Ayurveda into mainstream medicine. This will require long-term cooperation from Ayurvedic physicians, government support, and evidence-based practices. It may take several decades, but it would be welcomed by the public, insurance companies, and new Ayurveda graduates (Joshi et al., 2022; Lakhotia, 2020).

Preparation of Gritha kalpanas

Siddha Ghritas are medicated ghee preparations that involve boiling ghee with specific Kasayas (decoctions) and Kalkas (fine pastes) of specific medicinal herbs, according to defined proportions and processes. The boiling process allows the active therapeutic properties of the herbs to be absorbed into the oil base. There are three essential ingredients in these preparations: Sneha (ghee), Drava (liquid), which can be in the form of decoctions or expressed juices, and Kalka, the fine paste of the herbs.

The ratio of these ingredients, unless otherwise specified, is four parts ghee to one-part kalka and sixteen parts liquid. However, there are exceptions to this rule. During the preparation process, the fine paste and liquids are mixed together, and then ghee is added. The mixture is boiled over a low flame while continuously stirring to prevent the fine paste from sticking to the vessel.

Boiling continues until the liquid portion has evaporated, at which point the moisture of the fine paste starts to evaporate. The cooking stage is determined by testing the paste's consistency with a ladle. If the paste feels waxy when rubbed between the fingers, it is categorized as Mridu (soft), and if it is hard and burns without producing a cracking noise, it is considered Madhyama (moderate). The ideal condition of the medicated ghee is attained when uniform froth subsides and reaches the Madhyama Paka stage. This method of preparation ensures that the active properties of the medicinal herbs are absorbed by the ghee, making it a potent therapeutic agent(Ministry of Health and Family Welfare, 2000).

Saraswatha Gritha (SG)

It is prepared by the above procedure using the following contents It is to be noted that 14 different formulations with mild or major difference in ingredients have been mentioned in name of SG(Badal et al., 2021). The one we have used is prepared as described in Ashtanga Hridaya which is also the most commonly practiced formulation.

अजा-क्षीराभया-व्योष-पाठोग्रा-शिग्रु-सैन्धवैः ॥ ४५ ॥
सिद्धं सारस्वतं सर्पिर् वाङ्-मेधा-स्मृति-वह्नि-कृत् ।

AH. U. (1/35-36)

The above verse explains the SG is prepared with following ingredients;

Abhaya (*Terminalia chebula*), Sunthi (*Zingiber officinale*), Marica (*Piper nigrum*), Pippali (*Piper longum*), Patha (*Cyclea peltate*), Ugra (*Acorus calamus*), Sigru (*Moringa oleifera*), Ajaksira(Goat's milk) and Sarpi (Ghee)(Srikantha Murthy K.R, 2006).

Kalyanaka Gritha (KG)

Similarly, KG is also prepared with the above procedure using the following contents:

वरा-विशाला-भद्रैला-देवदार्व्-एलवालुकैः ॥ २६ ॥
द्वि-शारिवा-द्वि-रजनी-द्वि-स्थिरा-फलिनी-नतैः ।
बृहती-कुष्ठ-मञ्जिष्ठा-नागकेसर-दाडिमैः ॥ २७ ॥
वेल्ल-तालीश-पत्त्रैला-मालती-मुकुलोत्पलैः ।
स-दन्ती-पद्मक-हिमैः कर्षांशैः सर्पिषः पचेत् ॥ २८ ॥

AH. U (6/26b-28)

The above verse notes that the KG is prepared with following ingredients;

Ghee, Vishala (*Citrulus cholocynthis*), Elavaluka (*Prunus avium*), Sariva (*Hemidesmus indicus*), Bhadra ela (*Amomum subulatum*), Nata (*Valeriana wallichi*), Brihati (*Solanum indicum*), Devadaru (*Cedrus deodara*), Daruharidra (*Berberis aristate*), Shalaparni (*Desmodium gangeticum*), Haridra (Turmeric), Prishnaparni (*Uraria picta*), Kushta (*Saussurea leppa*), Manjishta (*Rubia cordifolia*), Vella (*Embelia ribes*), Talisapatra (*Abbies webbiana*), Phalini (*Callicarpa macrophylla*), Ela (Cardamom) Nagakeshara (*Mesua ferrea*), Dadimaphalatwak (*Punica granatum*), Danti (*Baliospermum montanum*), Padmaka (*Prunus poddum*), Malati (*Jasminum sambac*), Utpala (*Nymphea stellata*), Hima (*Santalum album*), Haritaki (*Terminalia chebula*), Bibhitaki (*Terminalia bellirica*), and Amla (*Emblica officinalis*), (Srikantha Murthy K.R, 2006).

KG is understood to have Tikta rasa (bitter taste), Laghu (easy to digest) property, and increase body heat (ushna veerya) These properties are able to generate due to 27 ingredients added in Gritha making it suitable in various disease conditions(Sinimol T P, 2019).

A clinical study in school children reported that consumption of KG for 90 days resulted in significant improvement in Binet Kamat test (BKT), and modified mini-mental status examination (MMMSE) scores (what they found in paper). Further, they concluded that it was safe to administer(Chaudhary et al., 2019).

Gritha and Medicated gritha

According to Ayurveda Gritha is believed to improve Dhi, Dhriti, and Smriti (Dhi refers to intelligence, Dhriti to self-control, will power and patience, and smriti to memory). Thus, Ghee has its prominent action on brain. It is stated to possess Gunas (qualities) that enhance Ojas, improve dhatu increase longevity etc.

Adding medicines to Ghee further enhances these gunas (qualities) and leads to their better action. Medicines in both SG and KG are understood to possess Ushna veerya (ability to improve body heat) and helps to overcome the Guru guna (Difficult to digest due to heaviness).

Model organism and its relevance

Drosophila melanogaster (Greek for dark-bellied dew lover), the common fruit fly, is a model system par excellence for genetic dissection of biological processes. Because of its small and well-studied genome, rapid life cycle and nonhazardous nature, it is immensely useful not only for genetic research, but also for exploring various pathways of development, physiology, reproduction, behavior, aging etc. In addition, it is also a good model to address complex biological disorders like neuro-degenerative diseases, heart diseases, cancer and in designing their therapeutic strategies. *Drosophila melanogaster* has only four chromosomes, because of which, it is amenable to large scale genetic screening, to saturate the whole genome with mutations, in an attempt to identify genes responsible for specific phenotypes. *Drosophila* also has the advantage of having a wealth of genetic resources like molecularly defined deletions and insertions of P elements that span the entire genome. Further, the availability of balancer chromosomes allows mutations to be stably maintained and enables the manipulation of entire chromosomes to construct "designer genotypes", facilitating the studies on functional genomics.

Understanding lifecycle of *Drosophila*

Drosophila melanogaster, a dipteran insect, undergoes a distinct four-stage life cycle consisting of the embryo, larva, pupa, and adult stages. Each stage can be identified by specific physical characteristics. The eggs of *Drosophila* are oval and whitish, measuring approximately 0.5mm in length. After about 22-24 hours at a temperature of 24°C, the egg hatches into a first instar larva, completing the embryonic development. Subsequently, the first instar larva molts after 24 hours, transforming into a second instar larva. After another 24 hours, the second instar larva undergoes another molt, becoming a third instar larva (Figure 1). During the following 48 hours, the 3rd instar larva feeds extensively, reaching its maximum size, and then molts into a pupa, which is a stationary stage where feeding does not occur. The newly formed pupa is initially whitish-yellow and gradually darkens through a process known as tanning. Within the pupal case, most of the larval tissues undergo metamorphosis, including the development of adult structures from imaginal discs and histoblasts. After 4-5 days, the adult fly emerges from the pupa.

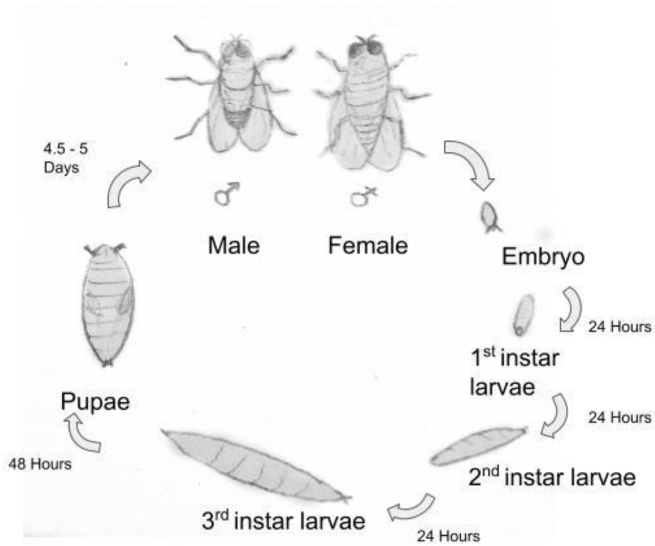

Fig 1. Life cycle of *Drosophila melanogaster* at 24-25°C

The development of *Drosophila* is influenced by temperature, with higher temperatures shortening the life cycle and lower temperatures extending it.

Advantages of *Drosophila melanogaster* as model organism

Being small in size, growing rapidly, producing many progenies and being readily available are crucial in terms of using *Drosophila* as an organism to study. It is a boon for research laboratories since compared to other models, it needs less space and cost for maintenance. In addition, there are fewer ethical constraints encountered when using flies for experiments.

Sequencing of the Drosophila genome (Adams et al., 2000) allowed the identification of most coding sequences, highlighting the necessity for a functional assignation of the annotated genes. There are an estimated 13000 genes in *Drosophila*, the structure, function and regulation of several of which are yet to be experimentally studied (Graveley et al., 2011). Interestingly, various biological processes, from simpler organisms like fruit fly to complex higher organisms like human beings utilize same or closely related genes working in highly conserved regulatory networks. Hence, advances made in the knowledge regarding developmental pathways in lower organisms could be applied to human beings as well(Jha et al., 2022).

The 60% similarity in genes and 75% of the human disease associated genes being shared between the two, makes this organism a good model for studying Human diseases (Lloyd & Taylor, 2010; Pandey & Nichols, 2011; Reiter et al., 2001). Availability of complete genome sequence and a huge number of mutants, makes *Drosophila melanogaster* an attractive and versatile model organism.

Powerful genetic strategies available for the fly model

Markers and Balancer Chromosomes

In fruit fly research, markers and balancer chromosomes are important tools. Markers are visible traits or characteristics associated with specific genes, helping researchers identify flies carrying those particular genetic mutations. Balancer chromosomes, on the other hand, are specialized chromosomes that maintain genetic stability during breeding experiments. They carry multiple inversions to prevent recombination with homologous chromosomes and have visible markers for easy identification. Balancer chromosomes are useful for preserving linkage of specific mutations by inhibiting production of recombinant prohgenies. Examples of balancer chromosomes include CyO, TM6B etc. The commonly used markers affect colour of eye or body, appearance of wing, bristle morphology, body shape or expression of GFP fluorescence etc.

GAL4-UAS system

The GAL4-UAS system is a widely used genetic tool in *Drosophila* research that allows precise spatiotemporal control of gene expression. Fly geneticists (Brand and Perrimon, 1993) have genetically engineered the yeast's binary regulatory system for galactose utilization to regulate expression of the desired transgene in the desired tissue at the time of interest. GAL4 is a yeast transcription factor that has been engineered to expressed under a specific *Drosophila* promoter to activate expression of the target gene which is placed under the yeast UAS sequence (the Gal4-responder) so that the target gene in expressed in specific tissues or cell types. This system thus includes two components: the Gal4 transcription factor produced under a specific fly promoter, and the target trans-gene placed under the yeast UAS (upstream activating sequences) promoter, which can respond to the Gal4 transcription factor see Fig. 3).

In many studies, *Drosophila* eye is the preferred target tissue for expression of the desired transgene. The *GMR-GAL4* or *eye-GAL4* drivers are most widely used for this purpose since the *GMR* (*Glass Multiple Reporter*) and *eye* (*eyeless*) promoters widely express in the developing eye discs.

The GMR enhancer/promoter contains binding sites for the transcription factor Glass, which is expressed in the developing eye. The *GMR-Gal4* transgene contains the GMR enhancer/promoter driving the expression of the Gal4 protein, which in turn binds to the UAS promoter sequences to activate expression of the downstream gene(Brand & Perrimon, 1993).

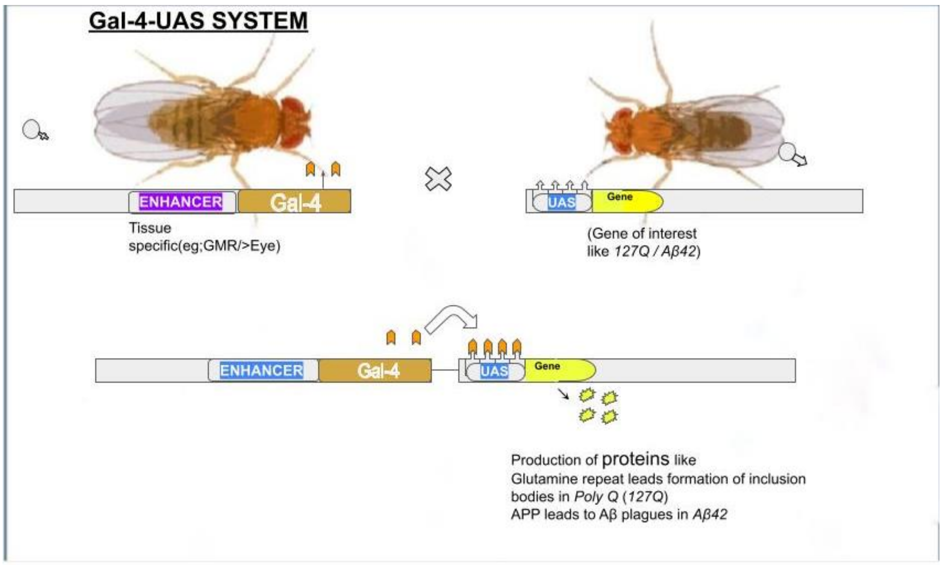

Figure 2. *Gal4-UAS* system

This system provides flexibility in controlling gene expression in specific tissues and developmental stages. By crossing a *GMR-Gal4* driver line with a *UAS-effector* line, researchers can express any gene of interest in a spatially and temporally regulated manner.

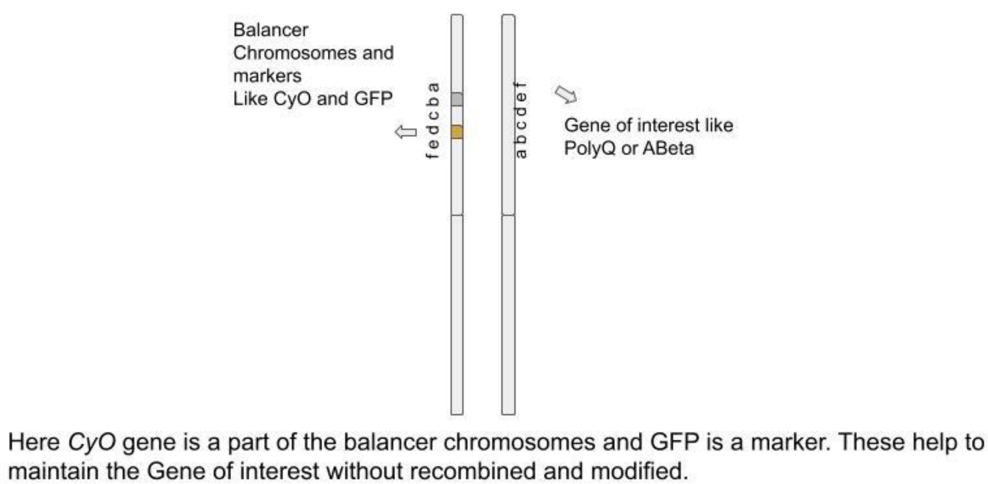

Fig.3 To understand Balancer Chromosomes and Markers arrangement in a chromosome

Eye imaginal disc of *Drosophila melanogaster*

In *Drosophila*, imaginal discs are compact collections of cells derived from the embryonic ectoderm. During metamorphosis, they transform into specific adult structures like compound eyes, wings, legs, antennae, halteres, and genitalia. While the wing discs give rise to the wings, the eye-antennal discs develop into the compound eyes, simple eyes (ocelli), and antennae. Other imaginal discs include the genital disc for the genitalia, the leg discs for the development of the legs, and the haltere disc for the formation of the haltere. The discs grow through cell divisions during the larval period, and differentiate during pupal period to generate the specific external structures in the adult fly.

Eye-antennal disc is a composite disc with its anterior part giving rise to the antennae and the posterior part to the eyes. The eye discpart comprises of anterior part, which is attached to antennal disc in front, and the posterior part where cells undergo further proliferation and differentiation into ommatidia and other components of the eyes. The Bolwig's nerve, associated with the eye-antennal discs is made up of a collection of sensory neurons that track light and transmit visual information to the brain. Larval phototaxis, or the capacity to move towards or away from light sources, depends on it. During the development of the eye, a wave of differentiation known as the morphogenetic furrow travels across the disc from posterior to anterior side. A boundary between differentiated cells behind it and undifferentiated cells in front of it is represented by the morphogenetic furrow. As the furrow deepens, cells behind it differentiate to generate various types of eye cells, including photoreceptor cells(Spratford & Kumar, 2014; Tsao et al., 2017).

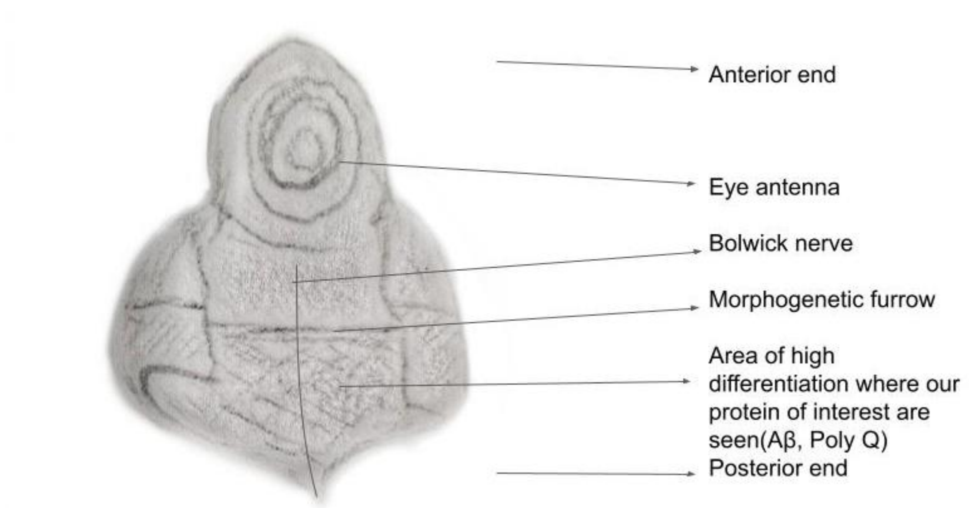

Fig. 4 Eye imaginal disc of 3rd instar *Drosophila melanogaster* larvae

Earlier studies on Actions of Ayurvedic formulations using *Drosophila model*

An attempt to understand the effect of ayurvedic formulations (Amlaki Rasayan and Rasasindoor was successful in improving the life span of flies as well as improving fecundity. These formulations were also used to understand their relevance in improving stress tolerance.(Dwivedi et al., 2012b)Since there is a relationship between cell stress and neurodegeneration these formulations were tested in PolyQ as well as Abeta diseased model flies and noticed to have good results.

In 2018 studies were started to understand effects of Saraswatharishta, Dhanwantharam kashayam, Saraswatha gritha as well as Kalyanaka gritha to understand their action in Poly Q and Alzheimer (Abeta) models of *Drosophila*. (Sharma et al., 2021; Swathi Sharma, 2021). These studies identified the apt dose of the medicines and examined their effects on parameters like lifespan. Confocal imaging was used to study the accumulation of defective PolyQ and AB protein aggregates in the eye discs.

Apt dose of the medicines and examined their effects on parameters like lifespan. Confocal imaging was used to study the accumulation of defective PolyQ and AB protein aggregates in the eye discs (Swathi Sharma, 2021).In the present study, I repeated some of the above results.

MATERIALS AND METHODS

Rearing of flies:

All flies were reared at $24^o \underline{+} 1^o$C on standard food. Standard fly food is prepared by mixing and boiling the following ingredients in 2.4L of water- Agar-Agar - 36g, Maize powder - 108g, Sugar - 96g, Dried Yeast - 36g, Nipagin - 8g. At the end of cooking, propanoic acid is added to impart a fruity smell. Nipagine (Methyl-4-hydroxybenzoate) helps to prevent fungal growth. The experimental food vials were prepared by thoroughly mixing the required quantity of the desired Rasayana (gritha) in the fully cooked standard food while it was still hot (~55^oC) and then dispensed in the vials.

The Saraswat Ghrita and Kalyanika Ghrita were obtained from Arya Vaidyasala, Kottakal (Kerala). The batch numbers were KG- 216001 for KG, and SG -212196 and SG-216678 for SG.

Fly stocks

All fly stocks and crosses were maintained on standard food as told above at 24°±1°C. The fly stocks used in the present study were:

Oregon R: D. E. Lancefield at Roseburg collected Oregon R from wild flies in 1925. This stock is one of the widely used wild type strain of *Drosophila melanogaster*.

***w^{1118}/w^{1118}; GMR-Gal4 UAS127Q/ CyO-GFP*; +/+:** This stock was generated in the laboratory by recombining the chromosome 2 linked *GMR-Gal4* and the *UAS127Q* transgenes on the same chromosome as a model for polyQ pathogenesis as the expanded polyQ (127Q) protein is expressed under the *GMR-Gal4* driver. This stock is homozygous lethal at larval stage; therefore, it is maintained with the CyO balancer chromosome. The *UAS127Q* transgene caries 127 CAG repeats downstream of the *UAS* promoter so that when activated by Gal4, it produces expanded polyQ protein, which simulates the Huntington disease. The larvae and adults show green fluorescence because of the Green Fluorescent Protein (GFP) marker carried by the *CyO* balancer. The adult flies have curly wings because of the dominant *Cy* marker on the CyO balancer chromosome.

***w*/ w*; >eye-Gal4 UASAβ42/CyO:* +/+:** These flies produce ABeta plaques in developing eye cells because the *UASAβ42* transgene is driven by the *eye-Gal4* transgene. They also show homozygous lethality.

***w*/ w*; GMR-Gal4/GMR-Gal4;* +/+**: Used as control for experiments with Poly Q flies

***w^+/ w^+; UASAβ42/UASAβ42;* +/+**: Used as control for experiments with Aβ expressing flies

Separation of Male and Female flies for behavioural assays

Male flies have rounded abdomen tip with darker pigmentation, sex combs on their front legs, and are usually smaller than females. The female flies have rounded abdomen tip with lighter pigmentation, no sex combs, and generally larger than males.

Based on the above features, male and female flies of defined ages (post-eclosion) were separated for phototaxis and climbing assays. Similarly male pupae and larvae were identified by the presence of sexcomb in late male pupae and large oval testes in male larvae, respectively.

Immunostaining and slide preparations.

Immunostaining of eye-discs of larvae requires materials like glass Maximov slides, dissection or insulin needles, glass slides, coverslips, Binocular microscope, Confocal microscope and following solutions.

Solutions:

PBS

Phosphate-Buffered Saline (PBS) is a buffer solution used to maintain the pH and osmolarity of the solution.1 litre 10x PBS contains 80g sodium chloride (NaCl), 2g potassium chloride (KCl), 14.4g sodium phosphate (Na_2HPO_4.), and 2.45g potassium phosphate (K_2HPO_4). Sufficient Quantity of NaOH or HCl is added to neutralize the pH of solution to pH7.

It was prepared by diluting the 10X stock solution.1X PBS is prepared by diluting 5 ml of 10XPBS with 45 ml distilled water. It is stored at 4^oC. The required quantity is taken out and brought to room temperature before use.

PBST

PBST contains Phosphate-Buffered Saline (PBS) and Triton X-100 and is used for washing the tissues. Triton X-100 is a nonionic detergent commonly used in biological research to permeabilize cell membranes and enhance the permeability of antibodies and other reagents. 0.1%PBST is prepared by adding 50µl Triton X-100 with PBS to make 50ml PBST solution. It is stored at 4^oC. The required quantity is taken out and brought to room temperature before use.

Paraformaldehyde (PFA)

Eye discs are fixed with 0.4% PFA for subsequent immunostaining. To prepare the 4% PFA solution, dissolve 0.4 g of PFA in 10 mL of PBS with constant stirring. Heat the mixture by keeping it at 60^oC and repeatedly shaking the solution every half hour until the paraformaldehyde is completely dissolved.

Solution is allowed to cool before use. Generally, it should be freshly prepared and used within a week of preparation, if stored at 4°C.

Blocking solution

A blocking buffer is a solution containing foreign proteins or compounds that saturates any remaining binding surfaces on a plate or slide to prevent non-specific binding of antibody. Its purpose is to reduce background interference, improve the signal-to-noise ratio, and enhance the sensitivity of the assay by minimizing non-specific binding events. It contains solutions Triton X100 0.1% v/v, BSA 0.1% v/v, FCS10% v/v, Deoxycholate0.1% v/v and Thiomersal 0.1%v/v all mixed in sufficient quantity of 1X PBS. After preparation these are stored at 4°C.

DABCO (Sigma)

DABCO (triethylenediamine or TEDA) is a bicyclic organic compound represented by the formula $N_2(C_2H_4)_3$. This is used as mounting medium to reduce quenching of fluorescence. It also slows down degradation of tissue.

Procedure for Immunostaining

Eye imaginal discs from late third instar larvae of desired genotypes were dissected out under a stereobinoclar in IX PBS and fixed in 4% paraformaldehyde for 25 mins at RT.

Tissues were rinsed in PBST (1X PBS, 0.1% Triton X-100) and kept in blocking solution (IX PBS, 0.1% Triton X-100, 0.1% BSA, 10% FCS, and 0.02% Thiomersal) for 2 hrs. at RT followed by incubation with the desired primary antibody at 4° C overnight.

Post antibody incubation, tissues were washed in 0.1% PBST (3 X 15 min each) and were again kept in blocking solution for 1 hour followed by incubation in the desired secondary antibody for 2 hrs at RT. Following this, the discs were washed in 0.1% PBST and counterstained with DAPI (1 µg/ml, Molecular Probe), for fluorescent staining of nuclei, for 25 min at room temperature. They were again rinsed in 0.1% PBST and mounted in DABCO (Sigma),

Antibodies

Primary antibodies

22C10: This mouse monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa) stains the cytoplasm and inner surface of cell membranes of all peripheral nervous system neurons and a subset of the central nervous system neurons. The working dilution for immunostaining was 1:100.

Anti-HA (Y-11, sc-805, Santa Cruz, USA): This is an affinity purified rabbit polyclonal antibody raised against a peptide mapping to an internal region of the influenza haemagglutinin (HA) protein. The working dilution for immunostaining was 1:400. This was used to detect the 127Q protein which carries the HA tag at its carboxy end.

Abeta antibody- Mouse monoclonal [DE2B4] against the beta Amyloid was used at dilution 1: 300.

Anti Elav 7E8A10 antibody DILUTED 1:50 raised in rat developed by Developmental Studies Hybridoma Bank. This used as nuclear marker for post mitotic neuron.

M 22 c10: It was used to see neuronal morphology and axonal projections. It is raised in mouse and is used with dilution 1;200.

Secondary antibodies

Cy3 conjugated anti-goat IgG (Whole molecule) (Sigma-Aldrich, India): This secondary antibody, generated in sheep and raised against mouse IgG and tagged with the Cy3 fluorochrome (from Invirogen) was used at a working dilution of 1:200. For primary AB m22c10

AF 488 Anti-rabbit: This antibody emits green fluorescence when excited 488nm. This was used against Anti Ha raised in rabbit. This was used in poly Q model against primary AB Anti-HA.

AF 647 Anti-mouse: This antibody was purchased from Invitrogen and was raised in Goat it was used for primary antibody M 22 c10 as well as for ABeta

Alexa Fluor 488 donkey anti-rat IgG (Molecular Probes, USA): This is an Alexa Fluor 488 fluorochrome labeled, affinity purified secondary antibody raised against mouse IgG in donkey. Its working dilution to detect primary antibodies was 1:200 for primary AB anti Elav.

Fluorescence Stains

DAPI (4, 6-diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich, India): This blue fluorescent dye preferentially stains dsDNA in live as well as fixed cells. Its absorption maxima and emission maxima are 351 nm and 461 nm, respectively. The working dilution was 1µg/ml. DAPI is a known mutagen and was handled with care.

Lethality Assay

For lethality assay, synchronized freshly hatched 1st instar larvae were collected at 2-hour interval and gently transferred to food vials containing either KG or SG supplemented food or to vials with the regular food (control) and reared at 24+1°C. The total numbers of larvae that pupated and subsequently emerged as flies were counted in each case. At least 5 replicates of 25 larvae each were examined in each case.

Microscopy and image analysis

For recording the external morphology of adult eyes, flies of the desired genotype and feeding regime were etherized and their eyes were photographed using a Sony Digital Camera (DSC-75) attached to a Zeiss Stemi SV6 stereo binocular microscope for examining the external morphology of adult eyes. For light microscopy, a Nikon E800 microscope was used with appropriate filter combinations. The images obtained were recorded with a Nikon DXM 1200 digital camera. The different objectives used were 10X (0.3NA. Plan Fluor), 20X (0.5NA, Plan, Fluor) or 60X oil (1.4NA, Plan, Apo).

Procedure of Confocal Scanning

1. Slide preparation was done as per procedure of immunostaining and kept in -$_{o}$ C inside an opaque box till we are able to get confocal scanning.
2. At time of confocal scanning machine is switched on step by step allowing sufficient time to get ready (warm up). Necessary lasers (Argon, green and far-red) are put in standby as well as switch on mode step by step.
3. With help of binoculars, we focus our eye disc tissue using coarse and fine focus knobs.
4. Then we switch to scanning mode where we set parameters Z stack, channels, laser strength, grain, zoom etc. to get a good image of our tissue.
5. These are set based on control and all the parameters are kept fixed and are repeated throughout the experiment.
6. Images are captured initially in 20x and later in 40x zoom. 40x zoom images are captured using cedar-oil and after capturing all images oil is wiped off from eyepiece and machine is switched off in order.

Later image processing is done with help of ImageJ software where images of various z stacks are compiled and finally all channels are merged.

Phototaxis Assay

Figure 5. Phototaxis apparatus is shown in a, while.5b represents arrangement of light illumination of one arm during the phototaxis assay (the shaded arms are 'dark while the white arm of Y is the illuminated arm).

A group of 20 flies were put in a Y maze (Fig. 5). One of the arms was covered with black tape to make it the 'dark' arm. One side arm, which was not covered with black tape, was illuminated with external light. The stem of the Y maze was also covered with black tape (Fig. 5a). Flies of the desired genotype were introduced into stem of Y maze through ts bottom opening. After 120 seconds, flies were counted in the lighted arm. These experiments were performed in triplicates for each formulation concentration and genotype with 100 flies. Flies present in the lighted arm were represented as mean % ± standard deviation

Climbing Assay

Vertical climbing apparatus was prepared by joining 2 vials with markings at 5 and 10cm height from bottom (Fig. 6). Flies were etherized and transferred to respective formulation and normal food vials one day prior to experiment. Experiments were performed on 5- and 10-days old flies. *GMR-Gal4>127Q* flies reared on normal or formulation supplemented food vials (SG0.05, SG0.25, KG0.05, KG0.25) were experimental flies while the *GMR-Gal4/GMR-Gal4* flies served as negative control. Flies of the desired genotype and feeding regime were allowed to climb up after they were gently tapped down to the bottom of climbing apparatus. Using a stopwatch, the numbers of flies crossing the 10 cm marker line after 10 and 15 seconds were counted.

Fig.6 The climbing assay setup.

Statistical analyses

All Statistical significance tests were calculated with Excel and GraphPad Prism8 software, using two-tailed Student's t-test. Data are presented as mean ± standard deviation as values obtained from at least three independent experiments. P values <0.05 (*) were considered as statistically significant.

RESULTS

LethalityAssay

PolyQ fly larvae were reared under uncrowded condition in normal (Control) and formulation supplemented food (0.05 or 0.25% KG or SG supplement) and the proportion that successfully emerged from pupae were counted and expressed as % survival (Fig. 7). As seen from the data presented in Fig. 7, only 0.05% KG or SG supplemented food significantly improved survival of *GMR-Gal4>127Q* larvae to fly emergence stage (p value <0.05, Fig. 7).

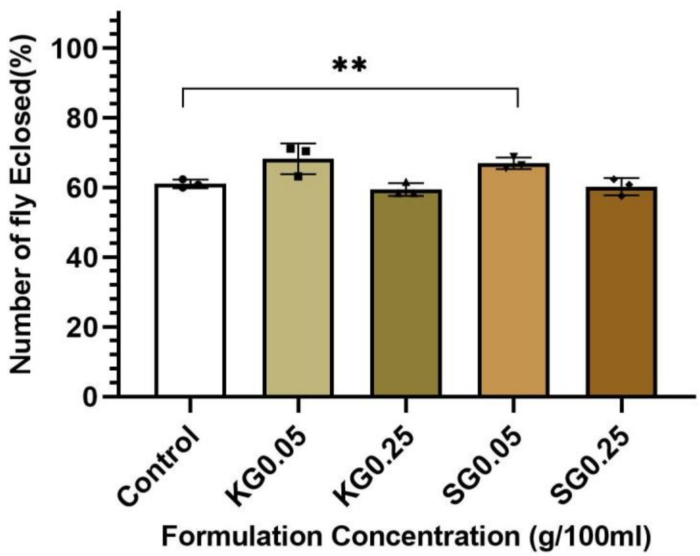

Fig 7. Graph showing results of lethality assay; N=125*3 for each data set; Y axis shows the Mean ± S. D. % fly emergence following rearing of larvae on different concentrations of supplemented formulations (X-axis).

Phototaxis assay

In agreement with earlier results(Dwivedi et al., 2012) it was observed that the positive phototaxis behavior of *GMR-Gal4>127Q* expressing flies reared on normal food (+ve control), was poorer than *GMR-Gal4* (-ve Control in Fig. 8) flies and decreased with age as the percentage of flies moving to lightened part of the Y maze was less in 10-days old flies compared 5-day old flies. Feeding the *GMR-Gal4>127Q* expressing flies reared since the larval stage on KG or SG formulation supplemented food further reduced their phototaxis in a dose-dependent manner, more so in female flies.

The data show that positive phototaxis of male flies is less affected than corresponding females.

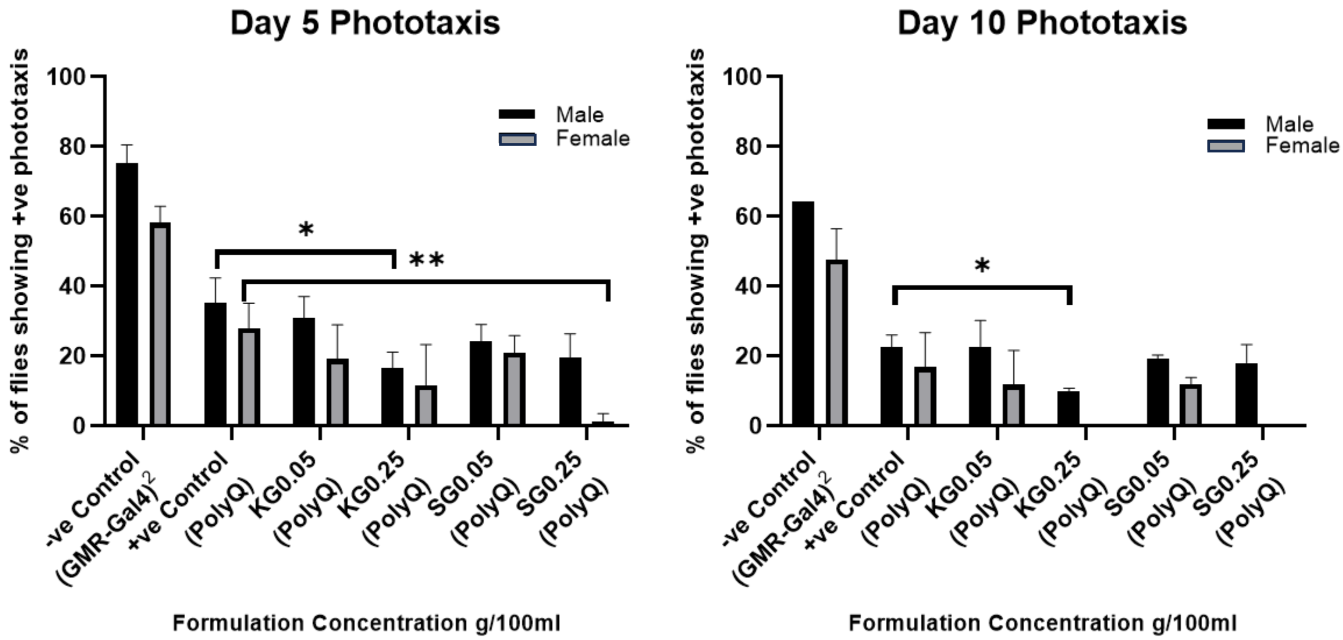

Fig.8 Histograms showing the phototactic response of 5 days (left) and 10 days (right) old GMR-Gal4>127Q flies phototaxis of 5 days old (left) and 10-day old (right) PolyQ flies. * and ** indicate $P<0.05$ or <0.01, respectively between the compared samples. In other cases, the mean values are not statistically different.

Climbing assay

The groups of flies that were used for phototaxis were also used for the climbing assay. As shown in Figs. 9 and 10, the *GMR-Gal4>polyQ* expressing male as well as female flies showed reduced ability to climb during 10 or 15 sec time. Formulation feeding further reduced their climbing ability, with females being affected much more than males.

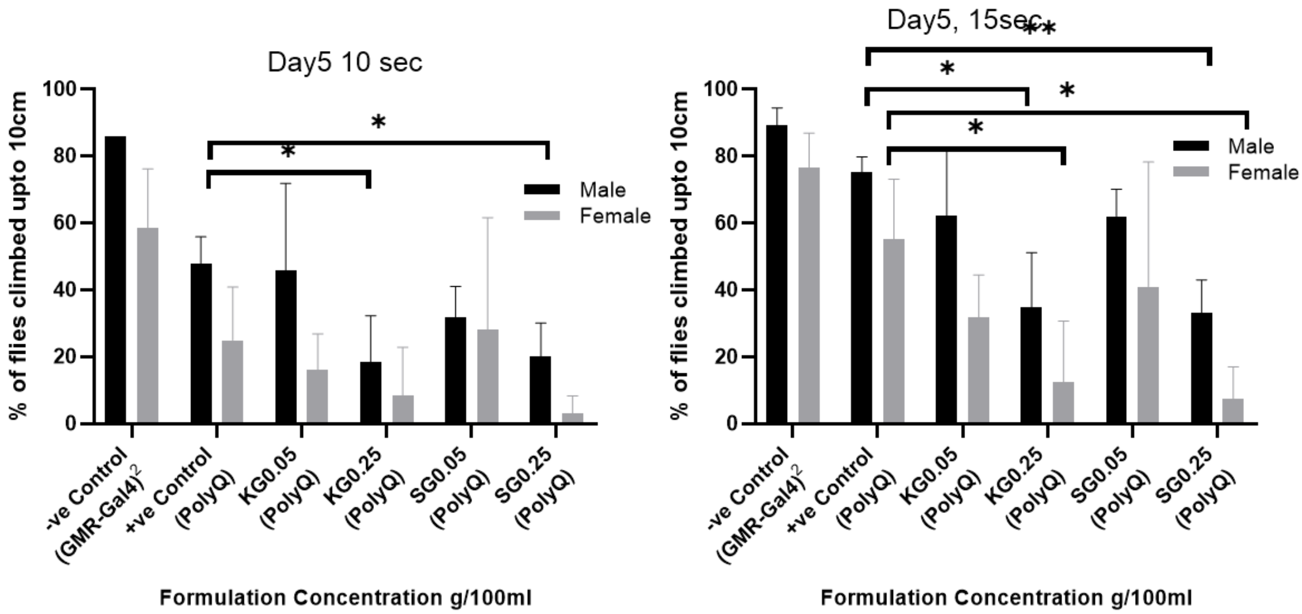

Fig.9. Histograms showing the climbing ability of 5 days old *GMR-Gal4>127Q* flies assayed for 10 sec (left) and 15 sec (right, respectively. * and ** indicate P<0.05 or <0.01, respectively between the compared samples. In other cases, the mean values are not statistically different.

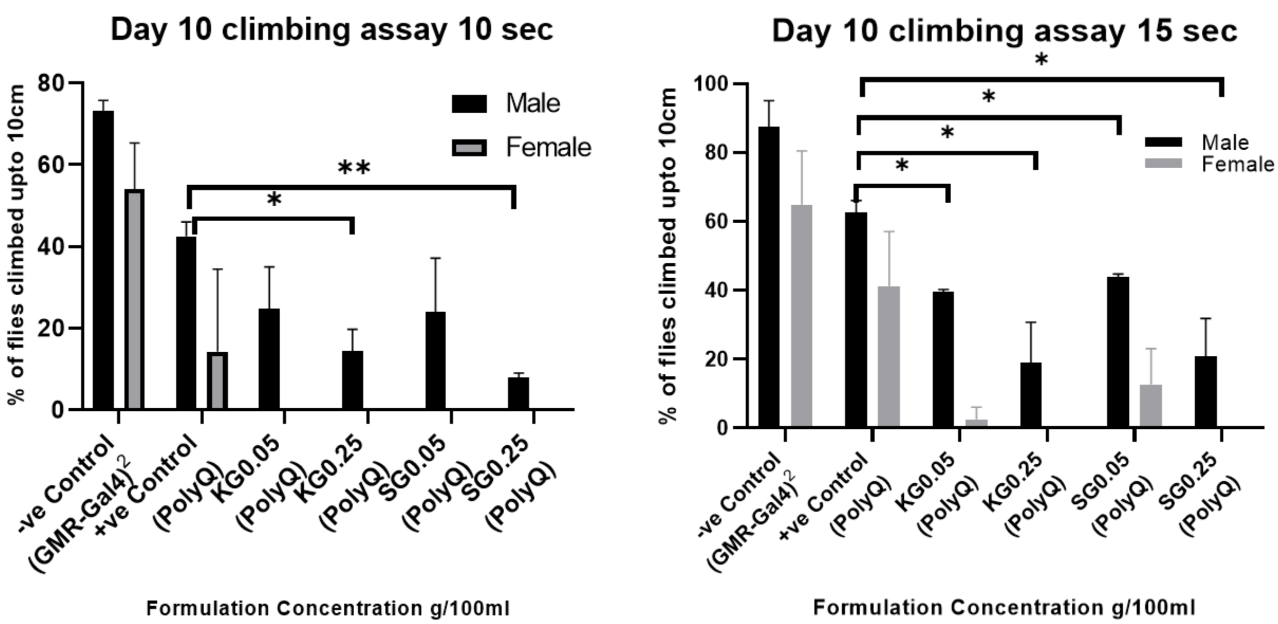

Fig.10. Histograms showing the climbing ability of 5 days old *GMR-Gal4>127Q* flies assayed for 10 sec (left) and 15 sec (right, respectively. * and ** indicate P<0.05 or <0.01, respectively between the compared samples. In other cases, the mean values are not statistically different.

In situ expression of *GMR-Gal4>127Q* or *>Eye-Gal4>Aβ42* transgenes following formulation feeding

Patterns of expression of *GMR-Gal4>127Q* or *>Eye -Gal4>Aβ42* transgenes following formulation feeding were examined by immunostaining of late third instar larval eye discs with antibody for HA tag to assay the levels of HA-tagged polyQ or Aβ42 proteins followed by confocal microscopy. The confocal images presented in Figs. 11-14 show the distribution of the Aβ42 protein (magenta in Figs. 11-12) or polyQ (green in Figs. 13, 14) proteins which are expressed in GMR-expressing neurons posterior to the morphogenetic furrow, which were identified either by immunostaining for Elav protein (green in Figs. 11, 12) or the 22C10 antibody reacting neuron-specific protein (magenta in Figs. 13-14). Images were collected with 20x objective to examine the overall organization of differentiating ommatidia in eye discs and with 40x objective to get a more detailed view of the ommatidial pattern. At least six imaginal discs were examined for each sample.

A visual comparison of staining intensity between control and experimental discs revealed an appreciable reduction in the Aβ plagues in eye discs form larvae reared on KG (Fig. 11) or SG (Fig. 12) supplemented food. However, the staining for PolyQ (Figs. 13, 14) did not reveal any detectable difference in staining intensity between control and SG or KG treated eye discs. This was possibly due to collection of the confocal images at high gain so that all the images had reached their saturation points. Unfortunately, due to shortage of time, this experiment could not be repeated.

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Immunostaining of Aβ42

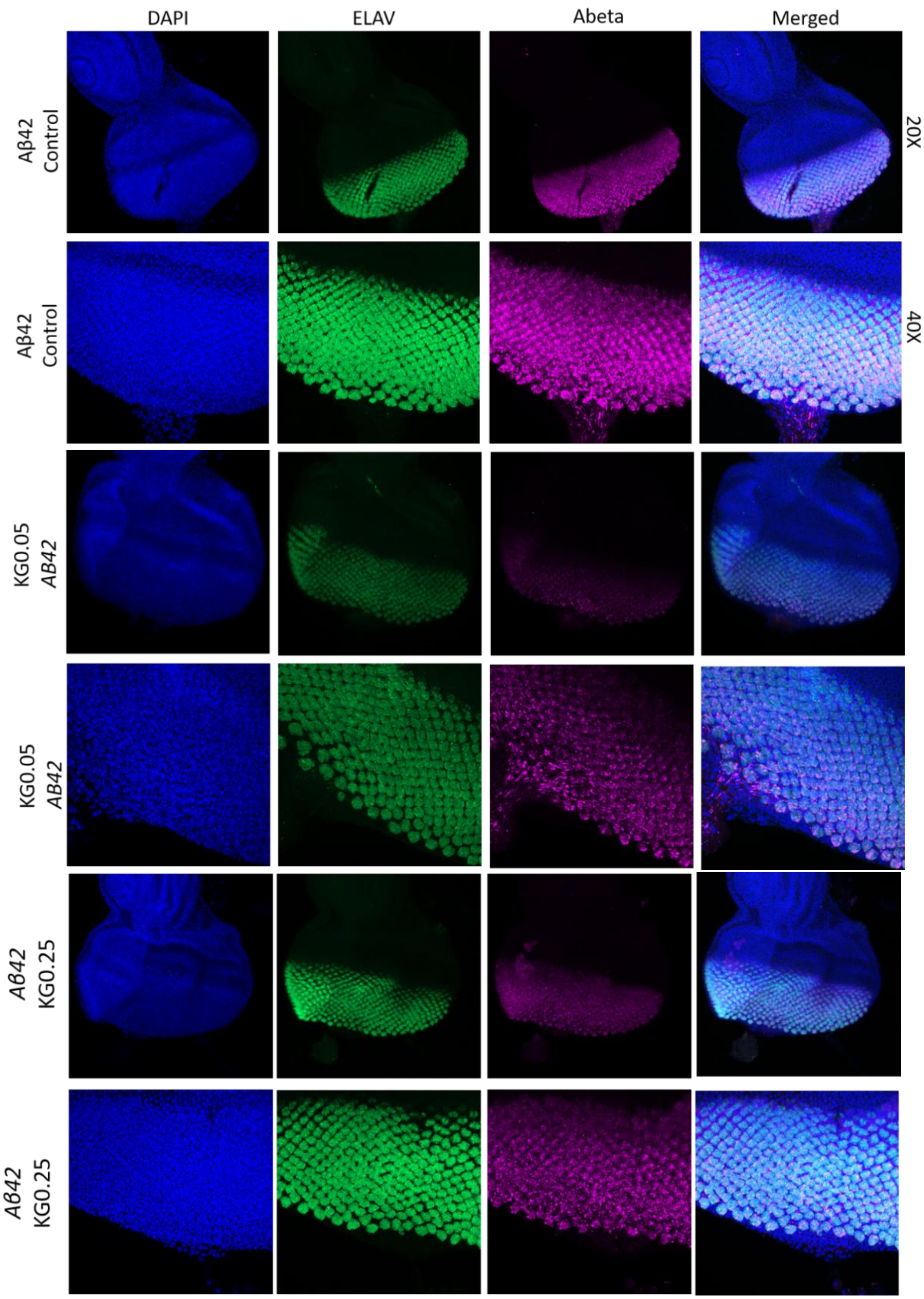

Fig. 11. Confocal projection images of eye discs immunostained for detecting expression of HA-tagged Aβ42 in larvae reared on normal food (Control, top two rows) and on food supplemented with KG (0.05% or 0.25%, noted on left of each row. Each pair of rows show images of eye discs collected using 20x or

40x objective respectively (as noted on right of the first two rows on top). Columns 1-4 show DAPI-stained DNA (Blue,), Elav protein (green), Aβ42 protein (magenta) and merge of all the three fluorescence patterns, respectively.

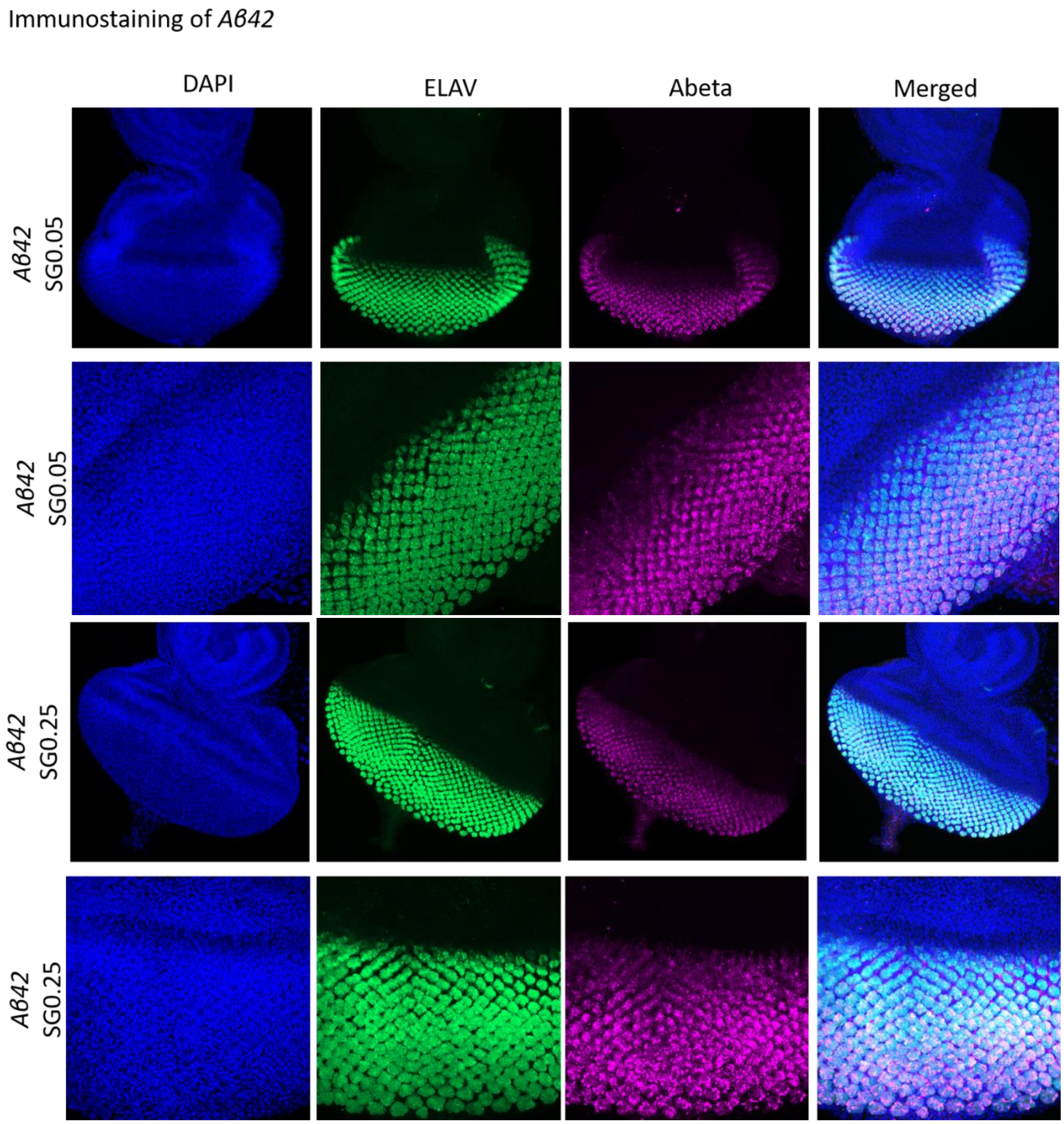

Fig. 12. Confocal projection images of eye discs immunostained for detecting expression of HA-tagged Aβ42 in larvae reared on food supplemented with SG (0.05% or 0.25%, noted on left of each row. Each pair of rows shows images of eye discs collected using 20x or 40x objective respectively (as noted on right of the first two rows on top). Columns 1-4 show DAPI-stained DNA (Blue), Elav protein (green), Aβ42 protein (magenta) and merge of all the three fluorescence patterns, respectively.

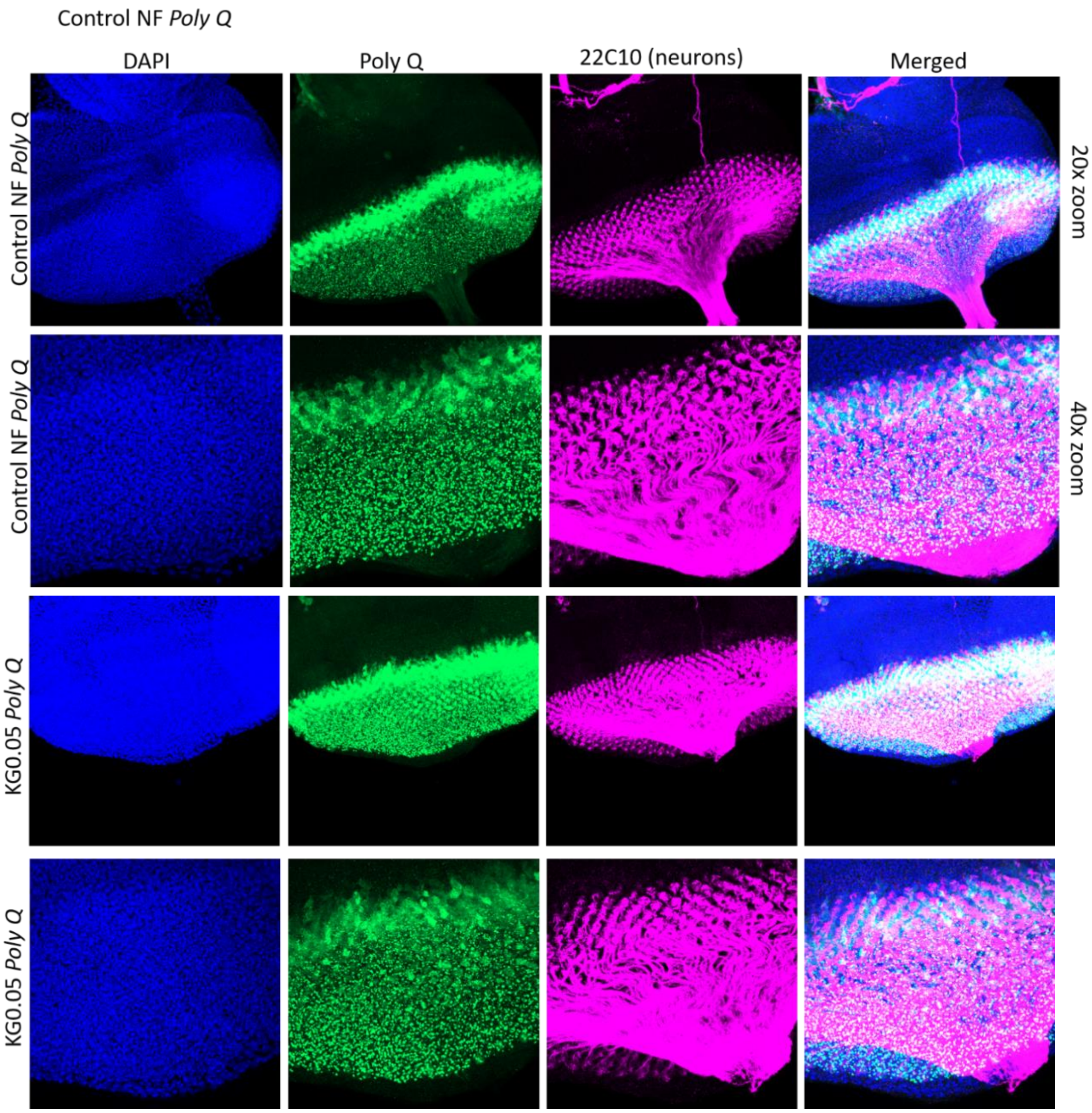

Fig.13. Confocal projection images of eye discs immunostained for detecting expression of HA-tagged 127Q in larvae reared on normal food (Control, top two rows) and on food supplemented with KG (0.05% or 0.25%, noted on left of each row. Each pair of rows shows images of eye discs collected using 20x or 40x objective respectively (as noted on right of the first two rows on top). Columns 1-4 show DAPI-stained DNA (Blue), 127Q protein (green), neuron-specific 22C10 reactive protein (magenta) and merge of all the three fluorescence patterns, respectively.

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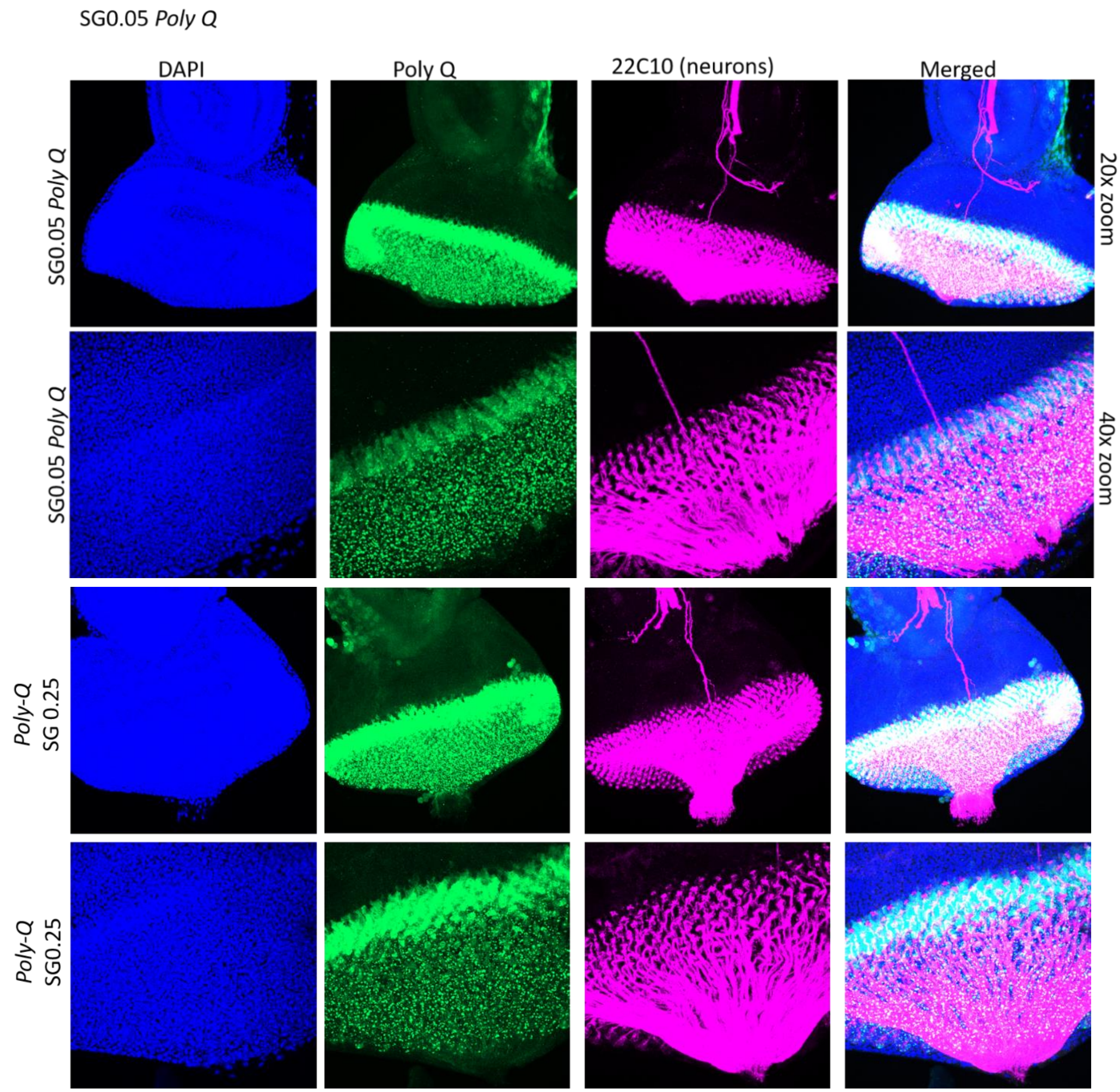

Fig. 14. Confocal projection images of eye discs immunostained for detecting expression of HA-tagged 127Q in larvae reared on food supplemented with SG (0.05% or 0.25%, noted on left of each row. Each pair of rows shows images of eye discs collected using 20x or 40x objective respectively (as noted on right of the first two rows on top). Columns 1-4 show DAPI-stained DNA (Blue), 127Q protein (green), neuron-specific 22C10 reactive protein (magenta) and merge of all the three fluorescence patterns, respectively.

DISCUSSION

In the current era, while we have made significant advancements in increasing life expectancy, there remains a pressing need to enhance the quality of life. Neurodegenerative diseases have become increasingly prevalent, serving as stark reminders of this imperative. Ancient Ayurvedic science has endeavored to comprehend the significance of these ailments, categorizing the head(shiras) as an essential part of the body known as the or "uthamanga." Ayurvedic medicines, many of which have exhibited positive outcomes in clinical practice, attribute a crucial role to ghee in promoting neuronal health.

During the course of this dissertation project, aimed to get trained in research methodology using advanced genetic and microscopic methods, re-investigated the impact of two Ayurvedic formulations, namely SG and KG, on fly models of Huntington's and Alzheimer's diseases. Following the results of an earlier study with these formulations (Sharma et al., 2021) .These formulations were administered at two different concentrations (0.05 and 0.25). To assess their effects, I examined various parameters, including lethality assay, climbing assay, phototaxis assay, and immunostaining for protein aggregates in the affected cells.

In agreement with the earlier results (Swathi Sharma, 2021), present results also revealed reduced expression of Aβ plaques in eye discs from the *>Eye-Gal4>Aβ42* expressing larvae when reared on KG or SG-supplemented food, especially at the lower concentration of each. Surprisingly, however, the polyQ aggregates in *GMR-Gal4>127Q* expressing larvae reared on KG or SG-supplemented food did not show any reduction. This is contrary to the earlier results (Swathi Sharma, 2021). In my first immunostaining set using similarly treated polyQ expressing larvae, I saw a reduction in polyQ expression in larvae reared on 0.5% KG or SG-reared food. However, in that initial study, the imaginal discs were not properly mounted, because of which I repeated the experiment with better mounted 6 imaginal discs. However, the confocal image collection parameters were not properly set so that the polyQ staining images of control treated samples were collected at saturation points which precluded any quantitative comparison. The experiments could not be repeated due to the limited time. Therefore, these experiments need to be repeated.

It was surprising that the behavioural assays (phototaxis and climbing assays) on adult flies showed adverse effects of KG or SG on the movement abilities of adult flies, especially of females. It appears that the continuous feeding of adult flies on ghrita containing food alters their physiology so that they become sluggish and incapable of active motility. The present results are limited because all the appropriate controls were not examined due to limited tie. In order to see if the larval feeding affected adult behaviour, the adults emerging from the supplement-fed larvae should have been reared on normal unsupplemented food. In addition, the *GMR-Gal4* flies (-ve control) should also have been fed on the KG- or SG-supplemented food during larval and adult stages to reveal the physiological effects of these gritha containing supplements. In addition, the other control should have been rearing of larvae and flies only on gritha-supplemented food. Further studies should keep such controls as part of the experiment.

A recent study in *C. elegans* has shown that *Terminalia chebula*, an important component of both the formulations, has potential effect in preventing Abeta toxicity. (Zhao et al., 2021). Other studies have shown that curcumin also improved brain health in multiple ways: it acts as an antioxidant, binds to amyloid β, reduces inflammation, inhibits tau, chelates metals, promotes neurogenesis, and enhances synaptogenesis. (Salehi et al., 2020). Similarly, *Cedrus deodara* has been shown to possess anticonvulsive and neuroprotective biomolecules research and to have cognitive enhancement and antidepressant activity in mouse model.(Sinha, 2019).

Another study explored the effectiveness of embelin, derived from the *Embelia ribes Burm*. plant, in protecting rat brains against damage caused by transient global ischemia. (Thippeswamy et al., 2011)Antidepressant activity of *Elletaria caraamum* oil was studied in a rat model.(Abdel-Rasoul et al., 2023)In another study using a rat model of chronic unpredictable mild stress (CUMS) to induce depression-like symptoms, researchers found that jasmine tea treatment improved depression-related behaviors and neurotransmitter levels. The study also investigated changes in gut microbiota, as revealed by 16S rRNA sequencing, since the diversity and richness of gut microbiota in the depressed rats was enhanced. The results suggest that jasmine tea may attenuate depression in rats by acting on the brain-gut-microbiome axis.(Zhang et al., 2022).

In a study, researchers investigated the neuroprotective properties of EISO (extracts from the Sandalwood tree) and its active components on *C. elegans* model. They found that EISO and two of its components, α- and β-santalol, were able to protect *C. elegans* from neurodegeneration caused by 6-OHDA, a toxin associated with Parkinson's disease. The protective effects were due to the ability of EISO and santalol to reduce intracellular levels of reactive oxygen species (ROS) and inhibit apoptosis (programmed cell death).(Mohankumar et al., 2020).

Limitations of the present study

The present study has several limitations, including smaller and variable numbers of flies utilized for behavioral studies and planning oversights, and the inability to produce more refined confocal scanning images for the polyQ phenotype. Obviously, it is essential to repeat the experiments using greater numbers of flies and multiple times to establish robust and valid results.

The relevance of such researches on Ayurvedic Medhyarasayana becomes more pronounced in the post-COVID era, where an increase in neurodegenerative cases has been observed. The prolonged human lifespan in current times has also contributed to a rise in individuals suffering from neurodegenerative conditions, such as dementia. It is worth noting that modern medicine does not focus on utilizing fat-based or similar formulations in the field of neurology, whereas Ayurveda emphasizes the importance of fat, particularly ghee, in improving neuronal health, memory, and intelligence. Ayurveda considers Gritha, processed with ushna veerya drugs like KG and SG, capable of reducing Vata and Kapha doshas and their associated complications, which can be explained by understanding the mechanisms underlying proteinopathies. These findings open up possibilities for further exploration and the development of more effective drugs in treatment of diseases like Alzheimer's which is need of the hour.(Kim et al., 2022) These aspects need further experimental studies in different model systems. Considering the adaptability of higher organisms like rat/mice to fat-containing diets, future research should consider conducting similar studies in these models to yield more applicable results. Molecular studies focusing on tau proteins (in relation to obesity) and genomic investigations can provide a more comprehensive understanding of the underlying mechanisms.

CONCLUSION

- *Drosophila melanogaster* serves the purpose of model organism for Huntington and AD.
- Low dose of KG and SG are able to decrease lethality of *PolyQ* flies with larval feeding.
- KG and SG are able to decrease *PolyQ* aggregates and *Abeta* plagues in HD and AD model flies in larval stage.
- Sluggish nature of *PolyQ* flies is seen to increase in continuous formulation feeding.
- Dosage of medicine have very important role in modeling the results, apt dose specific for disease and organism plays crucial role in giving results
- Female *PolyQ* flies were found to be poor climbers as compared with male
- Increased concentration of medicine was making flies calmer.
- There exist many limitations of choosing model organism and interpreting their results with limited span of time
- Analysis qualitative data and converting them into numbers are subjected to variations

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