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Nutritional assessment of a gemmotherapy extract with a presumed anti-inflammatory effect

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INTRODUCTION

Excessive sugar consumption is of rising concern as it is associated with many non-communicable diseases (NCD) including type 2 diabetes (T2DM), diet-induced obesity, cardiovascular diseases, hyperuricemia, and some forms of cancers (Huang *et al*., 2023). Recent studies report that increased intake of simple sugars, such as glucose and fructose, are closely linked with the development of low-grade chronic inflammation, autoimmune diseases, and even neuroinflammation (Ma *et al*., 2022).

In the case of chronic inflammation, the immune system secretes pro-inflammatory cytokines such as plasma C-reactive protein (CRP), interleukin-6 (IL-6), E-selectin (E-selectin), monocyte chemotactic protein 1 (McP-1), and tumor necrosis factor-α (TNF-α), plasminogen activator inhibitor 1 (PAI-1) and others (Ma *et al*., 2022). Persistent and prolonged production of such molecules may further induce complications in many systems including the inflammation of neurons and the brain cells. Such neuroinflammation is an extensive sign of many neurodegenerative disorders including Alzheimer's, Parkinson's, Huntington's disease, and Amyotrophic lateral sclerosis (Singh et al., 2021). Indeed, in a recent study it has been reported that high-glucose diet in *C.elegans* leads to α-synuclein aggregation and accelerates the aging progress which is strongly associated with neurodegeneration, particularly, with Parkinson's disease (de Guzman *et al*.,2022). Another study demonstrated that high-sucrose diet causes psychosis-related behavior like hyperactivity, impaired sensory gating, poor memory, and disrupted interneuron function in glyoxalase (GLO1) deficient mice (Hirai *et al*., 2021). It also caused brain angiopathy with impaired glucose uptake (Hirai *et al*., 2021). Excessive sugar intake in rats for less than 2 weeks was associated with increased expression of inflammation markers in the hippocampus, and rats fed liquid sugar demonstrated central and peripheral inflammation and showed memory deficits (Beilharz *et al*., 2016).

Current research has demonstrated that certain compounds from plants and herbs can delay neuronal damage and inhibit oxidative stress. Some of these compounds include quercetin, carnosic acid, rosmarinic acid, curcumin, amygdaline and others (Singh *et al*., 2021). Despite this, there is limited study in the field of gemmotherapy extracts and their anti-inflammatory effect on neuronal system, especially, *in vivo* experiments.

This study will investigate a gemmotherapy extract called Polygemma 28 (GTE 28) for its nutritional, anti-inflammatory and neuro-protective effect using *Drosophila melanogaster* as a basic model-system. The *Drosophila melanogaster* will be grown in three different media, which are the zero media (0M) to test the nutritive effect, normal media (NM) to test for

cytotoxicity of the plant extracts, and the high-sugar media (HS) to stimulate inflammation. Combination of HS-media with the GTE 28 will be used to test if the extract has a potential rescue and anti-inflammatory effect. In addition, brain dissections will be performed to compare if there is any difference between larvae fed on NM and HS-dietary conditions. This will be done to observe impact of HS-diet on brain of larvae and see if GTE 28 can have some neuro-protective role, and potentially restore the changes to the brain, if any.

The main objectives of the study are as following:

- to determine the total polyphenol content (TPC) of polygemma extract;
- to determine the total flavonoid content (TFC) of polygemma extract;
- to determine the antioxidant activity of the polygemma extract;
- to perform viability and cytotoxicity assays of *Drosophila melanogaster* in 0M and NM (5 different concentrations);
- to determine the effect of HS media on the flies' viability and the effect of the extract when combined with HS-diet;
- to perform quantification of the Red Eye Pigment of male adult flies to determine relation of diet with epigenetic regulation of the chromatin structure;
- to perform brain dissection of third instar larvae fed on HS-diet to observe and measure brain size.

LITERATURE REVIEW

1.Background on Gemmotherapy

Gemmotherapy is a field of phytotherapy that uses embryonic stem cells of plants such as buds, sprouts, and young stems by maceration in hydroglyceroalcoholic solutions (Grigoriu *et al*., 2021). The primary beneficial effect of such extracts are enhancement of organ function, potential elimination of toxins, and facilitation of tissue regeneration (UNDA, n.d.). The fundamental principal of gemmotherapy is a holistic approach characterised by preventative measures and an understanding of the imbalance affecting organs, rather than a simple treatment of pathological symptoms (Ionescu, 2018).

The first use of this homeopathic therapy was done by a Belgian doctor Dr. Pol Henry in 1950s (UNDA, n.d.). He conducted a preliminary research on the various properties of plant buds in Belgium and published his clinical findings in 1970, and named it 'Phytoembryotherapie'(UNDA, n.d.). The current name 'Gemmotherapy' was coined by a French doctor Dr. Max Tetau (UNDA, n.d.). The commercial gemmotherapy products are regulated by the European Union and the common distributors are Boiron, Herbal Gem, UNDA, and Plant Extrakt (Hubele, 2015). The modern laboratory studies in Italy by Dr. Fernando Piterà demonstrate the scientific basis and the growing implication of this field (Hubele, 2015). Dr. Piterà is an expert in the field of Homeopathy, Homotoxicology, Phytotherapy, and Gemmotherapy, with over 200 publications (Fernando Piterà, n.d.). Some of the notable books include *Compendio di Gemmoterapia Clinica* (1994, Ed. De Ferrari, Genoa), 12th volume of the "Health" Encyclopedia of "Le Grandi Opere del Corriere della Sera" (2006), 17th volume "Health for all" of the Medical Encyclopedia of the Umberto Veronesi Foundation of "La Gazzetta dello Sport" (2012), Homeopharm Therapeutic Handbook (2012) and many other articles in the field of Gemmotherapy and Homeopathy (Fernando Piterà, n.d.).

2. Polygemma 28 Neuro Protect extract

Polygemma 28-Neuro Protect is a gemmotherapy liquid extract (GTE) that is used in this research work to investigate its neuroprotective effect on *Drosophila melanogaster* as a basic model system. The given extract is from a brand Plant Extrakt and contains the combination of four plants: gemmotherapy extracts of buds of blackcurrant and shoots of black grass; and phytotherapeutic extracts of sage herbs and saffron stigmas (Figure 1.) (Plant Extrakt LTD, n.d.). It is claimed by the brand that this is a natural product which can help with concentration, relaxation, improve mental performance and emotional balance. Extract of sage

supports working memory, black grass has calming properties, saffron contributes to the emotional balance, while blackcurrant enhances resistance to stress. Including, the antioxidants can protect against free radicals (Plant Extrakt LTD, n.d.).

Figure 1. Polygemma 28 Neuro Protect (Plant Extrakt LTD, n.d)

2.1. Blackcurrant: General description and health benefits

Ribes nigrum, commonly known as blackcurrant (BC), is a species that belongs to the family of Grossulariaceae (Tian *et al*., 2019). It is a woody shrub with a small dark purple fruit

that has a sour, bitter, and an astringent taste. As shown in the Figure 2. below, they are small round and grow in groups.

Figure 2. *Ribes nigrum* shrub and fruit (Britannica, n.d.)

They are native to colder climate areas including northern Europe, northern Asia, and Central Asia (Cortez & Gonzalez, 2019). BC contains many bioactive compounds such as polyphenols, flavonoids, and flavour metabolites like sugars, acids, and phenolic compounds. These characteristics make it an excellent target for traditional herbal medicine including gemmotherapy. The full phytonutrient profile of BC extract has been published in a recent study by Téglás *et al* (2023).

One of the most studied chemical groups in BC are anthocyanins which are known to exert antioxidant, anti-inflammatory, and neuroprotective roles (Cao *et al*., 2021). Anthocyanins are contained not only in fruits but as well as in the buds of BC (Cao *et al*., 2021). In recent studies, it has been reported that the consumption of anthocyanins from bilberries and BC supress NF-κB pathway which causes inflammation in human subjects with metabolic syndrome (Cao *et al*., 2021). Another study conducted by Nanashima *et al* has presented that anthocyanins of BC induce G0/G1 cell cycle arrest and apoptosis in the breast epithelial cell line MCF10A (2017). It reduced breast cancer cell proliferation and can be a useful complement in breast cancer prevention (Nanashima *et al*., 2017). BC is also rich in flavonols such as quercetin, naringenin, myrectin, which decrease oxidative stress induced on neuronal cell membrane (Karjalainen *et al*., 2009). A study conducted on *Drosophila melanogaster* and Wistar rats has demonstrated a neuroprotective effect of blackcurrant that prevented microglial body swelling in hippocampus and reduced *TNF- α* serum levels (Téglás

et al., 2023). Overall, BC is known to have beneficial effects on supporting cardiovascular health, regulation of blood glucose levels, and can prevent risks associated with Non-Communicable Diseases (CND) (Laaksonen, 2014).

2.2. Common sage: General description and health benefits

Salvia officinalis, known as common sage, is a member of Lamiacea family that contains over 900 species all over the world (Hamidpour *et al*., 2014). It is an herbaceous, perennial plant with flowers in different colours (Figure 3). It is native to many Mediterranean countries (Hamidpour *et al*., 2014). This plant is widely used in many cuisines as a spice.

Figure 3. *Salvia officinalis* leaves and flowers (Hamidpour *et al*., 2014)

Sage is rich in phenolic compounds that have been extracted and characterised in many studies, which include the derivatives of phenolic acids (carnosol, rosmanol, epirosmanol, rosmadial, methyl carnosate), tannins, phenylpropanoid glycosides, triterpenoids, γ-tocopherol, α-tocopherol, carotenoids, gallic acid, 3-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, caffeic acid, hesperetin, hispidulin, and genkwanin (Živković *et al*.,2017). Phenolic compounds are excellent in downregulating pro-inflammatory cytokines, apoptosis regulation, and in preventing protein aggregation, which are commonly associated with progressive neurologic loss and complications (Rojas-García *et al*., 2023). In addition, *S.officinalis* is known to be useful in memory disorders, depression, and cerebral ischemia (Hamidpour *et al*., 2014). It also enhances mental functions and shows reparative effects in patients with Alzheimer's disease (AD). As was shown in a randomised trial, ethanolic extract of sage oil can have a positive effect in the management of mild to moderate forms of AD (Hamidpour *et al*., 2014). In other animal studies, essential oil of sage leaves demonstrated a hypoglycaemic

effect in diabetic rats, which has a potential role in the prevention of diabetes (Miraj & Kiani, 2016).

2.3. Common heather: General description and health benefits

Calluna vulgaris L. (heather) belongs to the family of Ericaceae Juss and is an evergreen perennial shrub (Chepel *et al*., 2020). This plant grows in Europe, the Mediterranean, some parts of western Siberia, Turkey, Iceland, New Zealand, Australia, Morocco and some parts of Asia (Kaunaite *et al*., 2022). The plant grows up to 20 to 50 cm in acidic soil, open sun, and in moderate shade conditions (iNaturalist, n.d). The flowers are small rose pink to purplish pink colours and appear in mid to late summer season (Figure 4.) (iNaturalist, n.d).

Figure 4. *Calluna vulgaris* flower

Heather is rich in proanthocyanidins, phenolic and triterpenic compounds that are known to be excellent free radical scavengers and antimicrobial agents (Kaunaite *et al*., 2022). Other bioactive compounds such as flavonoids, phenols, tannins, caffeic acid derivatives, triterpenes, and hydroquinones have been identified in *C.vulgaris* that have antioxidant, antimicrobial, anti-inflammatory, neurotropic, and chemoprotective properties (Cucu *et al*., 2022). Furthermore, common heather is widely used for urinary tract infections in traditional medicine (Vučić *et al*., 2014). For instance, *in vitro* studies of antibacterial activity against 30 strains of urinary

pathogens demonstrated that extracts of *C.vulgaris* leaves and flowers had inhibitory effect on them (Vučić *et al*., 2014).

2.4. Saffron: General description and health benefits

Crocus sativus, known as saffron, is commonly used as a flavouring spice in many cultures and it belongs to the family of Iridaceae (Mzabri *et al*., 2019). The saffron flowers start to appear at the beginning of autumn, flowers are purple colour composed of six petals (Figure 5.) (Mzabri *et al*., 2019). The stigma of saffron is in the middle of the flower and is used as a sweetener in many cuisines (Figure 5).

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Figure 5. *Crocus sativus* flowers (Britannica, n.d)

Figure 6. A pile of dried stigmas (Britannica, n.d)

The main chemical components that are biologically active include crocin, picrocrocin, safranal, along with these it contains carotenoids, flavonoids, and anthocyanins (Mzabri *et al*., 2019). Saffron has high radical scavenging activity and blocks pro-inflammatory cytokines (Ghaffari *et al*., 2019). Its mechanism is said to be through inhibition of transcription factor NF-κB which is involved in chronic inflammation (Ghaffari *et al*., 2019). Many studies report that saffron improves symptoms of mild depression. Short term administration of saffron (30 mg/day) capsules for six weeks has shown to improve conditions of patients with major depressive disorder (Khazdair *et al*., 2015). Also, saffron stigmas are reported to be effective in treating mild forms of depression, as well as can prevent aggregation of *β*-amyloid in the brain, thus, can be useful in Alzheimer's disease (Mzabri *et al*., 2019).

3. *Drosophila melanogaster* as a model organism

The common fruit fly, *Drosophila melanogaster,* is one of the extensively used model organisms for investigation of many biological processes, including nutritive research. Many studies have reported phenotyping *D.melanogaster* in the context of nutrition, especially, dietary interventions with high-sugar diet and high-fat diet types (Eickelberg *et al*., 2022).

There are several advantages to using fruit flies as a model to learn about dietary interventions and for the effect of inflammation. Firstly, it is of small size, low cost, and has short life cycle which allows rapid study. Secondly, *Drosophila* has a compact brain neurons and glial cells of which are similar in their function to those of vertebrates (Nitta & Sugie, 2022). This would allow rapid observation upon the effect of different diets such as the 0N, NM, and the HS-dietary interventions, as well as to observe the effect of HS-diet on the neurons and the brain of the fruit flies.

The lifecycle of *Drosophila* is divided into four stages, which are embryo, larvae, pupae, and adult (Figure 6). The egg stage lasts for 1 day and it transforms into 1st instar, 2nd instar, and 3rd instar larvae. The 3rd instar larvae feeds to then become a pupae. The pupae stage takes 3 days, which then hatches into an adult fly (Nitta & Sugie, 2022).

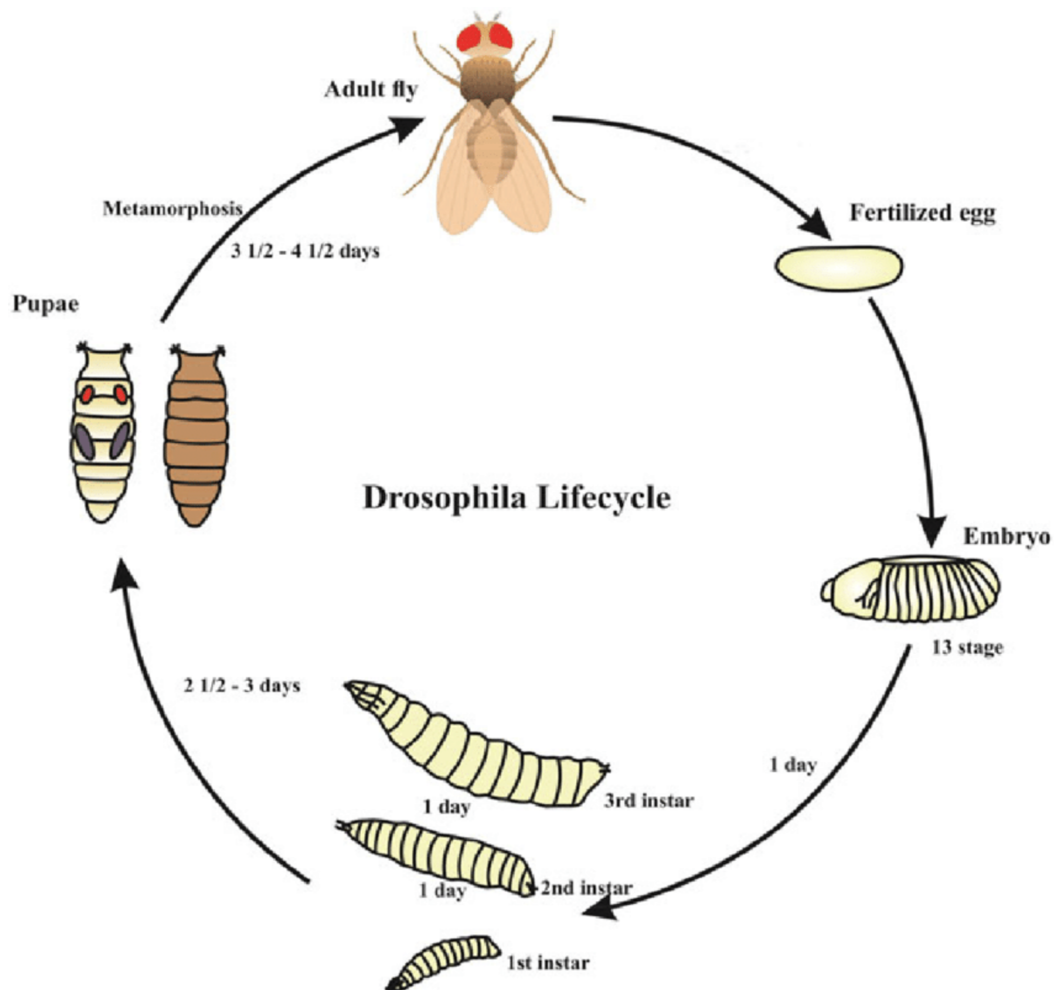

Figure 7. The lifecycle of *Drosophila melanogaster* (Barik & Mishra, 2018)

In this experimental work, a white-mottled (w^{m4h}) strain of *Drosophila melanogaster* was used. This strain is a genetic variant of the common fruit fly which is used for the study of underlying mechanisms of gene expression and parental imprinting (Hoyer-Fender, 2020). Commonly it is used as a model of position-effect variegation (PEV). PEV is a chromosomal inversion of the X chromosome in *Drosophila melanogaster,* which causes the inactivation of a wild-type euchromatic gene when it is relocated in or close to the heterochromatin by chromosomal rearrangement (Hoyer-Fender, 2020). The white gene, which encodes a pigment transporter for the red colour of the eye in wild type flies, is affected due to this rearrangement. The difference in condensed and inactive conformation of the heterochromatin leads to either expression or silencing of white in individual cells resulting in a mosaic eye phenotype, with patches of white and red (Berloco *et al*., 2014). This phenotypic characteristic can be seen in the figure below (Figure 8.), where wild-type would normally have red eyes, while the chromosomal rearrangement causes the eye to appear mottled (Singh, 1994). Such phenotype occurs because some of the dominant allele of white, w^+, get repressed due to their proximity

to the heterochromatin, allowing recessive alleles to be expressed. Thus, all parts of the eye with red colour represent pigmented wild-type phenotype and the non-pigmented are the mutant areas (Singh, 1994).

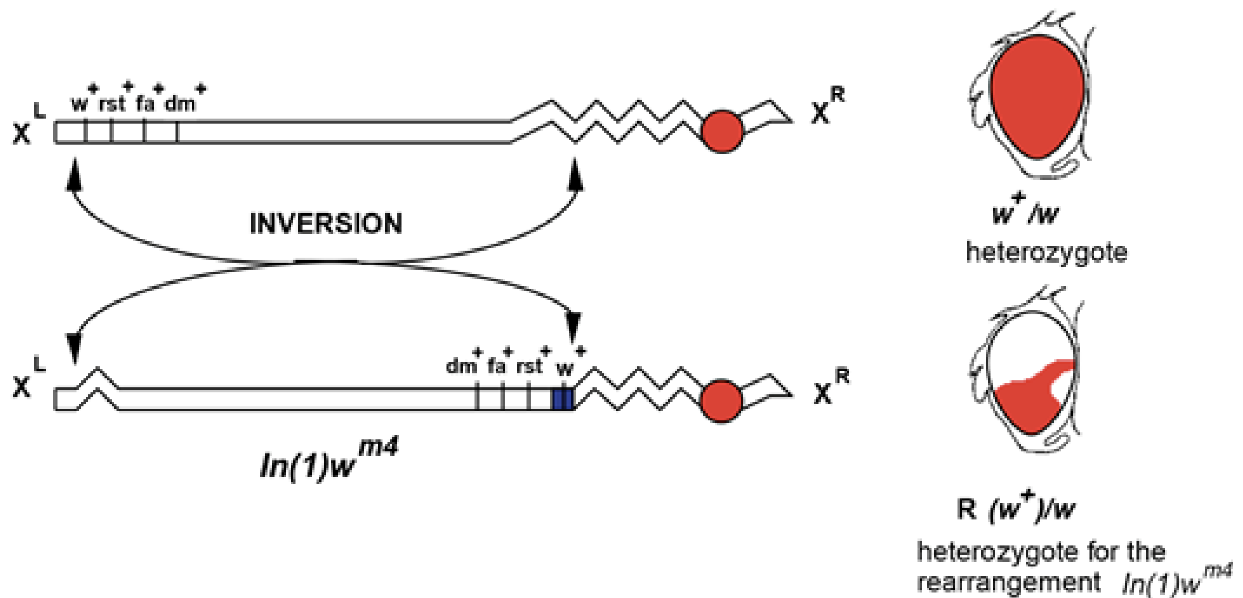

Figure 8. A schematic illustration of white variegation in the X chromosome inversion of ln(1)w^{m4} (Singh, 1994).

These modulations are regulated by transcriptional enhancers, E(*var*)s and suppressors, Su(*var*)s, which were isolated from w^{m4h} strain studies, and they affect either activation or the silencing of PEV (Hoyer-Fender, 2020). The inactivation of w^+ is stochastic, but once established, it is inherited to daughter cells. Such heritable phenotype without a change in DNA sequence is defined as epigenetic inheritance (Hoyer-Fender, 2020). Various environmental and external factors may have an inherited epigenetic effect on the eye colour of flies. For example, PEV regulation in *Drosophila melanogaster* is heat sensitive (Singh, 1994). Other factors such as parental stress conditions and diet may induce alteration of mottled eye colour (Singh, 1994).

There are different studies that have demonstrated epigenetic influence of dietary interventions. The study conducted on paternal diet and intergenerational obesity concluded that acute dietary interventions have the capacity to modify F1 offspring phenotype via the male germline (Öst *et al*., 2014). It also reported that high sugar diet acts as a physiological suppressor of variegation of eye color, allowing more of red pigmentation. Similar results were reported in a research studying epigenetic mechanisms of how high dietary sugar might induce tumor progression (Chang *et al*., 2021). According to it, PEV assay has shown that flies fed on high sugar had decreased levels of heterochromatin than the flies fed on normal sugar diet,

which resulted in a less mosaic eye color and more red pigmentation. This suggests that high sugar diet might reduce heterochromatin formation, meaning that it would decrease the expression of mosaic phenotype (Chang *et al*., 2021).

The current work will also utilize PEV assay to understand if there is any epigenetic influence of the dietary conditions, specifically, of high sugar diet, on the phenotypic appearance of the eye color and according modification of chromatin structure and its regulation.

4. High Sugar Media and Inflammation

Inflammation is a physiological immune response to infectious and non-infectious processes that involves innate, adaptive immune and the inflammatory mediators made to protect the body (Oransky *et al*., 2022). However, a persistent trigger of pro-inflammatory mediators leads to chronic inflammation.

Inflammatory responses can also occur in the brain or the spinal cord due to various injury, insults, or stress (DiSabato *et al*., 2016). This inflammation mediated by the cytokine and chemokine production by microglia and astrocyte cells of the brain is often referred to as neuroinflammation (DiSabato *et al*., 2016). Microglia are macrophages of the central nervous system (CNS), that acts as a phagocyte of harmful pathogens and cellular debris, and have a protective role for the brain (Muzio *et al*., 2021). But chronic activation of microglia can result in irreversible CNS damage. Persistent neuroinflammation in the brain can damage memory, cause loss of neuronal plasticity, and contribute to tissue damage in neurodegenerative disorders (Muzio *et al*., 2021). In this case microglia release inflammation activators such as TNF-α, IL-6, IL-1β, and ROS, which leads to chronic inflammation (Muzio *et al*., 2021). Studies have demonstrated that microglia and neuroinflammation contribute to the pathophysiology of neurodegenerative diseases. For example, a study in human tissue demonstrated an increased leukocyte infiltration to brain sites in AD patients, which implies a damage to blood-brain barrier (Woodburn *et al*., 2021). Some other studies of PD dementia found that activated microglia were prevalent in affected regions, and often in close proximity to α-synuclein and monoaminergic neuritis (Pasqualetti *et al*., 2015).

For the study of inflammation and associated complications, high-sugar diet experimental designs have been applied. Particularly, it has been used to model hyperglycemia, diabetes, and insulin resistance associated complications (van Dam *et al*., 2020). In other studies, the overconsumption of dietary sugars has been linked with the alteration of gut-brain axis, where high sugar can trigger both gut permeability and the blood-brain barrier (Ochoa *et*

al., 2015). Unaddressed chronic inflammation damages healthy cells and tissues in a long term and is associated with many illnesses like T2DM, obesity, cardiovascular complications and others. In the case of obesity, adipose tissue releases chemoattractant for macrophages that induces more inflammatory mediators to the cite, causing high degree of oxidative stress, hypoxia leading to metabolic and immunological diseases (Castro *et al*., 2017). A prolonged inflammation is also related to the damage in DNA repair and apoptotic pathway control, which increases the risk for the development of cancerous cells and their growth (Agresti *et al*., 2016).

In this experiment, HS-dietary intervention is used to stimulate inflammation conditions to observe its impact on the model organism. Particularly, it is focused on the effect of high sugar on neuronal system of the flies. The observations of neuron cells, nuclei and the brain size of the model organism is performed to understand the impact of HS and to investigate the potential rescue effect of the GTE 28, which is claimed to be anti-inflammatory for the nervous system by the brand.

MATERIALS AND METHODS

1.Preparation of GTE

The Polygemma 28-Neuro Protect was obtained from a brand Plant Extrakt, Romania. The vegetal materials and production were monitored to meet the quality standards at the laboratories of SC Plant Extrakt SRL QC.

The ingredients of the extract are as following: wetting agent – glycerin; glycero-ethanolic extracts from blackcurrant buds 1:20 (*Ribes nigrum* glycerinated macerates (MG)) – 20%; black grass shoots 1:20 (*Calluna vulgaris* MG) – 10%; hydroethanolic extracts of sage herb 1:1 (*Salvia officinalis*) – 10%; stigmas of saffron 1:10 (*Crocus sativus*) – 1%. Alcohol concentration is 25% v/v. All plants are organically harvested and ECOINSPECT Ro-008 certified. The listed ingredients are obtained from the instructions for use on the webpage of the brand (Plant Extrakt LTD, n.d).

2. Spectrophotometric Determination of Total Polyphenol Content (TPC) of the GTE 28

The TPC was determined quantitatively using the Folin-Ciocalteu reagent (a solution of phophomolybdic and phosphotungstenic acids) with Gallic acid as the standard (Singleton, & Rossi, 1965).

First, 0.3ml of the sample extract was diluted to a total of 15mL with the mixture of methanol-water (80:20). Then, to a 0.5ml of the diluted extract 2.7ml of 0.2N Folin-Ciocalteu reagent and 2.5ml of Na_2CO_3 (75g/L) solution were added and homogenized for 10s. The samples were incubated at room temperature in a dark area for 1.5 hours. The absorbance was measured at 760nm using a Ultraspec 2100 *pro* UV/Visible Spectrophotometer (Amersham Biosciences). The same protocol was applied for the gallic acid solutions in a concentration range of: 0, 5, 10, 20, 50, and 100mg/L. The calibration curve correlation factor was 0.9998. The TPC of the GTE was expressed in gallic acid equivalents (mg GAE)/mL of the extract. All reagents were purchased from Merck (Darmstadt, Germany). All analyses were performed in triplicate and the results were statistically evaluated using Excel Microsoft Office.

3. Spectrophotometric Determination of Total Flavonoid Content (TFC) of the GTE 28

The TFC was determined by a colorimetric assay modified from original paper using Catechin as a standard (Shin et.al., 2008).

In a 10mL graduated tube, 1mL of the diluted extract sample was mixed with 4mL of methanol-water (80:20) and 0.3mL of 5% $NaNO_2$, and was shaken for 2-3 min. After 5 minutes, 0.3mL of 10% $AlCl_3$ solution, after an additional 1 min, a 2mL of 1M NaOH was added. 2.4mL of methanol-water (80:20) was added to make a total of 10mL and was shaken thoroughly. The

absorbance was measured at 510nm using a Ultraspec 2100 *pro* UV/Visible Spectrophotometer (Amersham Biosciences). Catechin was used as standard in concentration range of 0, 5, 10, 20, 50, and100mg/L. The calibration curve correlation factor was 0.9974. The TFC of the GTE was expressed in catechin equivalent (mg CE)/mL. All reagents were purchased from Merck (Darmstadt, Germany). All analyses were performed in triplicate and the results were statistically evaluated using Excel Microsoft Office.

4. Determination of the antioxidant activity of the GTE 28

The antioxidant activity was measured on the basis of detection of the radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) at 517 nm method adapted from BLOIS (1958).

The sample preparation was done by mixing 1ml of the extract with 9ml of methanol and then was diluted five-fold. To a 100µl of the prepared sample, 1.4ml of methanol and 1.5ml of DPPH reagent were added and the mixture was incubated for 30 min in a dark place at room temperature. The spectrophotometric measurement was done at 517nm using a Ultraspec 2100 *pro* UV/Visible Spectrophotometer (Amersham Biosciences). Methanol was used as a blank. Trolox solution was used as a standard in concentration range of 0, 0.033, 0.05, 0.1, and 0.2 mg/mL. The calibration curve correlation factor was 0.9978. All reagents were purchased from Merck (Darmstadt, Germany). All analyses were performed in triplicate and the results were statistically evaluated using Excel Microsoft Office.

5. Cytotoxicity, Viability, and High-Sugar Diet studies on *Drosophila melanogaster*

In all *Dorosophila melanogaster*-based experiments the w^{m4h} (white mottled 4) mutant strain obtained from the Bloomington Stock Center was used. The flies were raised in three different dietary conditions: (1) zero media (0N), (2) normal media (NM), (3) high sugar media (HS). The 0N does not contain any nutrients and is used for the assessment of survivability. The NM is used to assess cytotoxicity of the extract. The HS media is used to simulate a condition of inflammation.

The 0N was prepared using 1g of carbon powder (VMR, No. 87126.230) and 1g of agar (VMR, No. 20767.298) in 100mL distilled water. It was brought to boil and cooled down to 45^0C, and aliquoted 5ml into vials.

For the preparation of NM and HS media, 70g of yeast paste was dissolved in 1.2L of hot water until homogeneity. Then 51.35g (for NM) and 256.75g (0.5M sucrose content) of sucrose and 30g of wheat flour were added. The mixture was continuously stirred and was brought to boil. 10g of agar powder was added slowly and thoroughly mixed. The boiling continued for over 30 min and the culture media reached a final volume of 1L. The culture

media was cooled to 50^{0}C in a water bath and 1g of Nipagin (ThermoFisher) was added and mixed in. Then, 5ml of media was aliquoted into each vial. Whenever appropriate, the extract sample was added and mixed thoroughly with the culture media. The GTE was set at five different concentrations: 5.4%, 2.7%, 1.35%, 0.675%, 0.3375%. These concentrations were defined through previous arbitrary experiments for cell cultures. All fruit fly experiments were carried out at 25^{0}C.

The collection of *Drosophila melanogaster* embryos:

Around two hundred five-day old female and male w^{m4h} *Drosophila melanogaster* were introduced into an embryo collecting cage placed over a plate with 0N media supplemented with yeast paste. After 48h, the plates from the egg collector were replaced every 2h and 0-2h old embryos could be obtained. The embryos were transferred using fine forceps under the microscope into vials of 0M, NM, or HS media supplemented with the extract and its corresponding concentrations. A total of 50 embryos were placed into each vial. The experiments were carried out at least in triplicates.

The monitoring of viability during *Drosophila melanogaster* development:

The experiments were performed at 25^{0}C and constant humidity. The number of third instar larvae and adults were monitored daily until no more hatched adults were found. The GTE specific viability tests were based on flies with the same genotype and age, all the experiments were carried in parallel at least in triplicates to obtain fully comparable results.

5. Quantitative measurement of red eye pigment with spectrophotometric analysis

This quantitative measurement of red eye pigment was adopted from a paper published by Evans and Howells (1977).

10 heads of adult male flies were homogenized in 300μL of 0.1% NH_4OH - $CHCl_3$ (1:1). The homogenates were centrifuged for 4 min at 400rpm. 200 μL of top aqueous layer from each tube was transferred into Eppendorf tubes to which 500 μL of 0.1% NH_4OH was added. The absorbance of each samples was read at 485nm using a Ultraspec 2100 *pro* UV/Visible Spectrophotometer (Amersham Biosciences).

6. Third instar larvae brain dissection and visualisation under fluorescence microscopy

Brain dissection of third instar larvae was performed for live visualization under microscopy following a protocol by Wu and Luo (2006).

Third instar larvae cultured in HS-dietary condition combined with different concentrations of GTE 28 extract were collected. Larvae of interest were placed on a slide with RINGER's solution under the microscope. The brain attached to the mouth hook was removed

gently with forceps. Excess tissue was removed and rinsed multiple times. Dissected brains were aligned using forceps for imaging under the microscopy (OLYMPUS MV PLAPO 1X, Japan). Brain sizes, where two lobes, of the samples were measured and reported.

RESULTS AND DISCUSSION

1.Assessment of the GTE related to TPC and TFC

Polyphenols and flavonoids are powerful antioxidants that can help reduce inflammation and promote beneficial health effects. Therefore, the TPC and TFC content of the GTE was determined. The GTE of interest contains four plants – *R.nigrum, S.officinalis*, *Calluna vulgaris* L., and *C.sativus*. The measurements of both TPC and TFC of the sample were compared to the values of some of the individual plants listed above and other GTE extracts such as *C.vesca*, *A.hippocastanum*, and *J.regia*. The results are presented in the figures below (Figure 9. and Figure 10.).

The TPC of GTE 28 extract is determined to be 568.83 mg GAE/100 ml and showed high value in comparison to the individual GTE extracts, especially, with its individual plant components such as *S.officinalis* and *C.sativus*.

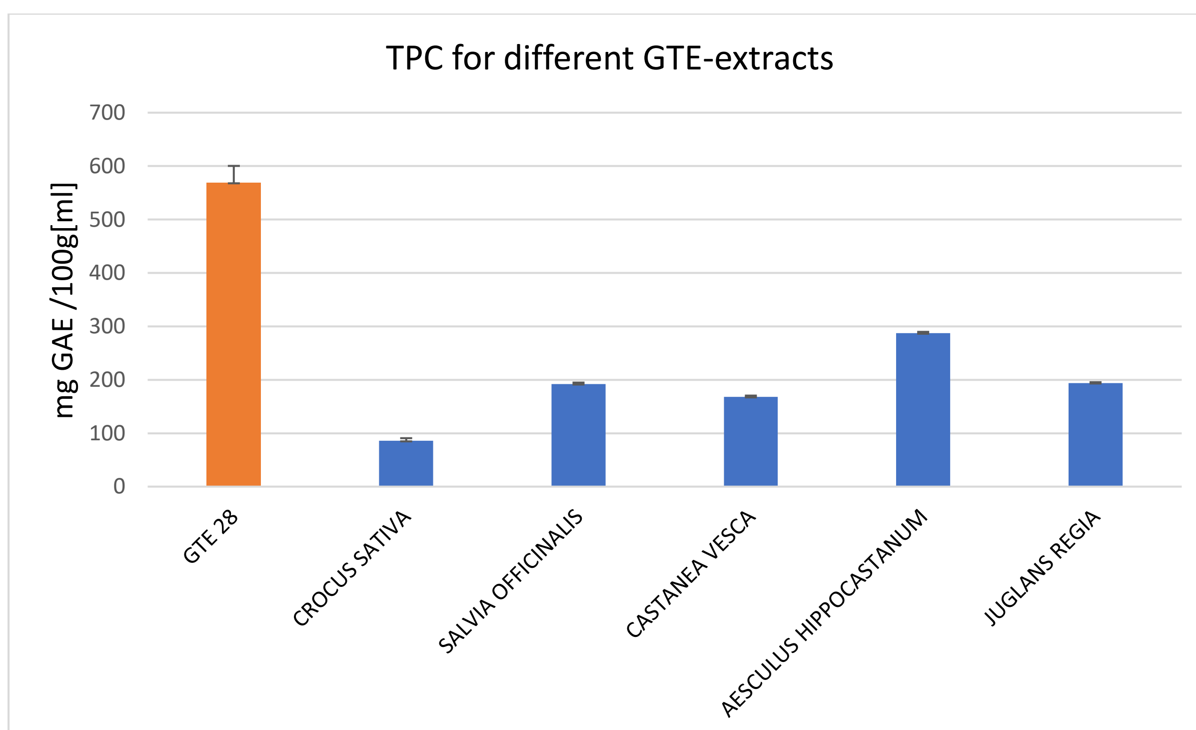

Figure 9. The TPC comparison of different GTE-extracts with the GTE 28

As for the TFC, the amount is determined to be 58.76 mg CE/100 ml. In comparison to other individual plant GTE extracts the value of TFC is low. This analysis reveals that GTE 28 majorly contains non-flavonoid type of polyphenols that could be responsible for antioxidant effects. It should also be noted that the full phytonutrient and HPLC-MS analysis for this GTE extract has not been completed and more details of other chemical compounds are unknown. Complete analysis of GTE extract of only *R.nigrum* has been published and can be accessed online (Téglás *et al*., 2023).

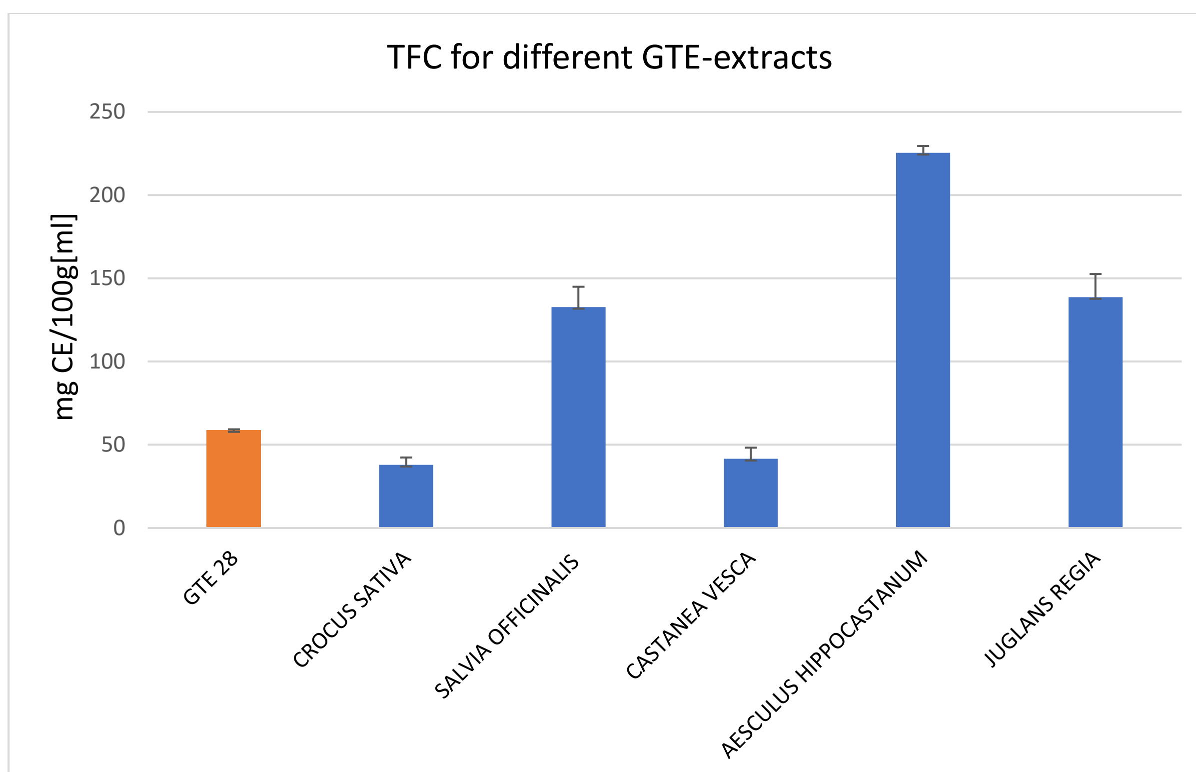

Figure 10. The TFC comparison of different GTE-extracts with the GTE 28

2.Assessment of the antioxidant capacity of GTE 28 with DPPH assay

Plant-derived polyphenols are powerful antioxidants that can protect against stress-induced or inflammation associated complications. Therefore, it is important to measure the antioxidant potential of GTEs and understand their free radical scavenging properties. For this purpose, the methodology of DPPH assay was utilised in this work. The DPPH is a free radical and its reduction level shows the antioxidant capacity of the given GTE (Lewis & Hamby, 2019). The result for the GTE 28 is reported below along with results for the individual plants - *C.sativus, S.officinalis, J.regia,* and *A.hippocastanum*.

Table1. The values of antioxidant capacity for different GTE extracts

Sample	**DPPH assay (µmol TE/ml)**
GTE 28	7.27 ± 1.00
C.sativus	5.24 ± 0.10
S.officinalis	30.03 ± 0.71
J.regia	42.54 ± 5.36
A.hippocastanum	69.42 ± 4.17

The value for GTE 28 extract is 7.27 ± 1.00 µmol TE/ml, which is lower compared to the other presented individual plants such as *S.officinalis, J.regia,* and *A.hippocastanum.* However,

it should be noted that solid weight of extracts added into GTE product are different and could have resulted in such differences. While the individual plants would be much more concentrated, mixture of different plants in GTE 28 might have resulted in a more diluted product, which resulted in such value.

3.Assessment of nutritive property of GTE using the *Drosophila melanogaster*-based viability at 0M and NM dietary conditions

In this experiment, different dietary conditions (0M, NM) were applied in combination with the extract to assess its effect on the survival of the larvae and the hatching of pupa into adult flies. Previous research has shown that even a minor deficiency in essential amino acids like arginine or isoleucine can significantly reduce lifespan by 30-70% of fruit flies (Piper *et al*., 2014). By assessing larval survival rates, adult hatching rates, and the duration of developmental stages insights can be gained into the nutritional quality of the diet being tested. This approach can provide valuable information on how dietary conditions and GTE influence the overall viability and development of fruit flies.

In the study of 0N diet, different concentrations of GTE 28 were added to the media to evaluate its potential nutritive effect. The results revealed that the sample extract did not support survival past 1st instar larvae across each given concentration.

Drosophila melanogaster species require a diet with essential nutrients that consist of proteins, carbohydrates, vitamins, and minerals for proper development. Behavioural studies have demonstrated that *Drosophila* larvae preferentially feed on protein-rich food sources and consume quantitates that are optimal for larval growth and fitness (Young *et al*., 2018). It was also demonstrated that absence of amino acids in the diet of fruit flies leads to growth inhibition due to regulation of Dilps, which are insulin-like peptides (Géminard *et al*., 2009). Decreased secretion of these regulators inhibits growth. Commonly, in such nutritive studies yeast provides a protein-rich environment, however, lack of it in 0N demonstrated absence of support for the development of larvae. Addition of GTE 28 extract in varying concentrations could not provide a substitute and is a rate-limiting step for the survival of larvae. The lack of essential nutrients in the media hindered the growth and development of eggs beyond the initial stage. Despite the extract being rich in antioxidants such as flavonoids and polyphenols, it does not have significant nutritive effects for the survival of larvae and pupa.

Next, different concentrations of GTE 28 were assessed in combination with the NM-dietary condition at 25^{0}C for cytotoxicity effect. The data are shown in Figure 11 below. The figure presents two graphs, where (left) demonstrates the pupariation time of the third larvae,

whereas (right) demonstrates the hatching time of the adult flies from the pupae or the adult survival. It can be observed that certain concentrations, specifically, 2.7ml and 1.35ml of GTE 28 extract in combination with NM, improve the survival of larvae and adults. The observed rise in percentage of survival was associated with shorter larval and pupal developmental stages, which could suggest a synergistic effect of the GTE 28. Overall, GTE 28 extract does not show cytotoxicity in any given concentrations as it appears similar to the controls.

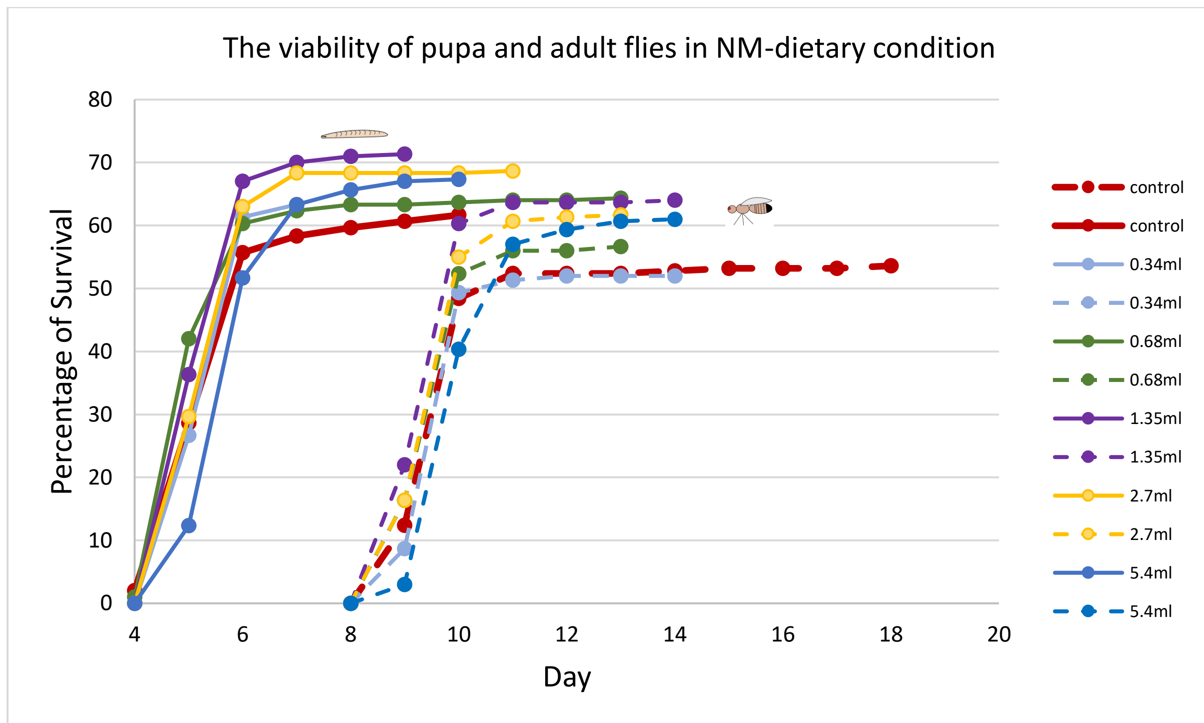

Figure 11. The pupa and fruit flies' viability in NM-dietary condition with 5 different concentrations of GTE 28 and a control, where NM means normal media.

Increase in percentage of survival of both third instar larvae and the adult hatching at the concentrations of 1.35ml and 2.7ml could be due to positive properties that promote health and vitality of flies, affecting overall physiological well-being. It is possible that the given concentrations could provide an optimal dose of bioactive compounds without any adverse effects.

3. Assessment of larval and adult viability in NM- and HS-dietary conditions

The assessment of larval and adult viability (%) are presented below to compare effect of different media. Statistical analysis using the Tukey Method and 95% Confidence has been applied. As mentioned previously, there is no significant difference in both larval and adult

hatch rate from the controls in NM-dietary conditions. This clearly shows that the given concentration range of the GTE 28 does not have cytotoxicity on the fly model.

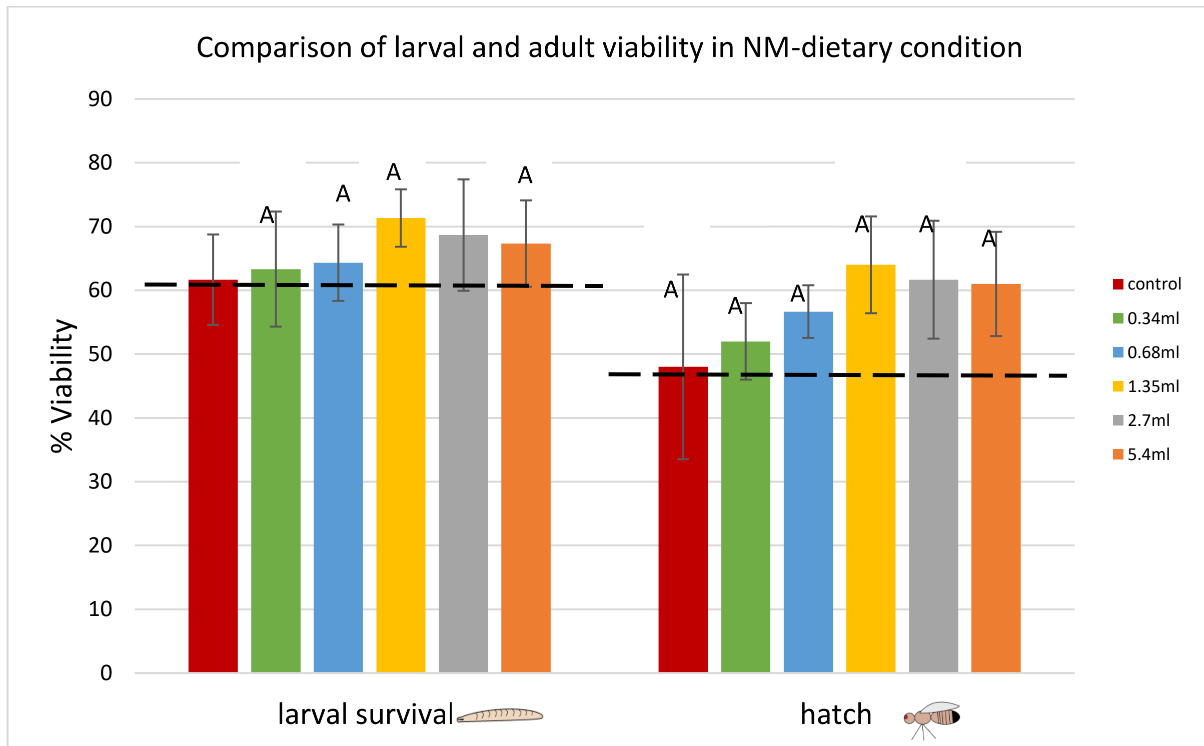

Figure 12. The comparison of larval survival and hatch rate in NM-diet

In the case of HS-dietary condition, the larval survival did not show a significant difference from the control, while for adult hatch there was a significant difference between concentrations 0.68ml and 5.4ml. However, both of them are not significantly different from the control. It is interesting, that there was a developmental delay in the case of HS-diet, but no notable effect on the viability. These could mean that application of HS-diet might have been counteracted with some compensatory mechanisms, which should be investigated further.

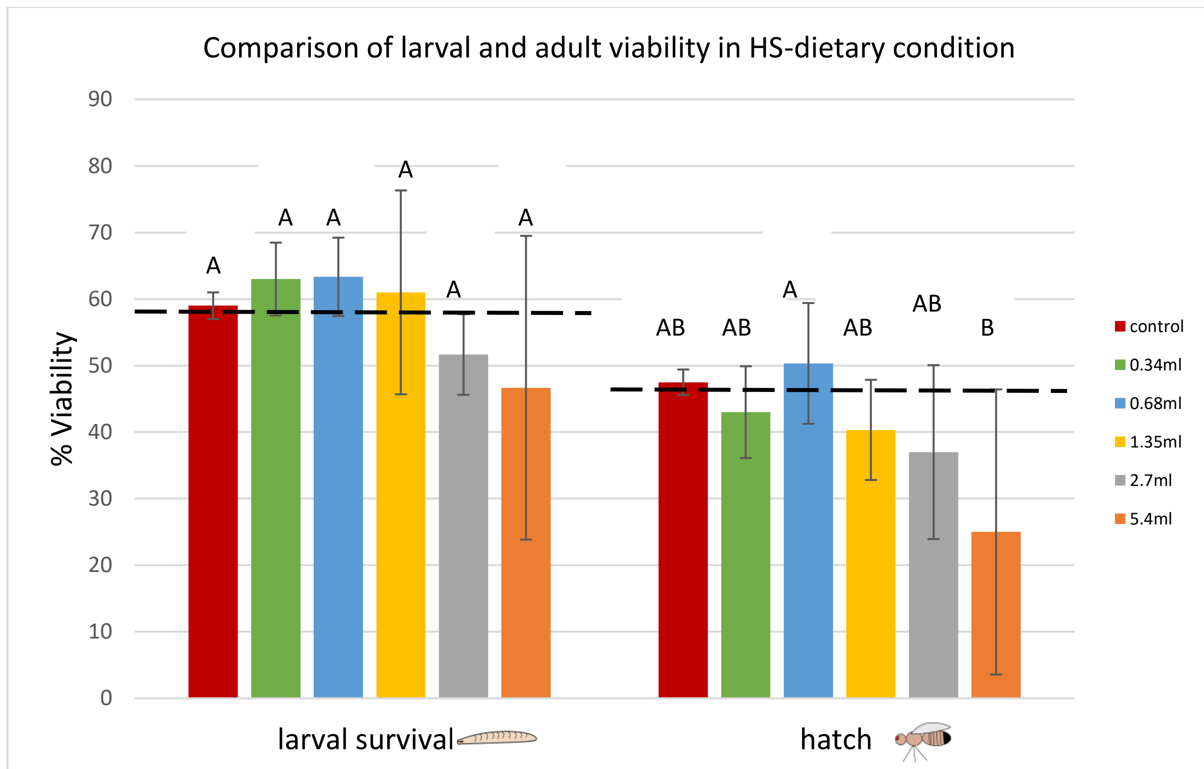

Figure 13. The comparison of larval survival and hatch rate in HS-diet

Statistical comparison between type of media showed significant difference between NM and HS-dietary conditions for both larval survival and adult hatching. It is interesting that. HS-diet caused a developmental delay, the viability of larval stage seemed almost similar to the NM-diet, while adult hatch rate showed decreasing tendency.

Overall, these observations suggest that the applied HS-diet is significantly different from the NM-dietary condition. The viability (%) of the system seemed to have insignificant difference between NM- and HS-diets, showing that there was no lethal effect upon model organism. However, there is still a certain effect of HS-diet on the fruit flies, possibly, a compensatory effect which could be related with the larval developmental delay. This difference requires further thorough studies.

4. Evaluation of the effect of HS media on the development of the w^{m4h} *Drosophila melanogaster* strain

To investigate how HS diet, containing 1.5 M sucrose, impacts the viability of third larvae and adult hatching, the developmental timeline of the w^{m4h} strain was examined under these conditions at a temperature of 25°C. The data are shown in the Figure 14 below, where (A) indicates the third larval survival and (B) indicates the hatching time of the adult flies.

Replacement of NM-diet with HS-dietary condition resulted in a developmental delay of the third instar larvae and respective hatching of the adult flies for about 3 days. The higher concentrations of the extract (2.7ml and 5.4ml) seem to interfere with the viability of both larval stage and adult hatching, as well as enhancing the developmental delay. On the other hand, lower concentrations (0.34ml, 0.68ml, and 1.35ml) do not interfere with the viability, although, demonstrate a slight delay in the development compared to the control.

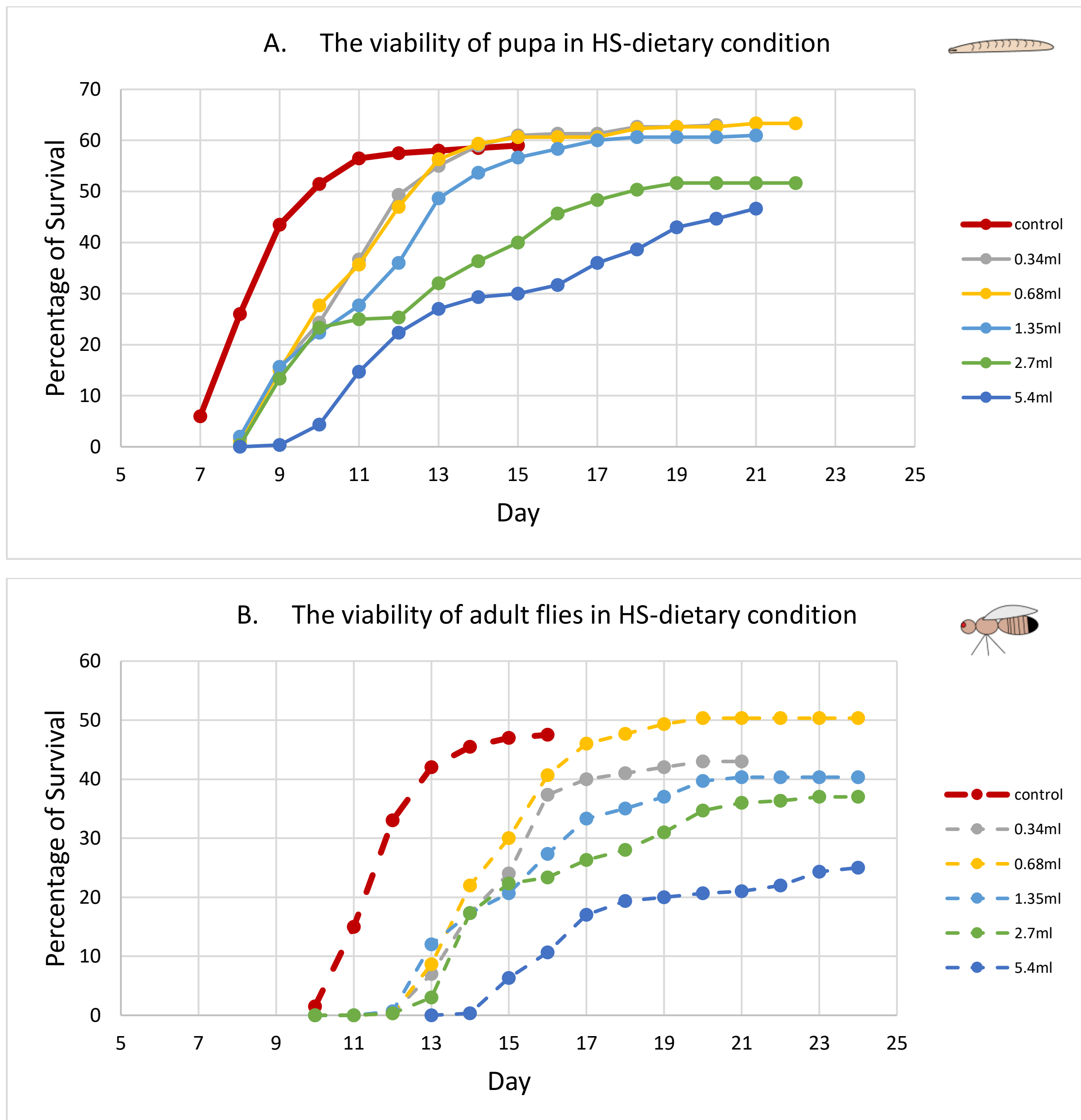

Figure 14. The pupa and fruit flies' viability in HS-dietary condition with 5 different concentrations of GTE 28 and a control, where HS means high sugar media.

Developmental delay in the HS-diet condition could likely be due to the impact of high sugar content on various physiological and metabolic processes in the fruit flies. High content

of sugar can disrupt certain metabolic pathways or energy metabolism, including insulin signaling pathways. In addition, excess sugar intake may have interfered with the uptake of essential nutrients needed for regular development leading to a delay in developmental stages of the flies. *Drosophila melanogaster* utilize a conserved insulin/insulin-like growth factor signaling (IIS) pathway which includes seven insulin-like peptides known as Dilps, which are responsible for different functions in cells such as growth, metabolism, stress response, reproduction and aging (Grönke *et al*., 2010). In early development, the function of ISS controls growth of tissue and fuel metabolism, while at the adult stage, these pathways are involved in fuel metabolism, stress resistance, fertility, and aging (Broughton & Partridge, 2009). Thus, high content of sucrose might have interfered with ISS signaling pathways inhibiting normal development. Additionally, similar study of larvae fed on high sugar diet has shown similar result as the current experiment. In this paper, it was demonstrated that high sugar diet accumulated high levels of circulating glucose and showed developmental delay of 3 days in larvae (Pasco & Léopold, 2012). This paper also demonstrated that growth inhibition resulted due to resistance to insulin-like peptides in the peripheral tissues.

For this reason, different concentrations of the GTE 28 extract were combined with the HS-dietary condition to observe potential rescue effect. This would mean that percentage of survival and the trend of the graphs shown in the Figure 14A-B should be similar to the graph of the control for NM-dietary condition in the Figure 11. However, no improvement or rescue effect could be observed for any of the given concentrations of GTE 28. Conversely, increasing concentration demonstrated an interference with the survival resulting in even lower viability level as compared to the control.

There are could be several factors that explain the lack of a rescue effect of the given extract. One possible reason is that the extract did not contain the necessary or enough of active compounds that could restore the desired phenotype or function in the flies. For this particular GTE 28 extract, a full HPLC-MS phytonutrient profile is still under process, so it would be reasonable to look at some of the major bioactive compounds present in its individual plant constituents. Study published by Téglás *et al* (2023), reported that the flavonoids are the most notable phytonutrients in the blackcurrant GTE. Particularly, the most important ones being luteolin, quercetin, and apigenin representing 91.7% of all flavonoids. As for the constituents of common sage extracts, derivatives of phenolic acids, tannins, triterpenoids, γ-tocopherol, α-tocopherol, carotenoids, gallic acid, 3-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, caffeic acid, hesperetin, hispidulin, and genkwanin are the most common (Živković *et al*., 2017). Similarly, saffron is rich in carotenoids that is considered to be its main bioactive compound,

including crocin and picrocrocin (Mzabri *et al*., 2019). On one hand, bioactive content of the extract might have been not enough to restore the damage introduced by HS-diet, on the other hand, there is no information known about the interaction of all the chemical components within the GTE 28. In general, interactions of multiple chemical compounds can be categorized as synergistic, additive/non-interactive, and antagonistic (Caesar & Cech, 2019). Additive and non-interactive combinations show a sum of their individual effects, while an antagonistic interaction leads to an effect that is less than additive effect or an expected mathematical sum (Caesar & Cech, 2019). Synergistic effect presents a greater positive effect than what would be expected from a simple additive effect (Caesar & Cech, 2019). In the case of GTE 28, it is possible that while some of the active compounds might possess synergistic effect, others have antagonistic interaction, which would decrease the benefits for the flies. For instance, in a study that investigated such interactions in food matrices, specifically, in tropical fruit juices, it was revealed that some combinations lead to increased antioxidant capacity. However, combination of camu-camu and acerola showed antagonistic interaction for antioxidant capacity (da Silva Pereira *et al*., 2015). Therefore, it is important to consider complexity of phytonutrient content of the given GTE and their interaction with each other, which would require more robust methodological studies in the future.

Another factor for the lack of rescue effect could be limited bioavailability of the phytonutrients. For instance, water-soluble phytonutrients like flavonoids, tannins, terpenoids are poorly absorbed due to their large molecular size and they cannot be diffused through cell membranes (Bhattacharya, 2009). The individual plant components of the GTE 28 are rich in tannins, phenolic acids, and carotenoids. Especially, saffron stigmas and common heather are rich in proanthocyanidins and anthocyanins (Mzabri *et al*., 2019; Kaunaite *et al*., 2022). However, anthocyanins have low stability and are easily influenced by light, pH, temperature, sugars, oxygen levels and other parameters. These factors and processes determine the bioavailability of anthocyanins (Enaru *et al*., 2021). According to recent studies, the bioavailability of anthocyanins does not exceed 1% (Enaru *et al*., 2021). Therefore, these factors need to be considered when interpreting the viability results of the fly experiments.

As for the interference with fly viability at higher concentrations, it could occur due to toxicity or adverse effects of certain components. Some of the chemical compounds could exert harmful effect, resulting in physiological stress, organ damage, as well as enhancing the damage caused by HS-diet. An assessment of toxicity of polyphenol-rich compounds has shown different toxicity profiles and they should be examined with caution due to their chemical heterogeneity (Boncler *et al.*, 2017). The study showed that some polyphenol-rich

compounds such as the spent hop extract, Aronox, and resveratrol were safe in regard to their cytotoxic effects on mitochondrial membrane potential and plasma membrane integrity, however, were found to be harmful in the analysis of nuclear area (in particular, resveratrol). In contrast, other polyphenol-rich compounds were quite cytotoxic on mitochondria and plasma membrane integrity (Boncler *et al.*, 2017). These results reveal that for the examination of plant extract toxicities a multiparametric analyses are required. GTE 28 is rich in polyphenols and biologically active compounds, however, at higher concentration this could mean that it might be toxic for some of the systems such as mitochondrial membrane potential or to plasma membrane integrity. This could mean that higher concentration of the extract would be exerting multiple effects on the model organism and enhancing the effect of HS-diet.

5. Quantitative estimation of the eye pigmentation of male w^{m4h} *Drosophila melanogaster* cultured in NM- and HS-dietary conditions

Quantitative spectrophotometric analysis of red eye pigmentation was carried out to determine the effect of dietary conditions on Position Effect Variegation (PEV). The difference in absorbance ($x10^{-3}$ nm) for both NM- and HS-dietary conditions are shown in figures below (Figure 15 - 18).

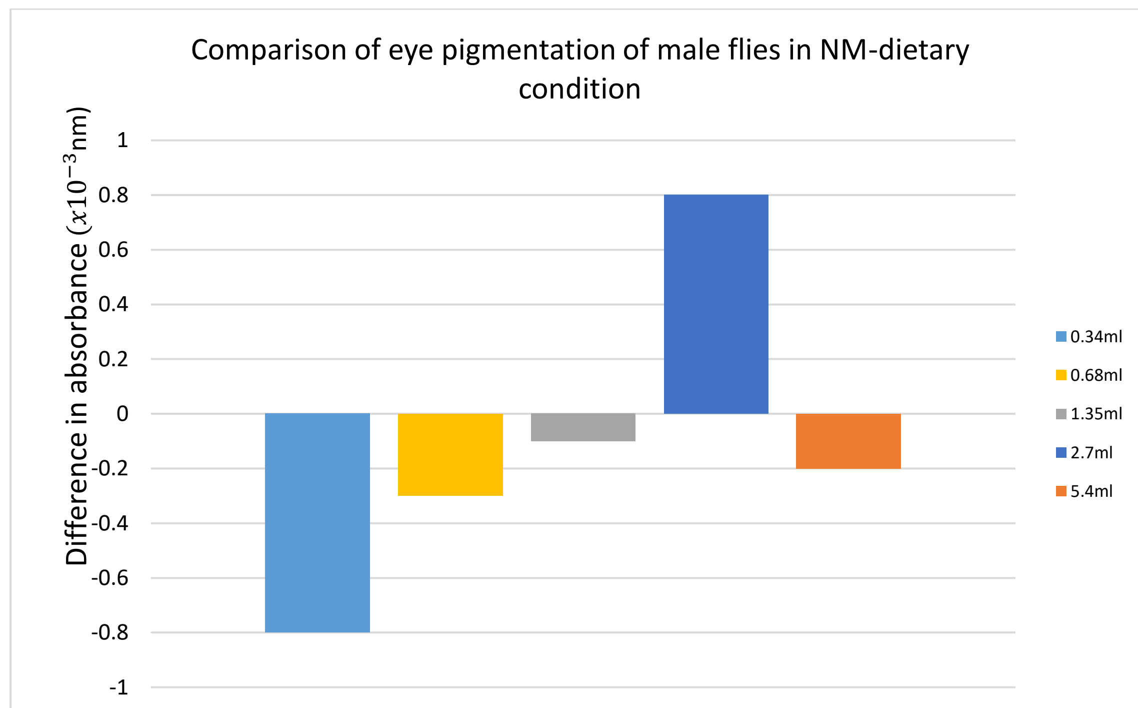

Figure 15. Comparison of eye pigmentation of male flies in NM-dietary condition presented as a difference in absorbance ($x10^{-3}$ nm) of different concentrations from the control.

Difference in absorbance of each concentration relative to the control was calculated to demonstrate either an increase or a decrease in intensity of the red eye pigment. In the case of

NM-dietary condition, lower concentrations (0.34ml, 0.68ml, and 1.35ml) and the highest concentration (5.4ml) showed a relative decrease in the absorbance compared to the control, whereas, the concentration of 2.7ml showed an increased absorbance. Noticeable difference in absorbance are apparent at the concentrations of 0.34ml and 2.7ml. The visual representation of the eye color of some of male adults for these corresponding concentrations and the control can be seen in the figure below (Figure 16).

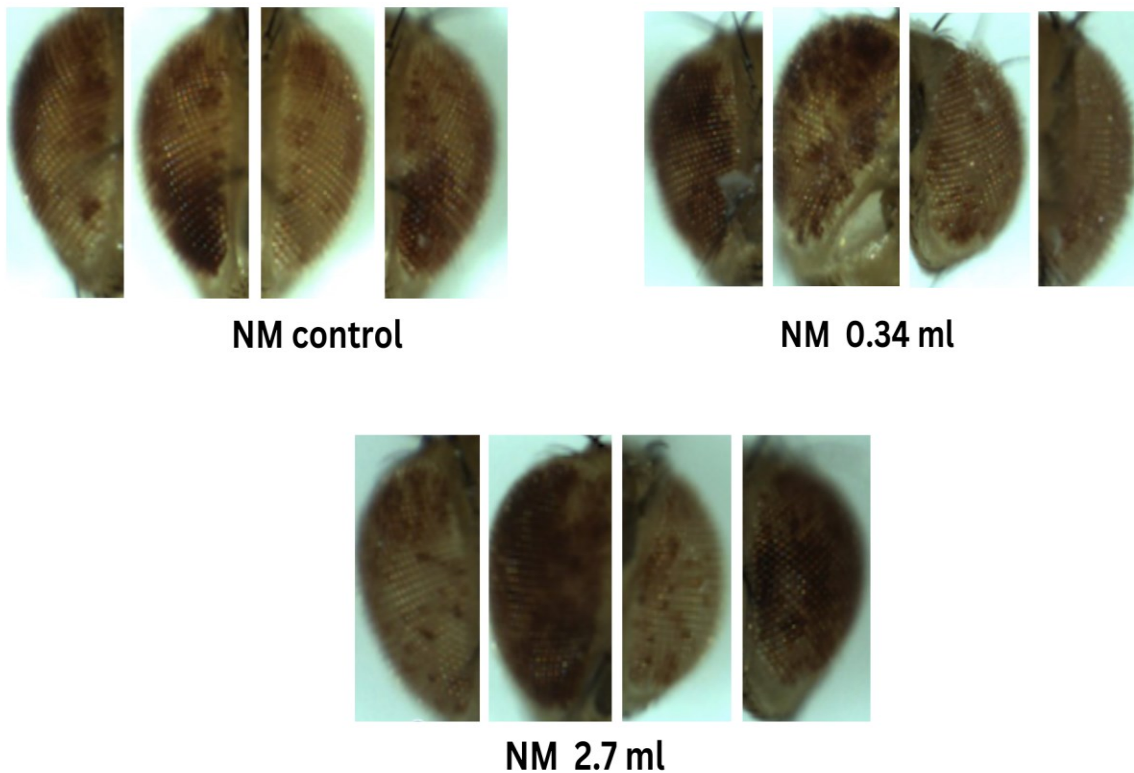

Figure 16. The pictures of eye pigmentation of male adult flies for NM control, NM 0.34ml and NM 2.7ml at magnification of x1.6 (OLYMPUS MV PLAPO 1X, Japan)

The microscopy images of eyes of 10 male flies were taken some of which are presented in the figure above. Visually no extreme differences could be distinguished as some of the eyes appeared to have more red pigment, while majority showed varying spots of red and white color similar to the NM control.

As for the HS-dietary condition, all the concentrations with an exception of the highest demonstrated an increase in the absorbance. The concentration of 2.7ml showed a noticeable difference relative to the rest.

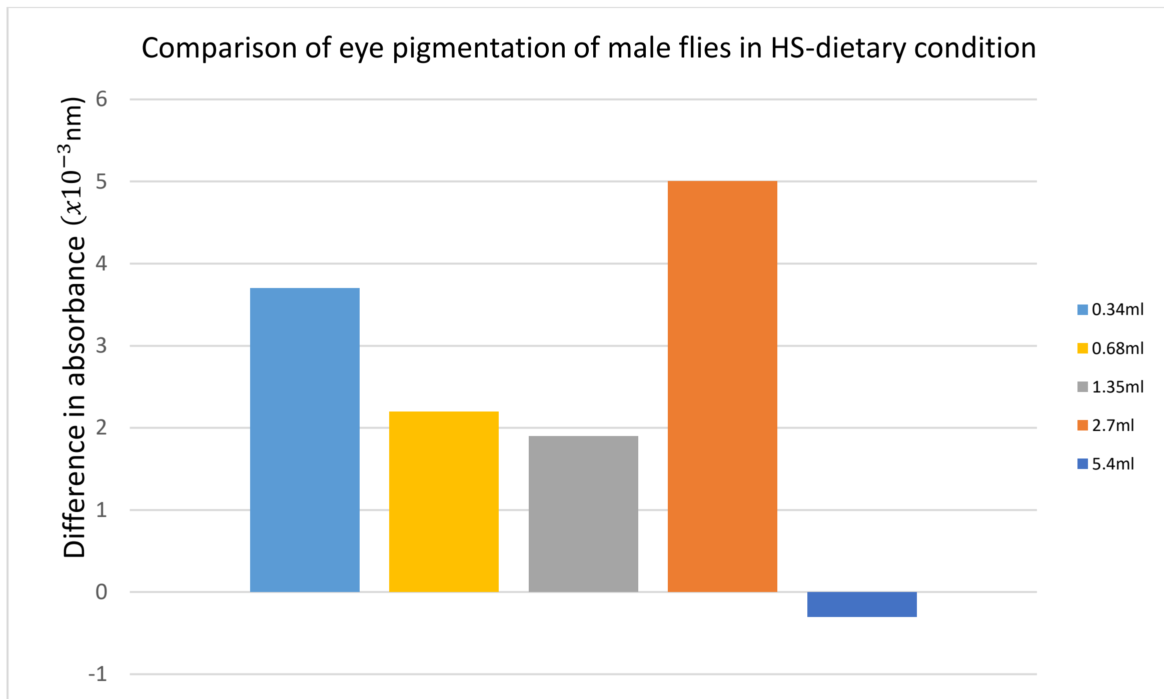

Figure 17. Comparison of eye pigmentation of male flies in HS-dietary condition presented as a difference in absorbance (x10^{-3} nm) of different concentrations from the control.

The visual representation of the eye color of some of the male adults for the concentrations 0.34ml, 2.7ml, and the control are shown in the figure below (Figure 16).

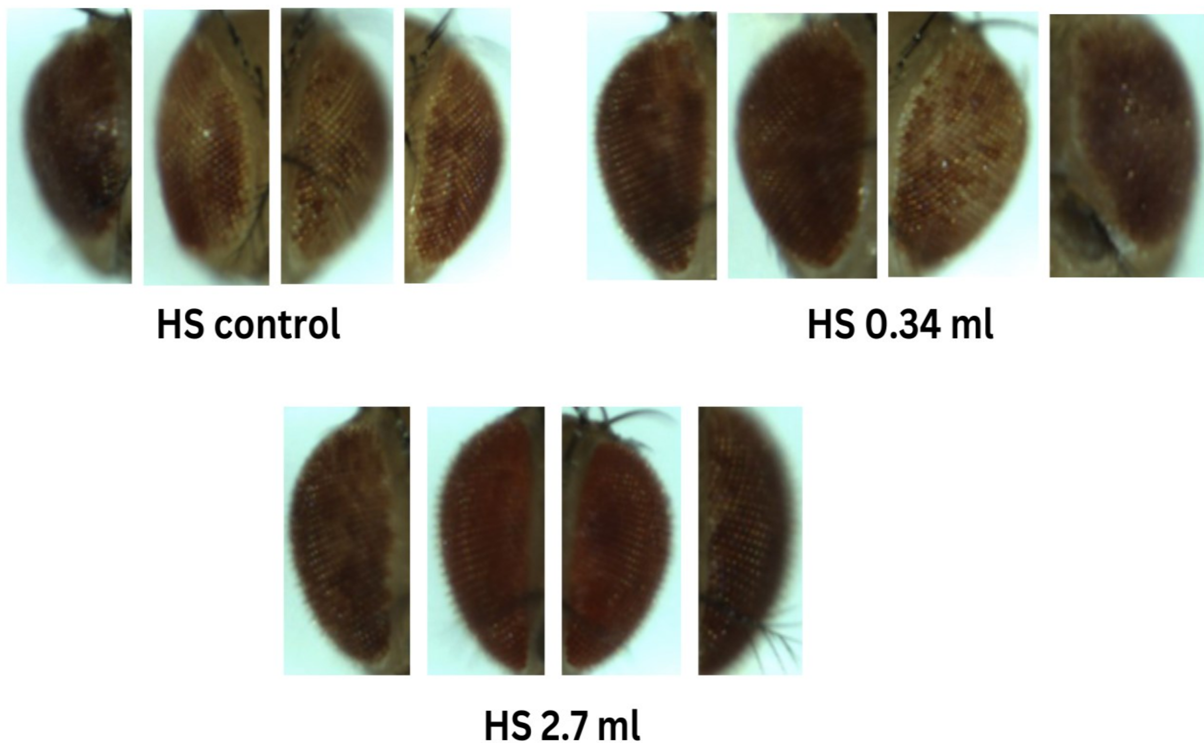

Figure 18. The pictures of eye pigmentation of some male adult flies for HS control, HS 0.34ml and HS 2.7ml at magnification of x1.6 (OLYMPUS MV PLAPO 1X, Japan)

Although, HS control did not show extreme difference from the NM control, combination of HS-diet with varying concentrations of the extract showed some noticeable difference. As can be seen from the microscopy images above, the concentrations of HS 0.34 ml and HS 2.7ml extract presented flies with more intense red eyes. Spectrophotometric absorbance also had a higher value in comparison to the HS control.

A PEV assay was performed using heads of 10 male flies of w^{m4h} strain for each given concentration of the extract in NM- and HS-conditions to observe impact of dietary intervention. For both NM and HS-diet, combination with an extract showed some difference from the control, however, no definitive conclusion can be drawn from them at this stage of the experiment. It can only be said that no distinct or noticeable phenotypic differences could be observed for NM-dietary condition between the control and the given range of extract concentrations. However, it was noted that HS-dietary condition combined with different concentrations of the extract had more number of male flies with red pigmentation and not many individuals had mosaic phenotype. Especially, the concentration of 2.7ml with HS-diet had intense red eyes, while 2.7ml with NM-diet mostly had individuals with mosaic eye type (Figures 16 and 18). Additionally, 2.7ml with HS-diet had the highest absorbance, corresponding to red pigmentation. Based on similar literature and experimental studies, it can be said that HS-diet and its combination with this given concentration of the extract might have a desilencing effect on the regulatory proteins such as H1a, meaning that it reduces the level of heterochromatin formation (Chang *et al*., 2021; Öst *et al*., 2014). However, specific effect of the extract is unknown and cannot be conclusive.

These observations demonstrate that there is an epigenetic influence of the diet on the regulation of the heterochromatin, however, its extent of influence, significance, and the mechanisms of modulation require research, particularly, further molecular biology laboratory techniques like RT-PCR and others. In addition, this experiment lacked a further follow up of offspring, where a generational impact HS-diet could have been observed similar to the mentioned studies.

6. Third instar larvae brain dissection and size measurement

Interrelation between high sugar diet and inflammation was extensively studied and reviewed from related literature. It was assumed that HS-media might induce inflammation and might have some adverse effects on the growth and development of fruit flies. In order to see if it had any difference between NM and HS-condition, brain of larvae was observed under the microscope.

Brain structure of a larvae is comprised of two lobes, which are populated by higher neurons, and of a ventral nerve cord (VNC), and of sensory input and motor outputs to the periphery as demonstrated in the figure below. Central brain consists of Type I and Type II neuroblasts, and of optic lobe neuroblasts (Hunter *et al*, 2021).

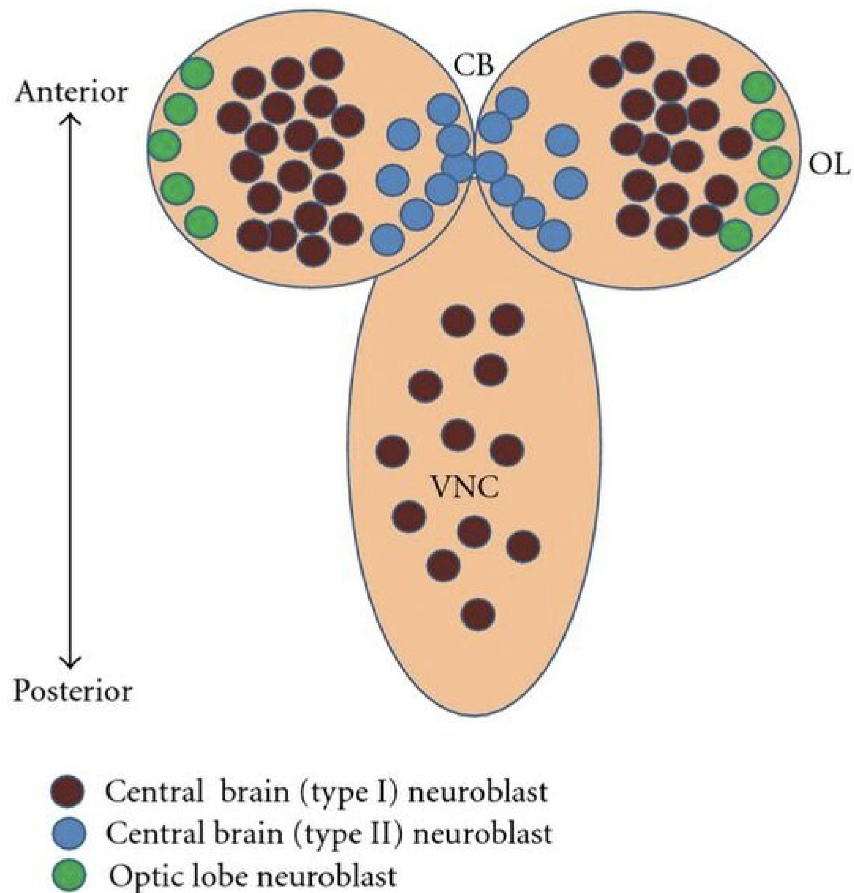

Figure 19. The basic anatomy of Nervous System of *Drosophila* Larvae (Reichert, 2012)

Brain dissection of 70 third instar larvae were performed and the brain size were measured. Two lobes of the central brain were measured and reported for each sample, for both NM and HS-dietary condition, which can be seen below.

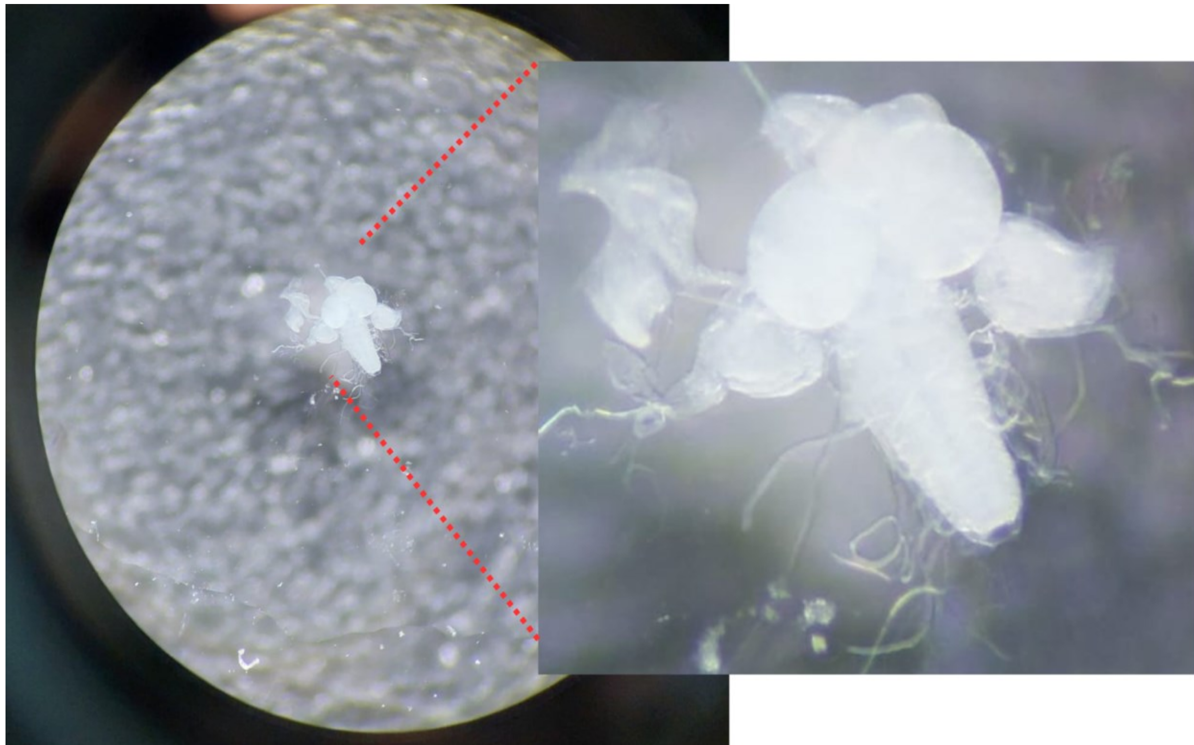

Figure 20. Dissected brain of third instar larvae, where two lobes of the brain and the trunk can be seen.

In the figure above, it can be seen that the brain is transparent, consists of two lobes and a trunk, containing nerve cells.
The measurement of two lobes were done and then averaged in between and reported in the table below, where sample number is the HS-media combined with different concentrations of GTE 28, HS is the control and NM is the control vial.
Table2. The brain size of the third instar larvae

Sample	length micrometer	stdev micrometer
HS 0.34ml	253.9354839	11.16189113
HS 0.68ml	221.7096774	26.91269184
HS 1.35ml	240.483871	17.29963506
HS 2.7ml	211.9354839	17.30347758
HS 5.4ml	250.0645161	15.26412165
HS control	216.6451613	21.87171841
NM control	246.2580645	16.9218754

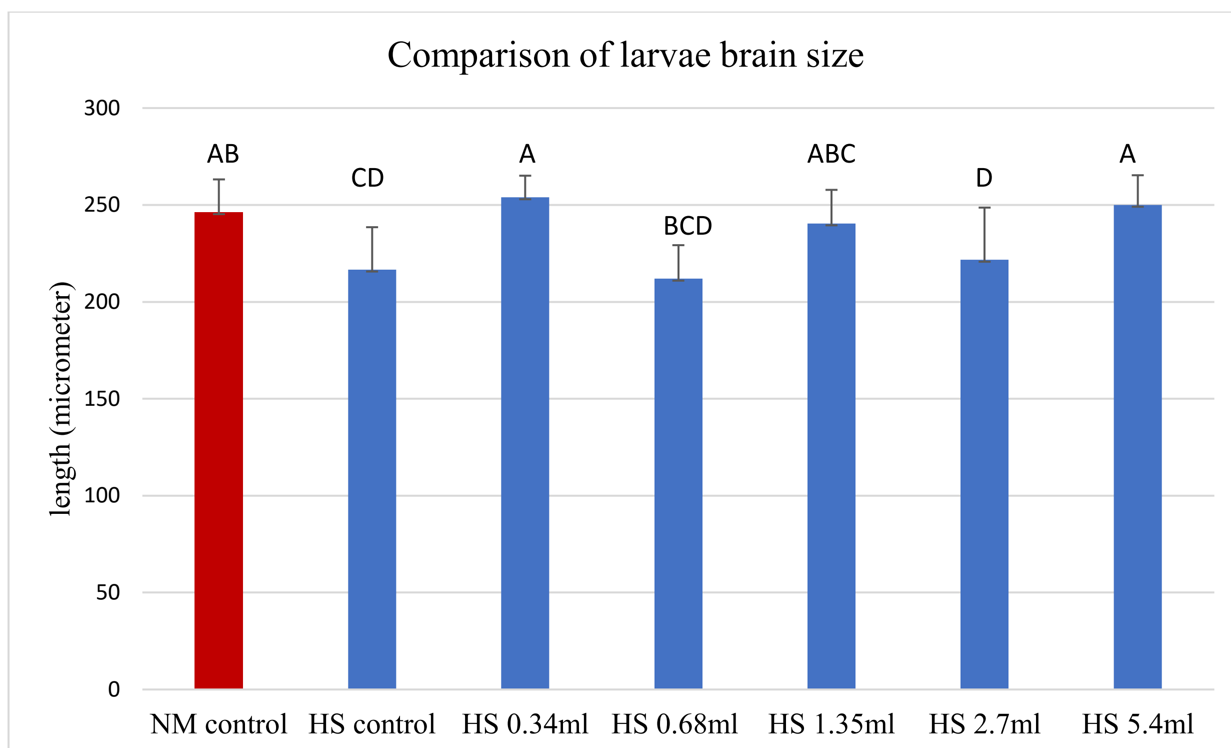

Figure 21. The comparison of larvae brain length (micrometer) with different dietary conditions and combination of GTE concentrations.

In the figure above, larvae fed on different concentrations of GTE in HS-media, HS control were compared to larvae fed on NM control. From observations under the microscope, it seems like that larvae fed on HS-diet had comparatively smaller brains than larvae fed on NM control. However, this was not true for all concentrations and no particular trend in brain size could be observed precisely.

As for the length of brain size, it could be noted that NM control and HS control are different. Tukey pairwise comparison with a 95% confidence showed that NM control and HS control are significantly different. Combining HS-media with different concentrations of GTE 28 did not show any particular trend or a potential rescue effect. It could be suggested that HS 0.34 ml and the HS 5.4 ml are similar to the NM control from the figure above. However, the results are not conclusive and require more number of samples to be investigated. Since this experiment is at its initial stages further thorough research is required to draw certain conclusions.

The significant difference in the brain size of the larvae fed on HS-media might indicate that it has a negative impact on neural development. This could occur due to factors such as

metabolic stress, increased oxidative stress and altered nutrient absorption, or alternatively, some compensatory mechanisms in the larval development.

On the other hand, combination of different concentrations of GTE with HS-media did not reveal any conclusive results. This might have several interpretations, that include dose-response issues, synergistic and antagonistic interactions, and some competing mechanisms. For example, the given concentrations of the GTE 28 might not have been sufficient to counteract the effects of high sugar. Testing a wider range of concentrations can be done to arrive to a definitive conclusion. Another reason could be that the plant extract may contain both beneficial and harmful metabolites, where some compounds could support and enhance brain development, while others might not be effective in a high sugar environment. This could result in an overall neutral effect. As mentioned previously, GTE 28 contains four different plants and each of them consist of hundreds of different compounds. These compounds might interact with the high sugar levels in unpredictable ways. Some might have synergistic or antagonistic interactions with each other as well. Some chemical compounds might exacerbate the negative impact of high sugar and this could neutralize any potential benefit effects.

In addition, brain dissection and observation of larvae were performed only in a short timeframe. Study of only first generation was observed, which might be too short and not capture the delayed effects of the plant extract. Long term studies as well as follow-up to the next generations could reveal different outcomes as the larvae continue to develop. This could also reveal other hints into the potential effects of the extract as we study offspring.

In summary, the lack of conclusive results when combining the plant extract with high sugar media likely is because of complex interactions between the compounds and sugar, insufficient dosages, or the overwhelming negative effects of the high sugar environment on brain development. Further investigation into these aspects could help clarify the relationship.

SUMMARY OF THE MAIN FINDINGS

A phytochemical screening of GTE 28, where the spectrophotometric assessment of total polyphenol and total flavonoid content, and the antioxidant capacity using DPPH assay have been performed. Results of TPC and TFC were comparatively high and revealed that it is majorly constituent of non-flavonoid type of polyphenols, which could be responsible for its antioxidant properties. DPPH assay showed a value of 7.27 ± 1.00 µmol TE/ml for its antioxidant capacity. In order to understand its antioxidant capacity other assays could be performed to obtain more comparable and relevant data. This will be processed in further studies of this extract.

Viability tests using different dietary conditions, 0N, NM, and HS-diet, combined with 0.34ml, 0.68ml, 1.35ml, 2.7ml, and 5.4ml revealed interesting results. In 0N media experiments, eggs did not survive past 1st or 2nd instar larval stage. This demonstrated that GTE 28 does not possess nutritive properties at any given concentration. In NM-dietary condition, all given concentrations did not show any cytotoxic effect on the growth and viability of flies. This revealed that the GTE 28 does not show adverse effect and is safe for use in the range of stated concentrations.

In this work, HS-dietary condition was used to stimulate a state of inflammation. This dietary condition showed an interesting result in the development of flies. There was a 3-day developmental delay in the third instar larvae and respective hatching of the adult flies compared to NM-media, but had no effect on viability. This could happen due to some compensatory mechanisms related to high-sugar content. In addition, combination with higher concentrations of the extract, 2.7ml and 5.4ml, interfered with the viability of both larval stage and adult hatching, while lower concentrations, 0.34ml, 0.68ml, and 1.35ml, did not interfere with the viability of flies. However, none of the given concentrations could demonstrate a possible rescue effect for this developmental delay. Thus, it was concluded that GTE 28 does not possess properties that could restore the damage induced by HS-diet.

Observations and quantitative measurement of the red eye pigment did not reveal any distinct phenotypic differences between NM and HS-dietary condition. Majority of the male adult flies had mosaic eye colour. However, HS-diet combined with 2.7ml demonstrated higher number of male individuals with intense red eye colour. This shows that diet has some impact on the chromatin structure responsible for PEV effect. Further molecular methodologies are required to study the effect of regulation, mechanism of action and their relation between HS diet and inflammation.

Similarly, third instar larvae brain dissection and brain length measurement did not reveal conclusive results. Statistical comparison demonstrated that there is a significant difference in the brain size of larvae fed on NM and HS-media, where brain of HS control appeared to be smaller compared to the NM control. However, HS-diet combined with different concentrations of GTE 28 did not demonstrate any rescue effect of the extract. It might be that this extract does not have an anti-inflammatory effect. However, more thorough studies are necessary to draw definitive conclusions since this is only initial stages of investigation.

CONCLUSION

This work investigated a nutritional effect and potential anti-inflammatory effect of a gemmotherapy extract, which contains four plants - *R.nigrum, S.officinalis*, *C. vulgaris* L., and *C.sativus*. The nutritional assessment was done with three different dietary conditions, which were 0N, NM, and HS-diet, each combined with 5 different concentrations of GTE 28 (0.34ml, 0.68ml, 1.35ml, 2.7ml, and 5.4ml). Results of 0N revealed that the extract does not possess essential nutrients to support the growth and development of larvae. Thus, it was concluded that it lacks nutritive properties on its own. Combination of different concentrations of the extract with the NM-diet demonstrated that neither of the given concentrations show cytotoxicity in the viability tests.

In the case of HS-dietary condition, both larvae development and adult hatching of flies demonstrated a developmental delay of about three days without affecting the viability. The developmental delay caused by high-sugar diet might indicate that there are some compensatory mechanisms and there also could be an adverse effect on the model organism. Therefore, in order to see if the plant extract has potential beneficial and restoring effect, different concentration of it was combined with HS-media. However, results did not show any rescue effect and could not counteract the negative impact of high sugar. Furthermore, higher concentrations, 2.7ml and 5.4ml, interfered with the growth and viability of both larval development and adult flies, which showed very poor results for viability of pupa and adult flies. This could occur because of physiological and oxidative stress caused by the high sugar content and extract lacked in necessary components to counteract and to have a rescue effect. On the other hand, the extract itself might have caused an additional stress.

In order to observe and identify what is the effect of growing flies in HS-dietary condition, specifically, if it had any adverse impact on the neural system of the flies, brain dissection of 70 third instar larvae were performed. They were performed on NM as a control, HS control, and other HS-conditions combined with 5 different concentrations of the GTE 28. Observations and measurement of the brain size of larvae grown on HS control and NM control diet showed a significant difference. Brains of larvae fed on HS control media was smaller than of larvae grown on NM control. This could indicate that high sugar levels do have some adverse effect on neuronal and brain development of the model organism. Different concentrations of GTE 28 were then combined with HS-media to observe its potential anti-inflammatory and some neuro-protective role. This, however, did not show any conclusive results. This could mean that more number of larvae need to be studied, as well as more robust models of analysing brain and neuronal development of larvae and adult flies need to be utilized in future studies.

Overall, this study revealed some interesting results and opened space for further research and areas to discover. It is still on its initial stages and requires more data and robust methods to arrive to conclusive results.

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