DOCTORAL (Ph.D) DISSERTATION

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DEBRECEN

2024

UNIVERSITY OF DEBRECEN
Doctoral School of Animal Science

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Comprehensive evaluation of plant extracts generated effects using multiple animal models for fish feed development

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Debrecen
2024

Comprehensive evaluation of plant extracts generated effects using multiple animal models for fish feed development

Dissertation submitted in partial fulfilment of requirements of the doctoral (PhD) degree in Animal Husbandry

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Prepared in the framework of the Doctoral School of Animal Science, University of Debrecen
(Animal genetics – gene conservation, animal ecology programme)

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The official opponents of the dissertation:

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Dr. …….. ……

The evaluation board:

Chairperson: Dr. …….. ……
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The date and venue of the dissertation defence:

Debrecen, ... 2024.

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ABBREVIATIONS

Acetyl coenzyme A - AcCoA

Acetyl Coenzyme A synthase – AcCoAS

Acetyl-CoA acetyltransferase1- ACAT1

Aldo-keto reductase 1B - Akr1B

alpha-linoleic acid - ALA

black mulberry gemmotherapy extract - BM-GTE

Black mulberry - BM

Black mulberry gemmotherapy extract- BM¬-GTE

2,2-diphenyl-1-picrylhydrazyl – DPPH

Caloric restriction – CR

Caloric restriction-mimetic – CRM

Essential amino acids - EAAs

Ferric Reducing Ability of Plasma – FRAP

Fourier Transform Infrared – FTIR

Gemmotherapy extract – GTE

glucose transporter - Glut

Histone deacetylase 4 and 1 - HDAC4 and 1

High sugar diet – HS diet

Linoleic acid - LA

Minimum bactericidal concentration – MBC

Minimum inhibitory concentration – MIC

Medium unsaturated fatty acid – MUFA

Normal media diet – NM diet

Olive – O

Olive Gemmotherapy extract – O-GTE

peroxisome proliferator-activated receptor gamma - PPARγ,

phosphoinositol 3 kinase - PI3K

protein kinase C - PKC

Polyunsaturated fatty acid – PUFA

sodium chloride – NaCl

sorbitol dehydrogenase genes 1 and 2 - Sodh1 and 2

Sweet almond - SA

Sweet almond gemmotherapy extract – SA-GTE

Total Flavonoid Content – TFC

Total Polyphenol Content – TPC

Ultra-High-Performance Liquid Chromatography–Electrospray Ionization-Mass Spectrometry- UHPLC–ESI-MS

zero-nutrient diet - 0N diet

1. INTRODUCTION

The use of plant extracts in medicine and healing dates to ancient times. Plants have been used by many cultures for medicinal purposes for thousands of years, and many traditional healing practices still use plant extracts today. The ancient Egyptians, for example, used various plant extracts, including aloe vera, frankincense, and myrrh, for their healing properties.

In traditional Chinese medicine, plant extracts are used to balance the body's energy and promote health and healing. Ayurveda, a traditional healing system from India, also uses plant extracts to treat a variety of ailments.

In the Islamic world, herbal medicine has a long history, and many traditional Islamic remedies are based on plant extracts. The Prophet Muhammad (peace be upon him) is reported to have used and recommended various plants and herbs for their medicinal properties.

In the Middle Ages, many of the Greek and Roman texts on medicine were translated into Arabic, and Arab physicians developed their own herbal remedies based on this knowledge. The Islamic Golden Age saw a flourishing of medical knowledge, and many important works on medicine and herbal remedies were written during this time.

In Europe, the use of plant extracts for medicinal purposes became popular during the Renaissance. Many of the medicinal plants used in Europe were introduced from other parts of the world, such as China and the Americas. By the 19th century, plant extracts were commonly used in Western medicine, and many of the drugs used today are still based on plant compounds.

A holistic approach to health and well-being is fundamental to the ideas of phytotherapy, which involves the use of entire plants or plant parts for medicinal reasons. This method is different from traditional pharmacology, which frequently concentrates on single active ingredients.

Phytotherapy is a combination of modern research and centuries-old traditions. Its practice includes a thorough study of plant biology, chemistry, pharmacology, and clinical sciences in addition to the use of plants for therapeutic reasons. With an emphasis on a more comprehensive approach to health and well-being, phytotherapy may provide viable substitutes and supplements to traditional medical treatments as research in this area expands. With the support of rigorous scientific research and ethical practices, phytotherapy has a bright future that might significantly contribute to the development of global healthcare solutions.

Throughout history, phytotherapy has been an essential component of conventional medical systems all throughout the world. Herbal medicine has been used for therapeutic purposes from ancient times, according to writings from Greek, Chinese, and Egyptian civilizations. A portion of the current interest in phytotherapy can be attributed to the realization of the drawbacks of synthetic medications, such as adverse effects and concerns regarding antibiotic resistance.

Contrary to traditional medications, which usually use single active ingredients, phytotherapy highlights the synergistic effects of several components found throughout the entire plant. This method is based on the idea that a complex combination of phytochemicals found in plants might offer a more comprehensive, balanced treatment with fewer adverse effects.

2. LITERATURE REVIEW

2.1.1. *Plant extracts study*

A multi-step procedure is involved in studying plant extracts, which involves selecting and gathering plant material, retrieving the extracts, and analyzing the compounds identified in them.

Another aspect of plant extract research involves enhancing plant growth and secondary metabolite production (Yadav et al., 2024). Technological advancements in phytochemical analysis and scientific research are critical to modern phytotherapy. To identify and quantify bioactive compounds in plants, methods including Nuclear Magnetic Resonance (NMR) spectroscopy, Gas Chromatography-Mass Spectrometry (GC-MS), and High-Performance Liquid Chromatography (HPLC) are implemented. Research on the antioxidant analysis of food and medicinal plants provides information on the phytochemical profiles of different species and their possible medical uses (Yu et al., 2021). Methods such as GC-MS and Fourier Transform Infrared (FTIR) spectrum analysis as well, can detect the different metabolites included in the plant extracts. Numerous chemicals with biological activity, including antioxidant, anticancer, antifungal, antibacterial, and antiviral qualities, were discovered to be present in the plant extracts (Mansoori et al., 2020).

Clinical applications and safety of phytotherapy research are crucial parts of plant studies as well. The effectiveness and safety of herbal remedies are of utmost importance. Rigorous clinical studies, extract standardization, and quality control are necessary to guarantee the efficacy and safety of herbal remedies.

The multi-faceted approach to plant extract research, from the meticulous selection and extraction processes to advanced analytical techniques, underscores the importance of this field in modern medicine. By leveraging technologies like NMR spectroscopy, GC-MS, and HPLC, researchers can uncover the vast potential of bioactive compounds, leading to discoveries that may revolutionize both preventive and therapeutic healthcare. Furthermore, ensuring the clinical efficacy and safety of these phytochemicals through stringent testing and quality control highlights the commitment to integrating traditional herbal knowledge with contemporary scientific rigor. This comprehensive methodology not only enhances our

understanding of plant-based treatments but also provides a robust framework for developing new medications and therapeutic strategies.

In addition, the continuous exploration of plant extracts and their properties can open new avenues in personalized medicine, where treatments are tailored to individual genetic profiles and specific health conditions. This personalized approach can significantly improve patient outcomes by targeting the unique biological pathways involved in various diseases. Moreover, the research can contribute to sustainable practices in agriculture and medicine, promoting the use of renewable plant resources and reducing the dependency on synthetic chemicals and pharmaceuticals.

In conclusion, phytotherapy is the result of combining innovative scientific studies with conventional medical techniques. Its principles focus on the synergistic effects of phytochemicals, clinical effectiveness, holistic treatment, strict quality control, and integration with contemporary pharmaceutical techniques. This method aims to offer safe, efficient, and comprehensive healthcare choices while still acknowledging the complexity of natural plant chemicals.

2.1.2. The health-promoting relevance of polyphenols and flavonoids

Polyphenols and flavonoids, which are naturally occurring substances that are found extensively within a wide range of plants, have drawn a lot of attention due to their potential health benefits. These substances are a focus of nutritional and medical study due to their potential importance in controlling and preventing several chronic illnesses.

Perhaps the most widely recognized advantage associated with polyphenols and flavonoids is their antioxidant capacity. They react by eliminating free radicals, which reduces the body's oxidative stress (Pandey and Rizvi, 2009). This is a significant mechanism since oxidative stress has been linked to the onset of many chronic illnesses, including acancer, heart disease, and neurological diseases (Pandey and Rizvi, 2009).

Consuming foods high in polyphenols has been associated with some heart-and-coronary protective advantages when it comes to cardiovascular health. Particularly flavonoids have been demonstrated to lower the risk of heart disease. They can reduce blood pressure and enhance endothelial function (Cassidy et al., 2011), which contributes to this. An increased

consumption of specific flavonoid types is linked to a lower risk of ischemic stroke (Cassidy et al., 2011).

A further important characteristic of polyphenols and flavonoids is their anti-inflammatory properties. Several diseases have chronic inflammation as their primary cause (Minihane et al., 2015), and these substances may be able to reduce inflammation. This is apparent in conditions including arthritis, inflammatory bowel disease, and some forms of cancer (Minihane et al., 2015).

Flavonoids and polyphenols have also been recognized for their neuroprotective properties (Mohammad et al., 2023, Giuseppe et al., 2021). These chemicals offer potential advantages in neurodegenerative illnesses (Giuseppe et al., 2021) such as Alzheimer's and Parkinson's, mostly due to their antioxidant and anti-inflammatory effects. Additionally, an extensive amount of research has been conducted on these compounds' anti-cancer properties (Khan et al., 2008, Wesam et al., 2023).

In summary, flavonoids and polyphenols offer numerous and diverse health benefits. Considering their significance in cardiovascular and neuroprotective wellness, as well as their anti-inflammatory and antioxidant properties, these compounds offer a natural way to prevent and control disease. Integrating them in the diet by consuming a range of plant-based meals may be an essential approach for preserving general health and well-being.

2.1.3. The Significance of Olive, Sweet Almond, and Black Mulberry studies

Olive (Olea europaea)

The olive tree belongs to the *Oleaceae* family, plants dicotyledons which include 900 species divided into 25 genres. The leaves are opposite (Gaussorgues, 2009).

Nowadays, the economic significance of olives is on the rise; however, the occurrence of *Xylella fastidiosa,* an invasive aerobic bacterium (Alaya et al., 2021), has negatively impacted thousands of hectares of olive plantations in Southern European countries, putting the EU's olive industry at risk (Alaya et al., 2021). The pathogenic bacteria are transmitted by insects, such as the meadow froghopper (*Philaenus spumarius*), that feed on plants,

causing bacterial leaf scorch-type diseases that are affecting not only olive trees but also other species such as oleander and almond trees (Saponari et al., 2013). Olive oil production is one of the most profitable agricultural activities in the Mediterranean region (Banias et al., 2017). Since ancient times, the olive fruit, oil, and leaves have been used for dietary, medicinal, and ceremonial purposes (Talhaoui et al., 2018), and they are an integral part of Mediterranean culture and cuisine.

The 16th century saw the most significant growth in olive cultivation, driven by the demands of soap factories and mechanical and textile industries. The 19th century marked the pinnacle of olive cultivation in terms of the total area dedicated to it. In the 20th century, cultivation areas gradually declined, but technological advancements in olive cultivation and oil extraction have led to a five-fold increase in olive oil production during this century.

Olive in Islam

In Islamic culture and religion, the olive tree and its products, particularly olive oil, are esteemed for their significant cultural, religious, and health-related values. The Quran and the Sunnah of the Prophet Muhammad (peace be upon him) feature multiple references to the olive, emphasizing its sacredness and utility.

The olive tree is revered in Islam as a blessed tree, with its oil believed to possess healing properties beneficial for both physical health and spiritual well-being. The Prophet Muhammad (peace be upon him) acknowledged the virtues of olive oil, advocating its use in various facets of daily life.

The Quran mentions the olive tree in Surah Al-Mu'minun, portraying it as a blessed tree from Mount Sinai that produces oil and serves as a relish for eaters (Quran 23:20). Another significant reference in the Quran describes olive oil as a symbol of divine light, nourishing and sacred (Quran 24:35).

In Islamic tradition, the use of olive oil extends beyond culinary applications to include roles in religious rituals and spiritual practices. The Prophet Muhammad (peace be upon him) is known to have applied olive oil on his head, recommending it to his companions for its curative properties. He also consumed olive oil himself and encouraged others to do so, citing its benefits in preventing illnesses.

The Prophet's Sunnah highlights several uses and advantages of olive oil:

- As a remedy: The Prophet Muhammad (peace be upon him) recommended olive oil for treating various ailments, reflecting its therapeutic significance.
- Dietary incorporation: He advocated the consumption of olive oil for its health benefits, considering it nourishing and preventive against diseases.
- Spiritual uses: Olive oil is utilized in Islamic rituals, including anointing the body during funeral rites, signifying its spiritual importance.
- Cooking: The Prophet endorsed olive oil as a healthier cooking option compared to other oils, indicating its nutritional value.
- Holistic benefits: He praised the olive tree, noting the medicinal properties of its leaves, bark, and fruit.

Overall, the olive tree and olive oil are integral to Islamic culture and tradition. Their significance spans health, spiritual, and religious aspects, making them vital elements in Islamic life and practices.

Olive oil

There are different qualities of olive oil, regulated by the Commission of the European Community:

- Extra Virgin Olive Oil: This highest quality category features no sensory defects and a fruity flavor. Its acidity level must not exceed 0.8 grams per 100 grams.

- Virgin Olive Oil: May contain minor sensory defects but maintains a very low level of flaws. Its acidity must not exceed 2 grams per 100 grams.

- Refined Olive Oil: Produced by refining lampante and sometimes virgin olive oil, this type has a maximum acidity of 0.3 grams per 100 grams. and is not intended for retail sale

- Refined Olive-Pomace Oil: Made by refining crude olive-pomace oil, it can have an acidity of up to 0.3 grams per 100 grams.

- Olive-Pomace Oil: A blend of refined olive-pomace oil with extra virgin or virgin olive oil, featuring an acidity up to 1 gram per 100 grams.

The positive impact of olive oil on human health has been demonstrated through relevant intervention studies, and it is widely recognized as a symbol of the Mediterranean diet.

According to a study conducted by Gorzynik-Debicka et al. (2018), extra virgin olive oil is one of the most effective resources against the development of chronic noncommunicable diseases such as cancer, metabolic syndrome, neurodegenerative diseases like Parkinson's and Alzheimer's, and other Noncommunicable diseases (NCDs) (Gorzynik-Debicka et al. 2018). The high content of oleic acid and various bioactive compounds, polyphenols, and vitamin E found in olive oil is responsible for its beneficial properties, as they possess antioxidant, anti-inflammatory, insulin-sensitizing, cardioprotective, antiatherogenic, neuroprotective, immunomodulatory, and anticancer activities (Reboredo-Rodriguez et al., 2018; Silenzi et al., 2020).

Olive leaves

The olive tree leaves are well-known for containing a variety of potentially bioactive secondary metabolites, including the secoiridoid derivatives oleacein and oleuropein, as noted by Edziri H. et al. (2019). Many studies have confirmed the health benefits of olive leaf extract, which may help to lower blood pressure, support cardiovascular and immune function, and increase energy levels (Edziri et al., 2019).

As a rich source of bioactive compounds, olive leaves have been shown to have antioxidant, antidiabetic, anti-inflammatory, anti-neuroinflammatory, cardiovascular-protective (Alaya et al., 2022), anti-cancer, and immunomodulatory effects (Alaya et al., 2021). Moreover, a study by Edziri et al. (2019) revealed that olive leaves have anti-biofilm activity against several microorganisms, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Candida albicans*, *Enterococcus faecalis*, and *Escherichia coli*, with inhibition percentages ranging from 83% to 93% at 2xMIC values. The olive leaf extracts also exhibited antimicrobial properties against foodborne pathogens, such as *S. aureus*, *E. coli*, *Salmonella spp*., and *L. monocytogenes* (Edziri et al., 2019). This biological activity can be attributed to the high content of oleuropein in the olive leaves.

Almond (Prunus amygdalus)

The almond is a deciduous fruit tree that belongs to the *Rosaceae* family, and is native to Asia Minor and Mesopotamia, growing up to 15 meters tall and bearing white and pink flowers. Its leaves are oblong, deciduous, and sharp-edged, growing to 8-12 cm long, light green above and grey-green below. The almond fruit is reminiscent of a small green peach, with a velvety green skin and a yellow, cracked, woody shell that contains one or two seeds known as "almonds".

The almond itself is oblong and flattened, covered in a fine brown skin that can be removed to produce a "pruned almond" or blanched almond. While wild almonds, or bitter almonds, are naturally toxic due to their high cyanide content, sweet almonds, which are the result of horticultural selection, are almost free of cyanide.

California is the largest producer of almonds, accounting for nearly 80% of the world's supply. Almond trees can be categorized into two varieties: sweet almond (*Prunus dulcis* variety *dulcis*) and bitter almond (*P. dulcis* variety *amara*), with sweet almonds being used as nuts, as a source of almond oil or almond powder, and in cooking, while bitter almonds provide bitter almond oil for the production of flavor extracts for foods and liqueurs.

Almonds are not technically nuts, but rather seeds encased in a hard fruit coating. They are high in protein and fat, and provide small amounts of iron, calcium, phosphorus, and vitamins A, B, and E. Almonds can be eaten raw, blanched, or roasted. Many parts of the almond fruit can be utilized for different purposes, and recent studies are yielding many papers describing challenging observations (Alaya et al., 2021).

Almond In Islam

Almonds are esteemed in Islamic culture and tradition for their diverse applications, encompassing nutritional, medicinal, and spiritual realms. While not explicitly named in the Quran, almonds are thought to be encompassed within the broader categorization of fruits and nuts. Verses like Surah Al-Anaam, 141, which mention gardens, palm trees, and a variety of foods, are interpreted to include almonds as part of Allah's bountiful provision (Quran, Surah Al-Anaam, 141). Similarly, almonds are believed to be part of the "good things" that Allah has provided, as referenced in Surah An-Nahl, verse 11 (Quran, Surah An-Nahl, 11).

In Islamic tradition and practices, almonds have a multifaceted significance:

- Nutritional Benefits: Almonds are treasured in Islamic nutrition for their healthful properties. The Prophet Muhammad (peace be upon him) advocated for the consumption of almonds, recognizing their nourishing benefits for the body.
- Medicinal Uses: Islamic medicine has long recognized almonds for their therapeutic properties. The Prophet Muhammad (peace be upon him) recommended almonds as a remedy for certain health issues, including constipation and anemia, highlighting their role in traditional healing practices.
- Symbolism and Hope: In Islamic literature, the almond, particularly its blooming tree, symbolizes hope and renewal. The blossoming of the almond tree in early spring, with its vibrant pink and white flowers, is seen as a sign of new beginnings and rejuvenation.
- Environmental Value: The almond tree is noted for its environmental friendliness in Islamic teachings. Its ability to withstand drought conditions and require minimal watering aligns with Islamic principles of sustainable and responsible stewardship of the earth. The Prophet Muhammad (peace be upon him) encouraged the planting of trees, including almonds, emphasizing that those who plant trees will be rewarded by Allah.

In summary, almonds hold a cherished position in Islamic history and culture. Their multifaceted roles, ranging from nutritional and medicinal to spiritual and environmental, make them a valued component of Islamic heritage and daily life.

Health-promoting activities of almond

In addition to its nutritional value, almond nut and skin have proven to have health-promoting and pharmacological activities such as anti-stress, immunostimulant, lipid lowering, laxative and anticancer effects (Alaya et al., 2021). The almond nut is also highly beneficial in preserving the metabiota of the colon (Alaya et al., 2021).

it has been demonstrated that replacing whole-wheat flour muffins with almonds, while keeping calories, Saturated fatty acid, Polyunsaturated fatty acid, and protein levels consistent, lowers lipid risk factors associated with coronary heart disease (CHD), and a daily

consumption of 7g portion of almonds reduces low-density lipoproteins (LDL) level by 1% (Jenkins et al., 2002). Furthermore, inducing almond hulls in the diet of broilers proved to have beneficial effect on intestinal development and liver antioxidant capacity (Wang et al., 2021). The impact of almonds on plasma cholesterol was examined by incorporating raw almonds into the diet and using almond oil exclusively for food preparation. This approach demonstrated a quick and lasting decrease in low-density lipoprotein and total cholesterol levels, without altering high-density lipoprotein cholesterol levels. (Spiller et al., 1992). Few studies have also shown the anti-microbial activity of almond. In research done by Musarra-Pizzo et al., (2019) the phenolic compounds present in the almond skin shown to cause damage to the cellular membrane, binding to the cell wall, inactivation of enzymes, and DNA damage within bacterial cells of Staphylococcus aurus. Also, selenium nanoparticles with tunable morphologies such as rods and brooms that was biogenically synthesized using almond skin has proven to have anti-microbial activity against B. subtilis (Priyadarshani S. et al., 2020). The antibacterial silver nanoparticles (AgNPs) synthesized from almond skin proved to be effective against gram-positive bacteria *B. subtilis* and *S. aureus* and gram-negative bacteria *S. typhimurium*, *E. coli* and *P. vulgaris* (Priyadarshani S. et al., 2020).

Black mulberry (Morus nigra)

The black mulberry is a deciduous tree belonging to the Moraceae family that can reach a height of 6-10 m and is characterized by slow growth. It is widely cultivated in tropical regions and is believed to have originated from various parts of the world, including Europe, the Near East, Asia, and some regions of Africa, as well as North and South America (Watson and Dallwitz, 2007; K. Browicz, 2000).

Black mulberry has a rich history of use in traditional medicine, particularly in Chinese and Uygur folk medicine, where it is valued for its antihypertensive, anti-diabetic, and anti-inflammatory properties (Zhou et al., 2019).

The nutritional research has focused on the bioactive compounds found in plants and their potential effects on human health, and the black mulberry has garnered attention. Nile and Park (2014) conducted a study that highlighted the plant's ability to impact human health and prevent diseases. The genus *Morus* includes about 16 species that are rich in isoprenylated phenolic compounds and alkaloids, which have a wide range of pharmacological properties

such as hypotensive, hypoglycemic, hypolipidemic, anti-inflammatory, and antitumor properties (Sung and Chang, 2019).

Black mulberry fruit

According to Huxley (1992), the black mulberry fruit can reach up to 25mm in diameter and is typically used when ripe from mid-August until September. The fruit can be consumed in various ways, including raw, cooked, as a preserve, or even dried and ground into a powder (Facciola, 1990). In addition to being a delicious dessert fruit, the black mulberry has been found to have several health benefits, as reported in traditional Chinese medicine, including hepatoprotective, hypotensive, antipyretic, analgesic, diuretic, expectorant, and anti-diabetic properties (Chen et al., 1995).

Researchers have shown great interest in the phytochemical profile of *Morus*, particularly the identification of phenolic compounds in the mulberry fruit. Eva M. Sánchez-Salcedo et al. (2015) found that the total phenolic content (TPC) of *Morus nigra* ranged from 7.0 to 13.6 mg GAE/g dw. However, Lin and Tang (2007) and Imran M. et al. (2010) reported higher TPC values in mulberry fruit of 1516 to 1650 mg/100 g fw, respectively. Zadernowski et al. (2005) suggested that the variation in phenolic compounds in the fruit is influenced by several factors, including genetic differences, environmental conditions during fruit development, and the degree of maturity at harvest. The black and red mulberry fruits are a rich source of phenolic compounds such as flavonols and phenolic acids, as well as anthocyanins in the case of black and red mulberry fruits (Eva M. Sánchez-Salcedo et al., 2015). Chen et al. (2017) evaluated the antinociceptive and antibacterial properties of anthocyanins and flavonols from black mulberry fruits. Additionally, Jiang et al. (2017) showed that black mulberry leaf extracts significantly reduced total cholesterol, triglyceride, and low-density lipoprotein-cholesterol levels, as well as atherogenic index in experimental atherosclerotic rats.

2.1.4. The putative relevance of gemmotherapy extracts

Gemmotherapy constitutes a branch of phytotherapy that employs extracts derived from the embryonic more exactly meristematic tissues of plants, such as buds, new shoots, and young

roots. It is a relatively new therapeutic technique that uses the medicinal properties of plant extracts obtained through maceration of fresh meristematic plant tissues, primarily buds and sprouts, in a solution of ethanol and glycerol (Donno et al., 2016). These extracts, commonly referred to as bud-preparations, are believed to contain a high concentration of bioactive compounds that work together to produce a synergistic pharmacological effect (Donno et al., 2016) and are rich in phytonutrients and can exhibit synergic, additive, or even antagonistic effects, like the multi-compound matrices found in whole plants or specific organs (Aleya et al., 2023).

In gemmotherapy, the utilized extracts are sourced from selected trees and shrubs, specifically targeting those components known for their strong medicinal qualities. These elements are responsibly harvested to obtain a botanical extract. This method seeks to capture the plant's health-promoting abilities at a point when its potential is at its peak.

The foundation of the gemmotherapy hypothesis is the idea that significant quantities of active compounds, thought to be more effective than those found in mature plants, are present in the tissues of developing plants. Research suggests that the phytochemical profile of plant embryonic tissues differs from that of the parent plant, hence providing acceptance to this theory.

In contrast to standard medicine, which often attributes the therapeutic effect to a single active compound, the benefits of gemmotherapy are thought to stem from a combination of different substances, including both active principles and other plant components. This is due to the large quantity of bioactive compounds present in herbal preparations, which can work together in complex ways to achieve a therapeutic effect (Donno et al., 2015).

In comparison to other well-established herbal medical practices, gemmotherapy is still regarded as a specialty area with fewer scientific studies, despite its increasing popularity. Nonetheless, early studies have shown encouraging findings in several areas.

In summary, although gemmotherapy is an emerging field that is gaining popularity and increasing evidence-based foundation, it is still not as well-researched as other well-established branches of herbal therapy. Gemmotherapy holds great potential due to its innovative use of plant embryonic tissues, which might provide distinct therapeutic

advantages that do not present in conventional herbal preparations. As the area progresses, it is expected to offer novel analysis and interesting insights about the healing mechanisms specific to multicellular organisms including the humans. The fact that phytochemical matrixes can generate biological activities is rather challenging for many reason but if these effects are reproducible it seems logic to continue the research efforts to understand these peculiarities of natural medicine and phytotherapy.

2.2. Objectives

Fruits and vegetables are widely recognized for their exceptional nutritional qualities, owing to their low calorie and fat content, as well as their rich composition of essential macro- and micronutrients. This makes them ideal for consumption by both humans and animals. Furthermore, the abundance of phytonutrients or phytoconstituents in these foods is known to enhance the quality of life for consumers by sustaining homeostasis at both the individual and cellular levels. Quite remarkably at the cellular level the homeostasis is maintained through cell cycle and metabolic profile regulations, while at a multicellular individual level by neurohormonal control. Given the abundance of phytonutrients, many of these plant-derived substances have been recognized as important micronutrients that can interfere with the efficacy of many life-related phenomena.

There is growing evidence to suggest that fruits and vegetables have significant health-promoting properties, yet the understanding of their specific cellular and/or physiological mechanisms together with antimicrobial properties remains limited. This gap in knowledge underscores the importance of research focused on the mechanisms of action of plant-derived bioactive compounds, as this research is fundamental to developing nutrition that supports health and high-quality animal feeding.

The current research project aims to:

- Investigate the nutritional properties of gemmotherapy extracts (GTEs) derived from olive (Olea europea), almond (Prunus amygdalus), and black mulberry (Morus nigra).
- Analyze the cellular mechanisms triggered by these GTEs to understand their impact on homeostasis at both cellular and multicellular levels.

- Examine the chemical composition of GTEs using advanced methods like Ultra-High-Performance Liquid Chromatography–Electrospray Ionization-Mass Spectrometry (UHPLC-ESI-MS).
- Evaluate the antimicrobial effects of GTEs against various bacteria and fungi species.
- Conduct a comprehensive nutritional assessment using fruit fly (*Drosophila melanogaster*) and carp (*Cyprinus carpio*) as model organisms.
- Develop a new translational model for fish feed prototypes by coupling results from fruit fly and carp studies.
- Perform a comparative study to assess the relative physiological effects of different GTEs under identical experimental conditions.
- Explore the diverse, non-linear effects of varying GTE concentrations, including potential complementary, synergistic, or antagonistic interactions.
- Understand the adaptive response capabilities of cells and multicellular organisms when exposed to different GTE concentrations.

In summary, this research is of a comparative multidisciplinary type, combining fundamental and applied studies to gain deeper insights into the analyzed GTEs phytochemical profile and the triggered biological effects. This comprehensive approach is also expected to significantly advance our knowledge in the field of health-oriented nutrition and animal feed development.

The findings from this project will not only contribute to the scientific understanding of the specific cellular and physiological mechanisms influenced by these GTEs but will also have practical implications for improving dietary practices and animal feed formulations. By providing a detailed comparison of the effects of olive, almond, and black mulberry extracts, this research has the potential to inform future studies and applications in both human nutrition and animal husbandry, ultimately promoting better health and well-being

3. MATERIALS AND METHODS

3.1. Determination of GTE-specific phytonutrient profiles

3.1.1. Preparation of gemmotherapy extracts (GTEs)

In the process of obtaining **g**emmo**t**herapy **e**xtracts (GTEs), specific harvesting and preparation methods were rigorously followed. Young olive shoots were gathered in June 2020, and sweet almond buds were collected in April of the same year. These were sourced from an organic farm located in Calabria, a region in the southern part of Italy. Meanwhile, black mulberry buds were harvested in April 2020 from an organic culture farm in Aşchileu Mare, Cluj, Romania, operated by SC PlantExtrakt SRL.

Post-harvest, these vegetal materials underwent strict quality monitoring at the laboratories of SC PlantExtrakt SRL quality control unit. To ensure accuracy and reference for future analysis, voucher specimens of each plant material were retained. The primary focus was to maintain the integrity and quality of the vegetal materials, aligning with the high standards set for GTEs.

The process of preparing the GTEs started immediately after the harvest, utilizing freshly collected vegetal materials. Preservation was critical and was achieved with a specially formulated mixture, consisting of a 1:1 ratio of 96% (v/v) ethanol and glycerol. This was combined with the plant material in a calculated ratio of 1:0.5 (plant-to-solvent). Before preservation, samples from each type of vegetal species were extracted and maintained at a controlled temperature of 4 °C, ensuring their stability until they reached the Quality Control Laboratory.

Moisture analysis was a key step in the preparation process, conducted using the previously mentioned samples. The outcome of this analysis informed the precise quantity of solvent needed to achieve an optimal dry plant-to-solvent ratio of 1:20. Following this, the initial volume of solvent used was adjusted accordingly.

The preservation method involved crushing the vegetal material into the ethanol-glycerol mixture, and afterward, the calculated amount of solvent was added to this blend. To facilitate the extraction process, this plant-solvent mixture was stirred periodically over a period of 20

days, for 20 minutes twice daily. After this period, the mixture was processed to separate the solid and liquid components. The extracted solid plant material was then subjected to additional pressing, enhancing the yield of the extraction process. This meticulous approach ensured that the GTEs were produced with the utmost care, preserving the natural integrity and potential therapeutic qualities of the plant materials.

3.1.2. The UHPLC-ESI-MS phytonutrient profile analysis of GTEs

The qualitative analysis of phytonutrient profiles of GTEs using Ultra-High-Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (UHPLC–ESI-MS) is an advanced approach, allowing for an in-depth exploration of their intricate chemical makeup. This process employs a high-tech Dionex Ultimate 3000RS ultrahigh-pressure liquid chromatography (UHPLC) system outfitted with a Thermo Scientific Accucore C18 column (Neamtu et al., 2020). This intricate analytical technique is essential for deciphering the complex phytonutrient profile of GTEs and identifying the exact phytonutrients that would contribute to their health-promoting effects. Employing this type of UHPLC–ESI-MS to analyze the phytonutrient profiles of these extracts represents by far the most advanced method applied these days.

The quantitative analysis of selected polyphenols in the current study was conducted using a high-end Shimadzu Nexera I LC-MS-8045 system, based in Kyoto, Japan. This system is an Ultra-High-Performance Liquid Chromatography (UHPLC) instrument, equipped with a sophisticated quaternary pump and an autosampler. These components are linked to an Electrospray Ionization (ESI) probe and a quadrupole rod mass spectrometer, facilitating precise detection and quantification of polyphenols. The chromatographic separation was carried out on a Luna C18 reversed-phase column, measuring 150 mm × 4.6 mm × 3 µm with 100 Å pore size, sourced from Phenomenex in Torrance, CA, USA. The temperature of the column was consistently maintained at 40 °C throughout the analysis.

The mobile phase used in this process, detailed in Supplementary Table 1, comprised a gradient mixture of methanol and ultra-purified water. The methanol, of LC/MS grade, was procured from Merck in Darmstadt, Germany. The ultra-purified water was prepared using the Simplicity Ultra Pure Water Purification System from Merck Millipore, located in Billerica, MA, USA. Additionally, formic acid, also purchased from Merck, was employed

as an organic modifier in the mobile phase. The flow rate applied during the analysis was set at 0.5 mL/min, and the total duration of each analytical run was 35 minutes.

Table 1. **The UHPLC-ESI-MS mobile phase gradient used for the quantitative analysis of GTE-specific polyphenols**

Time, min	**Methanol**	**Water**	**2 % formic acid in water**
0.00	5	90	5
3.00	15	70	15
6.00	15	70	15
9.00	21	58	21
13.00	21	58	21
18.00	30	41	29
22.00	30	41	29
26.00	50	0	50
29.00	50	0	50
29.01	5	90	5
35.00	5	90	5

Detection of polyphenols was executed using the quadrupole rod mass spectrometer, which operated with ESI in both negative and positive multiple reaction-monitoring (MRM) ion modes, as detailed in figures 1-6 and table 2. The interface temperature of the system was calibrated at 300 °C. Nitrogen was used both for vaporization and as a drying gas, at pressures and flow rates of 35 psi and 10 L/min, respectively. The capillary potential was fixed at +3000 V.

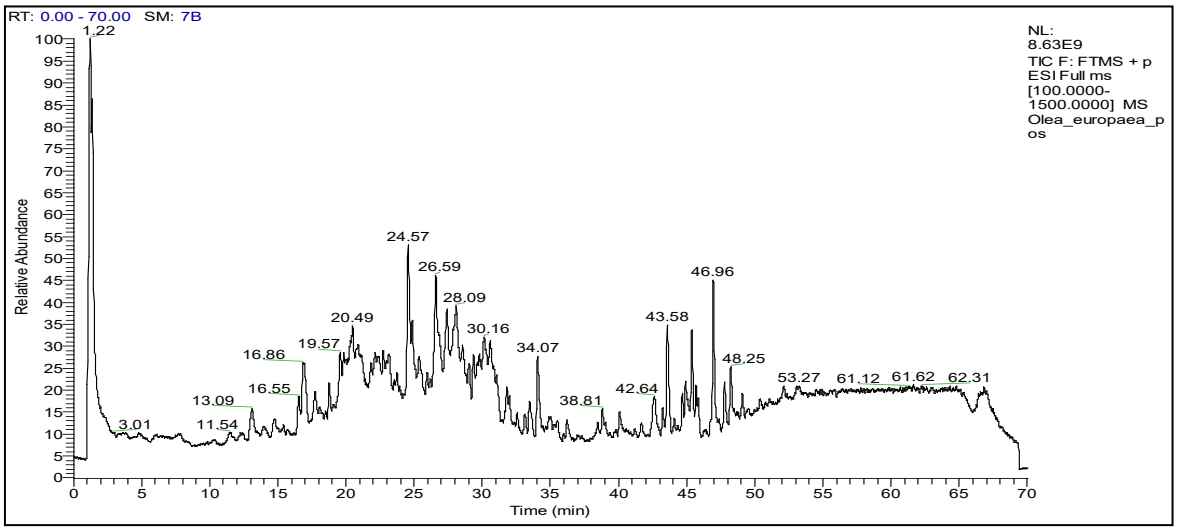

Figure 1. **Total ion chromatogram of *Olea europaea* GTE in positive ionization mode.**

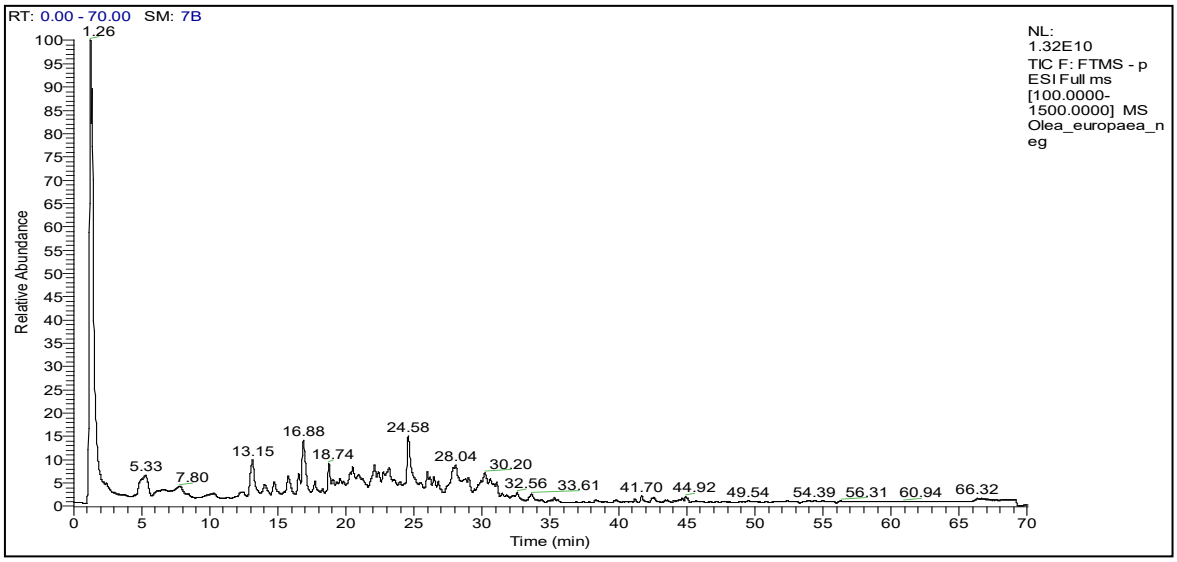

Figure 2. **Total ion chromatogram of *Olea europaea* GTE in negative ionization mode.**

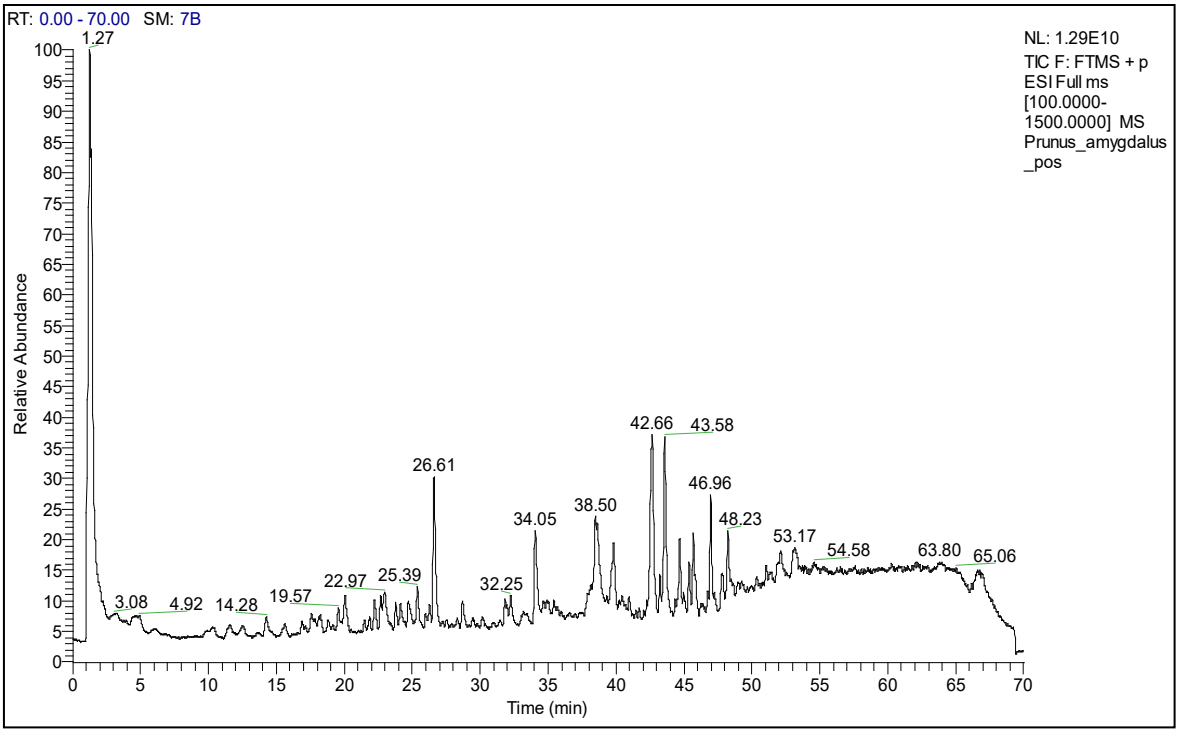

Figure 3. **Total ion chromatogram of *Prunus amygdalus* GTE in positive ionization mode.**

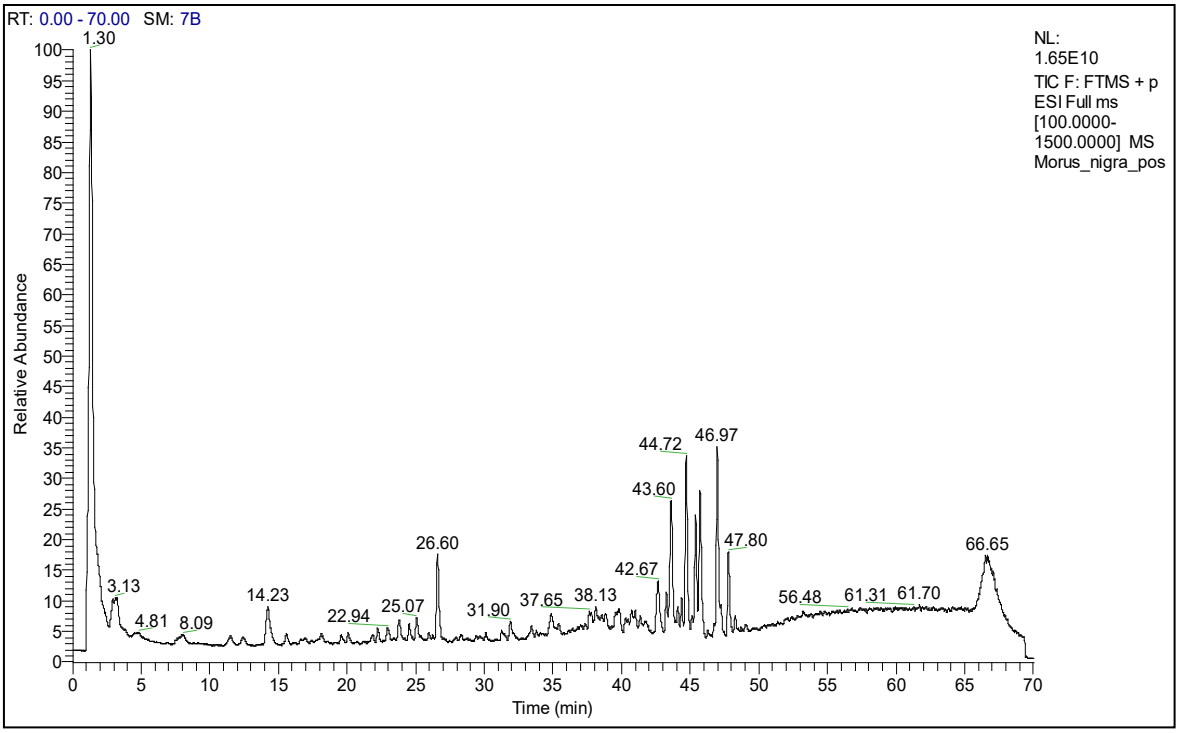

Figure 4. **Total ion chromatogram of *Prunus amygdalus* GTE in negative ionization mode.**

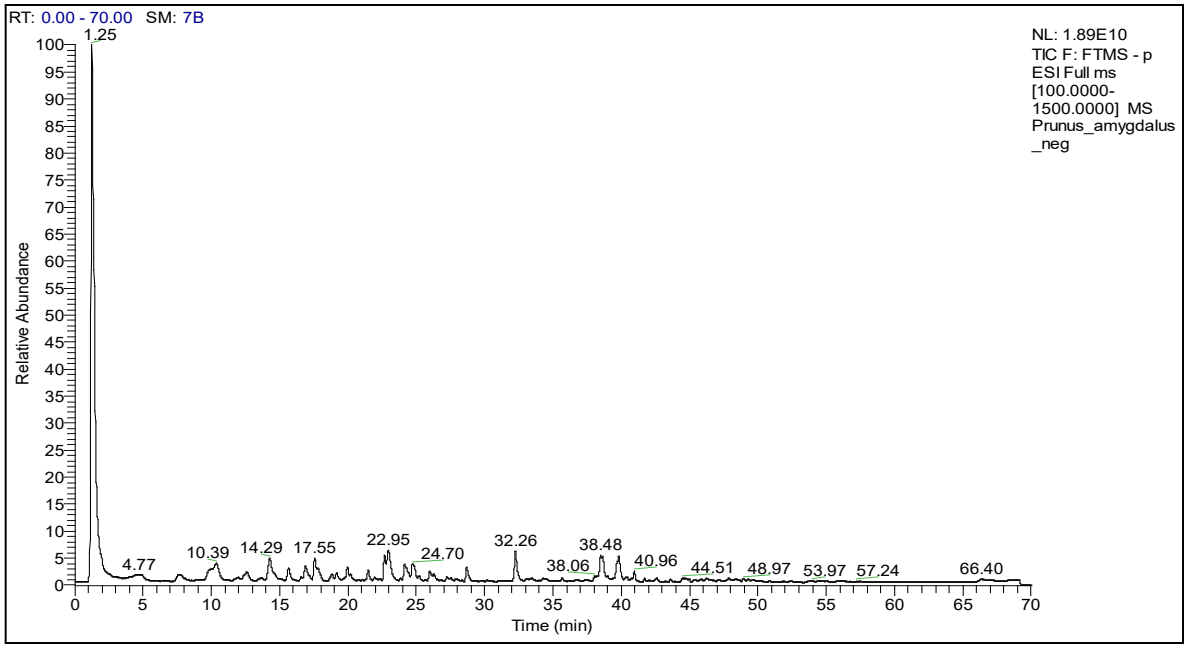

Figure 5**. Total ion chromatogram of *Morus nigra* GTE in positive ionization mode**.

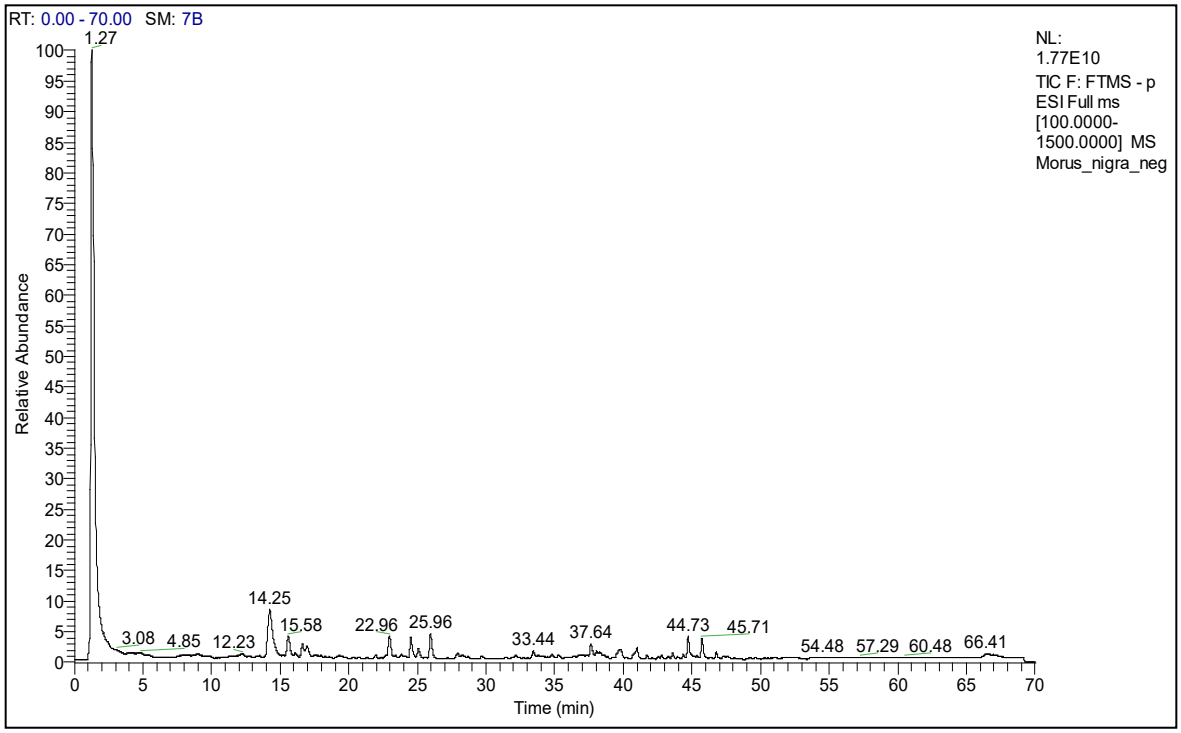

Figure 6**. Total ion chromatogram of *Morus nigra* GTE in negative ionization mode.**

For standardization and calibration, specific polyphenol standards were used. These standards, along with their calibration curve equations, correlation factors, and the limits of detection and quantification, are comprehensively presented in Table 2 and Table 3. The evaluation of the standards' specific concentrations involved the use of 1 μL of sample. The identification of the polyphenols was achieved by comparing the MS spectra and their transitions between the standards and the separated polyphenols. This identification and quantification were based on the primary transition observed in the MS spectra for each compound.

Table 2. **Standards used in the UHPLC–ESI-MS quantitative analysis of polyphenols of GTEs**

Name of Standard	Origin	Concentration Range, mg/mL	Calibration Curve Equation	Correlation Factor	Detection Limit, mg/mL	Quantification Limit, mg/mL
Caffeic acid	Phytolab, Vestenbergsgreuth, Germany	0.11–1.10	Area = 4 × 10^7 × conc[mg/mL] − 319,689	0.9998	3.20	4.80
Chlorogenic acid	Phytolab, Vestenbergsgreuth, Germany	0.13–1.30	Area = 2 × 10^8 × conc[mg/mL] − 269,699	0.9997	5.00	8.00
Apigenin	Phytolab, Vestenbergsgreuth, Germany	0.10–0.98	Area = 2 × 10^8 × conc[mg/mL] + 15,916	0.9999	0.20	0.30
Chrysin	Merck, Darmstadt, Germany	0.10–1.00	Area = 1 × 10^8 × conc[mg/mL] − 82,818	0.9997	3.00	5.00

Name of Standard	Origin	Concentration Range, mg/mL	Calibration Curve Equation	Correlation Factor	Detection Limit, mg/mL	Quantification Limit, mg/mL
Hyperoside	Phytolab, Vestenbergsgreuth, Germany	0.012–0.107	Area = $4 \times 10^8 \times$ conc[mg/mL] − 567,182	0.9986	0.60	0.90
Kaempferol	Phytolab, Vestenbergsgreuth, Germany	0.10–1.00	Area = $10^7 \times$ conc[mg/mL] − 20,574	0.9996	0.80	1.20
Luteolin	Phytolab, Vestenbergsgreuth, Germany	0.01–0.10	Area = $2 \times 10^8 \times$ conc[mg/mL] − 2295.4	0.9977	0.05	0.07
Luteolin-7-*O*-glucoside	Phytolab, Vestenbergsgreuth, Germany	0.07–0.70	Area = $1 \times 10^9 \times$ conc[mg/mL] − 700,317	0.9990	3.00	4.00
Naringenin	Phytolab, Vestenbergsgreuth, Germany	0.16–1.60	Area = $3 \times 10 \times$ conc[mg/mL] − 43,443	0.9999	0.60	0.90
Quercetin	Phytolab, Vestenbergsgreuth, Germany	0.09–0.91	Area = $5 \times 10^7 \times$ conc[mg/mL] − 9556	0.9964	0.80	1.10
Rutoside	Phytolab, Vestenbergsgreuth, Germany	0.17–1.70	Area = $2 \times 10^8 \times$ conc[mg/mL] − 191,937	0.9996	4.00	6.00

Name of Standard	Origin	Concentration Range, mg/mL	Calibration Curve Equation	Correlation Factor	Detection Limit, mg/mL	Quantification Limit, mg/mL
Vitexin	Phytolab, Vestenbergsgreuth, Germany	0.17–1.70	$\text{Area} = 3 \times 10^8 \times \text{conc[mg/mL]} - 10^6$	0.9996	1.30	2.00

Table 3. **Polyphenol standards used for the UHPLC–ESI-MS quantitative analysis.**

Name of Standard	**Retention Time, min**	***m*/*z*, and Main Transition**	**MRM**	**Other Transitions**
Caffeic acid	13.8	179.0 > 135.0	Negative	179.0 > 134.0 179.0 > 89.0
Chlorogenic acid	11.9	353.0 > 191.0	Negative	-
Apigenin	28.1	269.0 > 117.0	Negative	-
Chrysin	29.7	253.0 > 143.0	Negative	253.0 > 119.0 253.0 > 107.0
Hyperoside	20.3	463.1 > 300.0	Negative	463.1 > 301.0
Kaempferol	27.9	285.0 > 187.0	Negative	285.0 > 151.0 285.0 > 133.0
Luteolin	26.8	287.0 > 153.0	Positive	-
Luteolin-*7*-*O*-glucosid	19.9	447.0 > 284.9	Negative	-
Naringenin	26.2	271.0 > 119.0	Negative	271.0 > 107.0
Quercetin	25.4	300.9 > 151.0	Negative	300.9 > 121.0
Rutoside	20.2	609.0 > 300.0	Negative	609.0 > 301.0 609.0 > 271.0
Vitexin	18.4	431.0 > 311.0	Negative	-

The quantification process was further supported by the calibration curves, the equations of which are detailed in Table 2. Table 3 provides a comprehensive overview of the retention times and specific MS spectral data for the analyzed standards. This elaborate and meticulous

analytical process, combining advanced chromatographic techniques with mass spectrometry, ensures a thorough and accurate quantification and identification of polyphenols, contributing significantly to the understanding of their role and concentration in various samples.

3.1.3. The quantitative analysis of selective macronutrients from the GTEs

In this study, the evaluation of the protein content was conducted using the well-established Kjeldahl method. This method is recognized for its accuracy in determining the nitrogen content in organic substances, which is then used to estimate the protein content in the sample. It involves a process of digestion, distillation, and titration, and is widely regarded as a standard method in protein analysis.

The total carbohydrate content of the samples was determined through the phenol–sulfuric acid method (Nielsen, 2010). This method is known for its ability to detect a wide range of carbohydrate classes, encompassing mono-, di-, oligo-, and polysaccharides. It works by treating the sample with a mixture of phenol and sulfuric acid, leading to the development of a colored complex that can be quantitatively measured (Nielsen, 2010). This method is praised for its sensitivity and versatility in carbohydrate quantification, providing a comprehensive overview of the total carbohydrate content in the samples (Nielsen, 2010). Furthermore, the Luff–Schoorl method was employed for the determination of reducing sugars (Asquieri et al., 2019). This method is a classic analytical technique used to measure the concentration of reducing sugars, such as glucose and fructose, in a sample (Asquieri et al., 2019). It involves the reaction of reducing sugars with a copper reagent under alkaline conditions, resulting in the formation of a colored product (Asquieri et al., 2019). The intensity of the color is directly proportional to the concentration of reducing sugars present. This method is valued for its specificity and reliability in quantifying the reducing sugars, offering critical insights into the sugar composition of the samples (Asquieri et al., 2019).

Together, these methods provide a comprehensive analysis of the protein and carbohydrate content in the samples. The Kjeldahl method's precision in protein quantification, combined with the phenol–sulfuric acid method's ability to detect a wide range of carbohydrates, and the Luff–Schoorl method's specificity in measuring reducing sugars, ensures a thorough and accurate understanding of the nutritional composition of the samples under study. These

techniques, each with their unique analytical strengths, contribute significantly to the overall assessment of the biochemical constituents of the samples.

3.1.4. Determination of total polyphenol and flavonoid content of GTEs

To determine the Total Phenolic Content (TPC) of GTEs, a detailed and precise protocol was followed. Initially, a small volume of 0.1 mL of a GTE was taken to which 0.5 mL of phosphotungstenic reagent was added. This mixture was then diluted to a final volume of 25 mL using a 15% sodium carbonate solution. To ensure accuracy, the samples underwent an incubation period of 2 minutes. Following this, the absorbance of the solution was measured at a wavelength of 715 nm using a sophisticated Cintra 101 UV–Vis spectrophotometer, manufactured by GBC in Keysborough, Australia. For establishing a baseline, a blank sample was prepared. This consisted of 0.1 mL of the extract, diluted to 25 mL with the 15% sodium carbonate solution. The reading from this blank was then subtracted from the absorbance measurement of the test samples, ensuring more accurate results.

The same experimental procedure was applied to solutions of gallic acid, with concentrations ranging from 22 to 88 µg/mL. These measurements were essential for creating a calibration curve figure 6. The correlation factor of this calibration curve was found to be 0.9717, indicating a high level of reliability.

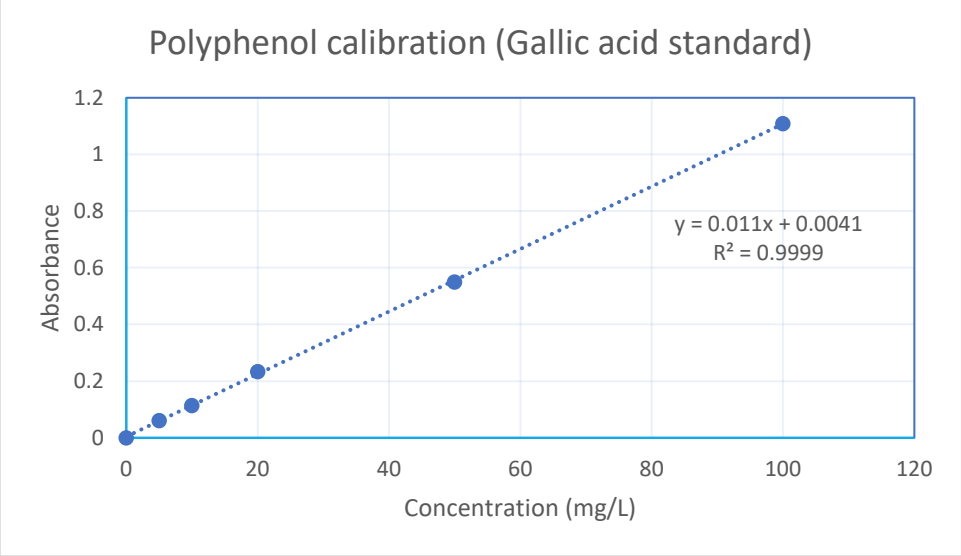

Figure 6. **Calibration curve for the spectrophotometric determination of Total Polyphenol Content (TPC) of GTEs.**

The TPC of the GTEs was quantified and expressed in terms of gallic acid equivalents (GAE) per gram of dry weight plant material. This methodological approach ensures a standardized measurement that is comparable across different samples. All the reagents used in this process were of analytical grade and were sourced from Merck, based in Darmstadt, Germany.

To ensure the robustness and reproducibility of the results, all analyses were performed in triplicate. The data obtained from these experiments were then statistically evaluated using Microsoft Excel, part of the Microsoft Office suite. This comprehensive approach to determining the TPC in GTEs not only underscores the meticulous nature of the process but also highlights the scientific rigor applied in assessing the phenolic content of these extracts.

The Total Flavonoid Content (TFC) of Gemmotherapy Extracts (GTEs) was measured using a spectrophotometric method, which has been adapted from the methodology outlined in the Romanian Pharmacopoea (Medical Publishing House, 1993). This process involves mixing 1 mL of the GTE with 5 mL of a sodium acetate solution (concentration of 100 g/L) and 3 mL of an aluminum chloride solution (25 g/L) in a 25 mL volumetric flask. The mixture is then brought to a final volume of 25 mL using methanol. After shaking, the solution is allowed to stand for 15 minutes at room temperature to ensure proper interaction of the reagents. The absorbance of the resultant solution is then measured at 430 nm using a Cintra 101 UV–Vis spectrophotometer, manufactured by GBC in Keysborough, Australia.

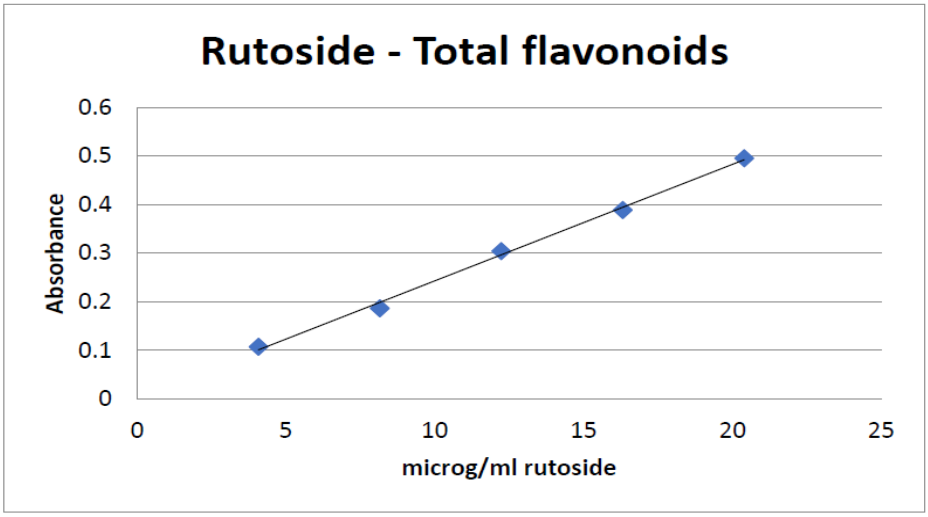

Figure 7. **Calibration curve for the spectrophotometric determination of Total Flavonoid Content (TFC) of GTEs**

For control purposes, a blank sample is prepared using 1 mL of the GTE, 8 mL of water, and methanol to make up a total volume of 25 mL. Rutoside is employed as the standard for this assay, with solutions ranging from 4 to 20 µg/mL being used to construct a calibration curve. The Figure 7, has demonstrated a high correlation factor of 0.9970, indicating the reliability of the measurements. The TFC of the GTEs is quantitatively expressed in terms of rutoside equivalent (RE) per milliliter. All reagents used in this assay are of analytical grade and are sourced from Merck, located in Darmstadt, Germany. The assay is performed in triplicate to ensure accuracy, and the results are statistically analyzed using Microsoft Excel.

3.1.5. Antioxidant potential analysis (DPPH)

Additionally, the antioxidant potential of the GTEs was assessed through several spectrophotometric methods using the same Cintra 101 UV–Vis spectrophotometer. These methods include the DPPH, Ferric Reducing Antioxidant Power (FRAP), and Xanthine Oxidase inhibition assays, all of which are integral in determining the antioxidant capacity of the extracts.

In the DPPH assay, samples prepared with varying concentrations of GTEs are mixed with DPPH reagent and incubated at 40 °C for 30 minutes. The absorbance is measured at 517 nm, and the percentage inhibition of each sample is calculated. This method, Figures8–S10, allows for the establishment of a correlation between % inhibition and concentration, from which the IC50 values are derived.

The FRAP assay involves mixing a small volume of GTE with methanol, water, and FRAP reagent, followed by a short incubation and measurement at 593 nm. A Trolox calibration, within a concentration range of 12.5–50.0 µg/mL, is used to express the antioxidant potential of the GTEs in µM Trolox equivalent per 100 mL of extract as describes in the research of Alam N. et al. (2013).

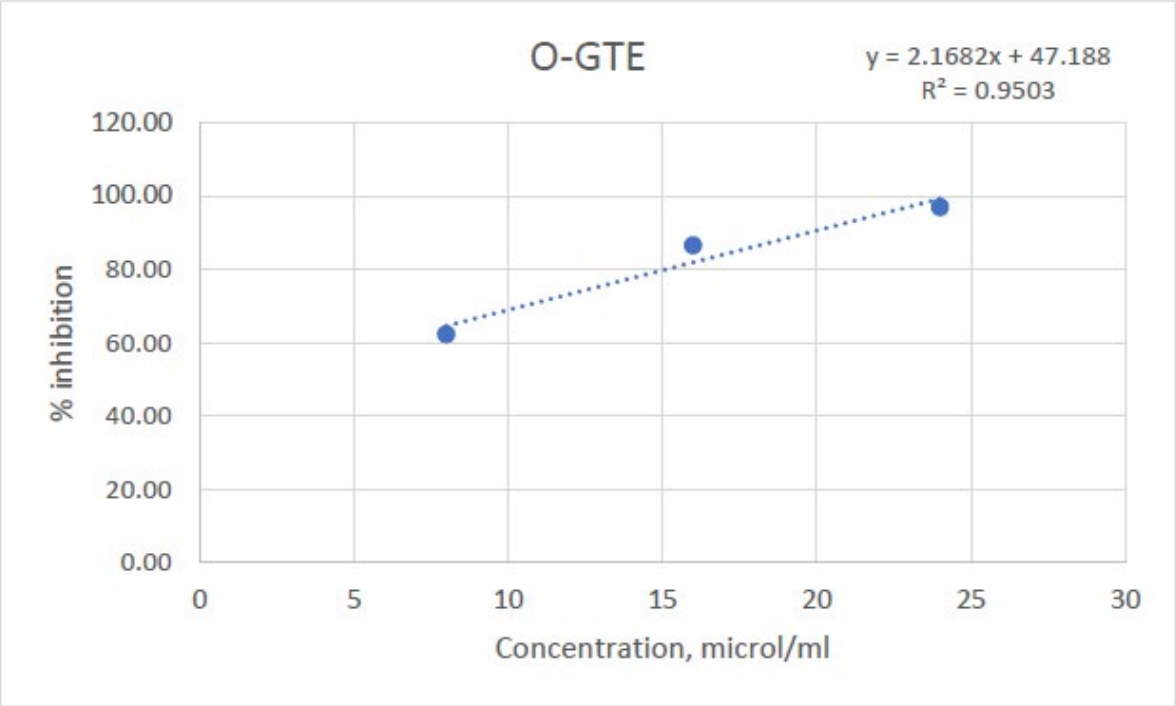

Figure 8. **Calibration curve for the DPPH assay of the O-GTE (olive)**

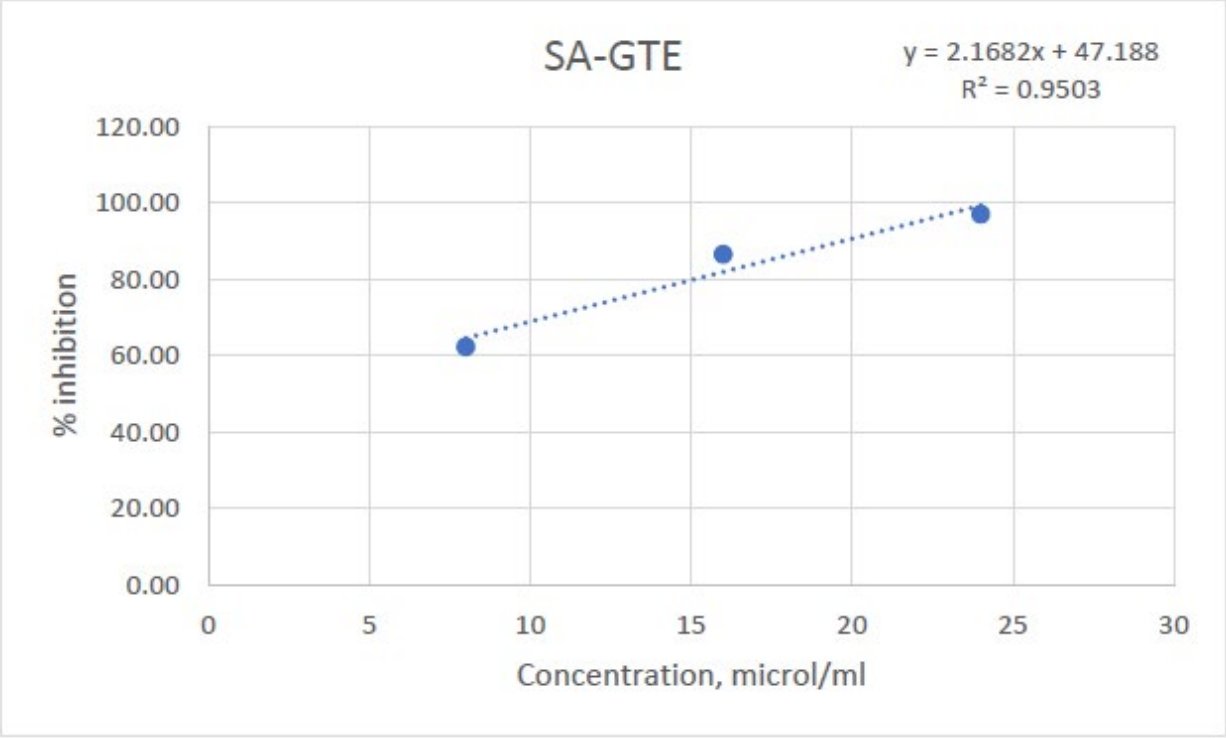

Figure 9. **Calibration curve for the DPPH assay of the SA-GTE (sweet almond)**

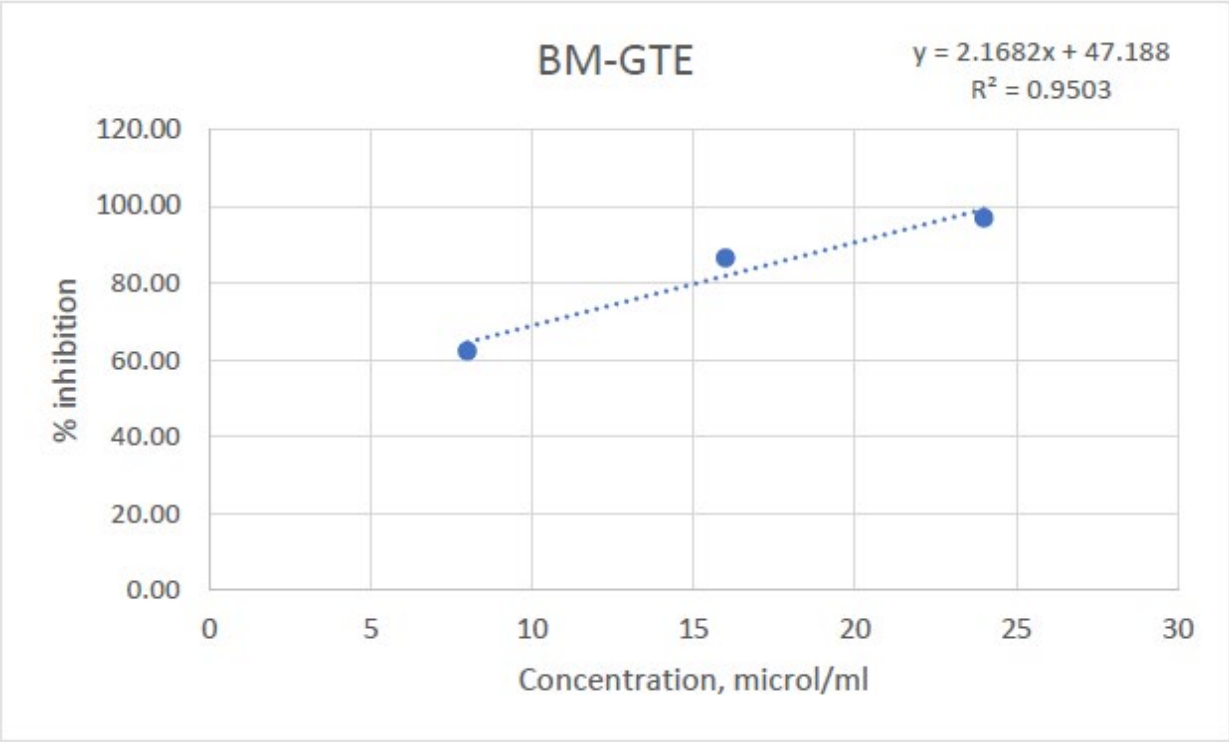

Figure 10. **Calibration curve for the DPPH assay of the BM-GTE (black mulberry)**

Lastly, the Xanthine Oxidase inhibition assay utilizes a mixture of GTE, phosphate buffer, and xanthine oxidase enzyme, incubated at 25 °C. The absorbance is measured at 293 nm after the addition of xanthine, and the percentage inhibition is calculated similarly to the DPPH assay (Alam N. et al., 2013).

Each of these assays provides a comprehensive view of the antioxidant properties of GTEs, underscoring their potential as natural antioxidants. The meticulous nature of these tests, coupled with the use of high-quality reagents and precise spectrophotometric measurements, ensures that the results are both reliable and indicative of the true antioxidant capacity of these plant extracts.

3.1.6. Analysis of the GTE-specific macronutrient profiles

The plant extracts are known to feature a complex composition and therefore, an assessment has been carried out to analyze the macronutrient content of GTEs. Initially, the carbohydrate content was determined which followed the protein content evaluation based on the Kjeldahl method. Alternatively, it had been used the phenol–sulfuric acid method [Nielsen, 2010] to

evaluate the total carbohydrate content that detected both mono-, di-, oligo-, and polysaccharides. The Luff–Schoorl method had been applied to determine the so-called reducing sugar content [Asquieri et al., 2019].

3.2. Analysis of the GTE-specific antimicrobial activities

3.2.1. The studied microbial strains

In this study, the bacterial and fungal strains utilized were sourced from the National Collection of Agricultural and Industrial Microorganisms (NCAIM). The evaluation of the antimicrobial efficacy of various bud extracts (GTEs) was conducted using eight bacterial strains, which include *Escherichia coli* B.00200, *Pseudomonas aeruginosa* B.01064, *Salmonella enterica subsp. enterica* B.00834, *Proteus vulgaris* B.00642 (representing Gram-negative bacteria), along with *Bacillus cereus* B.00076, *Staphylococcus aureus* B.01055, and *Enterococcus faecalis* B.01054 (as examples of Gram-positive bacteria). Additionally, the study involved five mycotoxigenic fungi, namely *Aspergillus flavus* F.00048, *A. niger* F.00071*, A. ochraceus* F.00850, *Penicillium citrinum* F.00815, *P. expansum* F.00601, and a yeast strain, *Saccharomyces cerevisiae* Y.00481. For the cultivation process, bacterial strains were grown on Nutrient agar comprising peptone, meat extract, NaCl (sodium chloride), agar, and distilled water, maintained at 37°C for 24 hours. In contrast, molds and yeast were cultivated on a complex medium containing peptone, yeast extract, glucose, agar, and distilled water at 28°C for 72 hours. These media were supplied by VWR International L.L.C., based in Debrecen, Hungary.

3.2.2. The agar-diffusion method

The extensively utilized agar diffusion method in microbiology is a technique used to evaluate the effectiveness of antimicrobial agents against a range of microorganisms Determining the sensitivity or resistance of bacteria or fungi to antibiotics, antiseptics, or disinfectants may be done easily and effectively with this approach.

The investigation into the specific antimicrobial properties of Gemmotherapy Extracts (GTEs) was conducted using the agar-well diffusion method. Initially, ethanol present in the extracts was eliminated using a rotavapor, which operated at a maximum temperature of 40°C and at 200 mbar. This careful process ensured that the bioactive compounds in the extracts

did not degrade or get lost. Subsequently, the ethanol removed through evaporation was promptly replaced with purified water. These prepared samples were then refrigerated at 4°C until the time of the study (Héjja et al., 2024).

For the analysis, a range of GTE concentrations, varying from 0% to 100% (v/v), were prepared. Here, 100% represented the fully concentrated GTE, while the lower concentrations were achieved by dilution with sterile distilled water. The specific concentrations of the diluted GTE solutions were prepared as follows: 100% concentration equated to 50 mg/mL, with subsequent concentrations (90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, and 10%) each reduced proportionally (45 mg/ml, 40 mg/ml, and so forth down to 5 mg/ml). Additionally, to explore their combined effects on specific microorganisms, some of these concentrated solutions were also mixed in a 1:1 ratio. These mixed GTE combinations were then tested to assess their collective effectiveness in antimicrobial activity.

A bacterial and fungal suspension with an optical density (OD) of 1 was prepared in a turbidity tube. From this, 0.1 mL of the microbial suspension (108 CFU/mL) was applied to the nutrient medium's surface. Subsequently, an 8 mm hole was made in the center of this medium, into which 0.1 mL of the extract at varying concentrations was introduced. The plates were then incubated for 24 hours at 37°C for strains including *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica subsp. enterica*, *Proteus vulgaris*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Listeria monocytogenes*. Post-incubation, the diameters of the inhibition zones, including the hole, were measured using a digital caliper. The average of three parallel measurements was calculated for accurate evaluation. This methodology was similarly applied to yeast and molds like *Saccharomyces cerevisiae, Aspergillus niger, Aspergillus flavus, Aspergillus ochraceus, Penicillium citrinum*, and *Penicillium expansum*, with the difference being the use of a complex medium and an incubation period of 48 hours at 28°C.

3.2.3. The analysis of minimum inhibitory concentration (MIC)

The methodology adopted to determine the Minimum Inhibitory Concentrations (MICs) of GTEs was based on techniques that have been described by El Baabouaa et al. (2022) and Agbeby et al. (2022). This method involved preparing an overnight culture of the test

microorganism, which was then diluted to achieve an optical density (OD) of 1. This dilution corresponded to an inoculum size of approximately 1.0×108 CFU/mL. In the testing process, a microtiter plate was employed, where each well was filled with 100 µL of different concentrations of the GTEs. The specific GTE concentrations used were as previously detailed, ranging from 100% to 10% concentration levels. To this, 20 µL of the test microorganisms, which included eight bacterial strains and one yeast strain, were introduced into each well.

For the incubation phase, the plates containing bacteria were maintained at 37°C, while those with yeast were kept at 28°C. This incubation lasted for 24 hours to allow for potential microbial growth. Subsequently, a crucial step involved the addition of 10 µL of 0.01 mg/mL resazurin to each well. After an additional incubation period of two hours, the presence or absence of microbial growth was indicated by a color change in the resazurin, shifting from purple to pink.

The MIC was identified as the lowest concentration of each extract that showed no visible microbial growth. To further validate the results, samples from various wells were also inoculated on nutrient medium, and the formation of colonies was monitored. This step was crucial to confirm the accuracy of the color change as an indicator of microbial growth inhibition, ensuring the reliability of the MIC determination.

Overall, this method offers a meticulous approach to evaluating the antimicrobial efficacy of GTEs, encompassing careful preparation, precise measurement of extract concentrations, and thorough incubation and observation procedures. The additional verification step provides a robust check to authenticate the results obtained from the resazurin color change, enhancing the credibility of the MIC values determined for each extract against the respective microbial isolates.

3.2.4. The analysis of minimum bactericidal concentration (MBC)

The process for determining the Minimum Bactericidal Concentration (MBC) of GTEs expanded upon the methodology used in assessing the Minimum Inhibitory Concentrations (MIC). This approach involved using the same microtiter plate method as in the MIC determination but with an additional step to assess the bactericidal properties of the extracts.

After conducting the MIC assays, the wells of the microtiter plate that exhibited no growth were further processed to determine the MBC. Nutrient broth from these non-growth wells was carefully diluted and then spread onto nutrient agar plates. These plates were then incubated under specific conditions: for bacterial samples, the incubation was at 37°C for 24 hours, while yeast samples were incubated at 28°C for 48 hours. The plates were placed in an inverted position during this period to promote optimal growth conditions and to prevent moisture from condensing on the agar surface, which could interfere with the accurate observation of microbial growth.

The MBC is defined as the lowest concentration of the extract that results in no visible growth on the nutrient agar following the stipulated incubation period. Essentially, while MIC determines the concentration at which microbial growth is inhibited, MBC identifies the concentration at which the microorganisms are killed. This distinction is crucial in antimicrobial studies, as some agents may only inhibit growth without killing the bacteria or yeast, which is a critical consideration in clinical settings when selecting appropriate antimicrobial therapies. This extended method provides a more comprehensive understanding of the antimicrobial efficacy of the GTEs. It not only evaluates their ability to inhibit microbial growth but also their potential to eradicate the microorganisms. This dual approach is essential in developing effective antimicrobial treatments, especially in the context of increasing antibiotic resistance.

3.3. The Drosophila melanogaster based experiments

The fruit fly is considered an accomplished model organism to study the genetic basis of many lives related phenomena ever since the chromosomal location of genes has been demonstrated. Moreover, its implication in chromosomal and genomic mutation, maternal inheritance, embryonic development, diurnal cycle, and innate immunity have been other topics that yield novel Nobel prizes. Recently the *Drosophila melanogaster* seems to prove its usefulness with respect to dietary assessments.

3.3.1. Fruit fly strains and different dietary culture conditions

For the *Drosophila melanogaster* experiments, we used the wm4h (white mottled 4) strain, sourced from the Bloomington Stock Center. The fruit flies were cultured under three distinct

dietary conditions: (1) zero nutrient (0N), (2) normal media (NM), and (3) high-sugar media (HS). The 0N media, resembling a minimal larval media without nutrients [19], was composed of 1 g carbon powder and 1 g agar (VWR, No. 20767.298) dissolved in 100 mL water, boiled for 10 seconds, cooled to 45 °C, and then distributed into vials, each containing 3 mL.

For the NM and HS media, 70 g of yeast paste was first blended with 1.2 liters of water. To this mixture, 51.35 g of sucrose for NM or a substantially higher 513.45 g for HS was added along with 30 g of wheat flour, and the mixture was brought to a boil. After adding 10 g of agar powder and ensuring thorough mixing, the boiling was continued for over 30 minutes until the volume was reduced to 1 liter. The culture media was then cooled to 50 °C in a water bath. Following this, 1 g of NIPAGIN, obtained from ThermoFisher, was mixed in thoroughly. The media was then aliquoted, with 4 mL per culture vial. To some of these vials, various volumes of GTE were added and mixed well to achieve different concentrations of 11%, 20%, 33.3%, 42.8%, and 50%. These concentrations were obtained by mixing 0.5 mL, 1 mL, 2 mL, 3 mL, and 4 mL of GTEs with 4 mL of culture media. All *Drosophila melanogaster* experiments were conducted at a stable temperature of 25 °C and 28°C.

3.3.2. Staged embryo collection and monitoring of viability

Collecting 0–2 hour-old embryos of *Drosophila melanogaster*:

To collect early-stage embryos (0-2 hours), we introduced approximately two hundred 5-day old w^{m4h} *Drosophila melanogaster*, both females and males, into an embryo collection cage positioned atop of a plate with 0N (zero nutrient) media enriched with yeast paste. Over a period of 48 hours, and with the egg collection plates being replaced every 2 hours, we were able to gather embryos that were between 0 and 2 hours old. These embryos were delicately extracted using fine forceps under a microscope from the collection plate and then transferred into vials containing the appropriate 0N, NM (normal nutrient), or HS (high sugar) media, each supplemented with varying concentrations of GTEs. In each vial, a total of 50 embryos were placed, and for each concentration under consideration, 5 vials were prepared. The entire process was replicated three times, and the results were averaged to ensure accuracy and consistency in the experiment.

Assessing viability throughout *Drosophila melanogaster* development:

These experiments were conducted under controlled conditions of 25°C and 28°C and consistent humidity. We diligently counted the number of third instar larvae and adults daily, continuing until no additional adults emerged. To gain a deeper understanding of the impact on viability, these experiments were not only repeated three times but also included an expanded range of GTE-specific concentrations.

It's important to note that in these GTE-specific viability assessments, we used flies of identical genotype and age. Furthermore, all experiments were carried out simultaneously, ensuring that the results obtained were directly comparable and devoid of discrepancies that might arise from variations in experimental conditions or developmental stages of the flies. This parallel execution of the experiments was crucial for obtaining reliable and accurate data regarding the impact of GTE concentrations on the viability of *Drosophila melanogaster*.

3.3.3. The assessment of larval ATP content

To assess the ATP content in the fish larvae, we systematically selected 10 larvae from each aquarium for analysis. These larvae were placed in a 1.5 mL Eppendorf tube, which contained 250 µL of Phosphate-Buffered Saline (PBS). A gentle tapping on the tube ensured that the larvae were thoroughly rinsed in the PBS, after which the PBS was carefully removed using a Pasteur pipette. Next, 50 µL of PBS was added back into the tube. Using the Micro-Vial Homogenizer System (Wilmad LabGlass Motor & Adapter, BP-7005-000, by ATS Life Sciences Wilmad, Vineland, NJ, USA) equipped with sterilized pestles, the larvae within the tube were then homogenized. Following the homogenization, the mixture was centrifuged at 7000 rpm at a temperature of 4 °C. The supernatant, containing the larval extract, was then carefully pipetted into an ATP test tube (Hygiena, UltraSnap, No.US 2020). The ATP content in this extract was measured using a luminometer (Hygiena, EnSURE V.2). To interpret the results accurately, the readings, initially obtained in Relative Light Units (RLU), were converted into picograms (pg) of ATP. This conversion utilized the formula: $m = (RLU \times 0.507)/2$.

By conducting this meticulous process, we could accurately quantify the ATP content in the larvae, offering valuable insights into their metabolic activity and overall health. This method of ATP measurement is crucial in understanding the impact of different diets, including the GTE-infused feed, on the metabolic health of the fish larvae. It provides a direct measure of the ATP production to cover energetic expenses within the larval development, which is a critical factor in assessing their growth, development, and viability.

3.4. The carp (Cyprinus carpio) based experiments

3.4.1. Carp fish larval culturing conditions

These experiments took place in the Fish Laboratory Unit at the Institute of Animal Science, Biotechnology, and Nature Conservation, University of Debrecen. Following the successful artificial propagation of common carp, fertilized eggs were placed in Zuger glasses, each connected to an independent mechanical and biological filter within a recirculation system. Within 48 hours, the carp larvae began to hatch, at which point the viable, non-feeding fry were transferred to a specialized fry-rearing unit, also linked to a recirculation system.

For the initial 48 hours, the larvae were kept in the rearing unit until they achieved their first respiratory activity, necessary for filling their swim bladders. Subsequently, about 200 larvae were introduced into each unit of a modular aquarium system, which served as the experimental setup. This system had a total water capacity of approximately 800 liters, comprising 12 individual aquariums with a usable volume of 20 liters each, and a buffer tank with a 320-liter capacity. In a randomized block arrangement, each aquarium housed 200 non-feeding larvae.

Mechanical filtration in the system was accomplished using a ceramic medium with a 20-liter volume and a 40 m^2 surface area, while biological filtration utilized a 50-liter volume sponge system with a 20 μm particle size. Oxygen supply to maintain the desired saturation level was ensured by a JEBAO AIR PUMP compressor. Additionally, 500 W Aqua L GOLD 500 aquarist heating elements regulated the water temperature. Both temperature (22.0 ± 0.5 °C) and dissolved oxygen concentration (79.3 ± 0.6%) were consistently monitored daily using a HACH HQ30d device, remaining stable throughout the experiment.

The larval groups were fed ad libitum during the experiment. Excess feed and waste were vacuumed from the aquariums daily. Four different treatment conditions were implemented, as detailed in table 4, and the entire experimental setup was replicated three times to ensure the validity and reliability of the results.

Table 4. **Scheme of the carp larval-seeding experiment**

1	2	3	4	5	6	7	8	9	10	11	12
O-GTE	SA-GTE	Control *Artemia s.*	BM-GTE	Control *Artemia s.*	SA-GTE	BM-GTE	O-GTE	BM-GTE	O-GTE	SA-GTE	Control *Artemia s.*

For the duration of the experiment, the control group of fish larvae was exclusively fed with brine shrimp (*Artemia salina*) as their live food source. Meanwhile, in the other aquariums, the carp larvae were provided with a diet infused with the Gemmotherapy Extracts (GTEs) under study. Throughout the experimental period, we closely monitored the larvae for their growth in terms of length and ATP content. At the conclusion of the experiment, the number of surviving larvae was counted, allowing us to calculate the overall survival rate.

3.4.2. Production of carp larval fed containing GTEs

To create a fishmeal specifically tailored for carp larvae, we mixed Fibersol-2, a water-soluble dietary fiber, with the designated GTE. The preparation involved adding 0.2 mL of GTE to every 1 gram of fiber, ensuring the mixture attained a uniform consistency. This blend was then left to dry for a period of 2-3 days in an incubator maintained at 35 °C. After the drying process, the GTE-Fibersol-2 mixture was finely ground to achieve a consistent grain size. The resulting feed was then stored at room temperature in airtight containers, away from light, until it was needed for feeding the larvae. This method was aimed at ensuring that the fish larvae received a nutritionally enriched diet, potentially enhancing their development and overall health.

3.4.3. The assessment of fish larval ATP content and growing parameters

To assess the ATP content in the fish larvae, we systematically selected 10 larvae from each aquarium for analysis. These larvae were placed in a 1.5 mL Eppendorf tube, which contained 250 μL of Phosphate-Buffered Saline (PBS). A gentle tapping on the tube ensured that the larvae were thoroughly rinsed in the PBS, after which the PBS was carefully removed using a Pasteur pipette. Next, 50 μL of PBS was added back into the tube. Using the Micro-Vial Homogenizer System (Wilmad LabGlass Motor & Adapter, BP-7005-000, by ATS Life Sciences Wilmad, Vineland, NJ, USA) equipped with sterilized pestles, the larvae within the tube were then homogenized.

Following the homogenization, the mixture was centrifuged at 7000 rpm at a temperature of 4 °C. The supernatant, containing the larval extract, was then carefully pipetted into an ATP test tube (Hygiena, UltraSnap, No.US 2020). The ATP content in this extract was measured using a luminometer (Hygiena, EnSURE V.2). To interpret the results accurately, the readings, initially obtained in Relative Light Units (RLU), were converted into picograms (pg) of ATP. This conversion utilized the formula: $m = (RLU \times 0.507)/2$.

By conducting this meticulous process, we could accurately quantify the ATP content in the larvae, offering valuable insights into their metabolic activity and overall health. This method of ATP measurement is crucial in understanding the impact of different diets, including the GTE-infused feed, on the metabolic health of the fish larvae. It provides a direct measure of the energy available within the larvae, which is a critical factor in assessing their growth, development, and viability.

4. RESULTS

4.1.1. The phytonutrient profiles of GTEs

The analysis of phytonutrient profiles is a paramount feature of our studies because the GTEs are complex matrixes that would contain several components. Therefore, a qualitative and quantitative analytical chemistry-based assessment would confer an unprecedented strength to such an analysis especially if the GTE-generated biological effects are to be evaluated. It is also true that many phytonutrients were studied individually for their induced physiological effects that offers an evidence-based wealth of knowledge. Moreover, this comprehensive analysis not only enhances our understanding of the chemical makeup of these extracts but also opens new avenues for exploring their potential health benefits. By categorizing these bioactive compounds, we gain valuable insights into the possible mechanisms through which GTEs exert their therapeutic effects. Knowing what to expect allows us to design more straightforward experiments that will eventually create the premises for the identification of the action mechanisms and their genetic control.

This extensive analytical chemistry characterization of the phytonutrient profiles of GTEs is a significant step forward in the field of natural health products and herbal medicine. It lays the foundation for further research into the specific health benefits and therapeutic applications of these extracts, potentially leading to the development of novel natural health solutions and supplements based on the rich phytochemical composition of GTEs.

4.1.2. The olive -specific GTE phytonutrient profile

In this detailed investigation, the phytonutrient composition of the olive GTE (O-GTE) was meticulously analyzed using UHPLC–ESI-MS (for details see Material and Methods). This advanced qualitative analysis technique enabled the identification of a diverse array of chemical constituents within various GTEs. In the O-GTE, there were identified 45 distinct chemical components that would belong to several compound types (Table 5).

Table 5**. Olea europea GTE specific bioactive compounds**

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
1	Flavonoid	Quercetin-di-O-hexoside	17.17		625.1405
2		Taxifolin (Dihydroquercetin)	19.32		303.0505
3		Dihydrokaempferol (Aromadendrin, Katuranin)	21.92		287.0556
4		Luteolin-7-O-glucoside (Cynaroside)	22.36		447.0927
5		Luteolin-O-rutinoside isomer	22.45		593.1507
6		Isoquercitrin (Hirsutrin, Quercetin-3-O-glucoside)	22.93		463.0877
7		Rutin (Quercetin-3-O-rutinoside)	23	611.1612	
8		Luteolin-7-O-rutinoside (Scolymoside)	23.07		593.1507
9		Cosmosiin (Apigetrin, Apigenin-7-O-glucoside)	23.98	433.1135	
10		Chrysoeriol-O-hexoside	24.25		461.1084
11		Luteolin-3'-O-glucoside or Luteolin-5-O-glucoside	24.67		447.0927
12		Luteolin-4'-O-glucoside	25.89		447.0927
13		Quercetin	26.99	303.0505	
14		Naringenin	27.21		271.0607
15		Luteolin (3',4',5,7-Tetrahydroxyflavone)	27.85		285.0399
16		Scutellarein-7-O-(6-O-feruloyl)glucoside	28.79		623.1401
17		Apigenin	29.67		269.0445
18		Isorhamnetin	29.84		315.0505
19		Chrysoeriol	29.9		299.0556
20		Pinocembrin (5,7-Dihydroxyflavanone)	32.25		255.0657

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
21	Polyphenol	Hydroxytyrosol (3,4-Dihydroxyphenylethanol)	5.34		153.0552
22		Dihydroxycoumarin-O-hexoside	12.24	341.0873	
23		Esculetin (6,7-dihydroxycoumarin)	14.04		179.0344
24		Chlorogenic acid (3-O-Caffeoylquinic acid)	14.28	355.1029	
25		Scopoletin (7-Hydroxy-6-methoxycoumarin)	18.55	193.0501	
26		Verbascoside	22.01		623.1976
27	Lignan Polyphenol	8-Acetoxypinoresinol-4-O-glucoside	22.75		577.1921
28					
		8-Hydroxypinoresinol-4-O or 4'-O-glucoside	23.37		535.1816
29	Carboxylic acid	Kynurenic acid	13.18	190.0504	
30		12-Hydroxyjasmonic acid glucoside or Tuberonic acid	17.59		387.1655
		glucoside			
31		12-Hydroxyjasmonic acid or Tuberonic acid	18.26		225.1127
32		Ginkgoic acid	47.4		345.243
33	Iridoid	Oleoside	16.88		389.1084
34		Neonuzhenide	21.54		701.2293
35		Nuzhenide dihydroxyphenylacetic acid isomer	21.66		715.2086
36		Oleuropein hexoside isomer 1	22.89		701.2293
37		Nuzhenide	23.07		685.2344
38		Oleuropein hexoside isomer 2	23.32		701.2293
39		Oleuropein hexoside isomer 3	24.07		701.2293
40		Oleuropein	24.57		539.1765
41		Ligstroside (Ligustroside)	26.44		523.1816

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
42	Terpenoid	Uvaol	45.11	443.3889	
43	Vitamin	Adenine (B4)	1.29	136.0623	
44		Nicotinamide	1.4	123.0558	
45		**Nicotinic acid (Niacin /B3)**	**1.4**	**124.0399**	

The profile of bioactive compounds discovered in the case of O-GTE (Figure 11) spanned several categories, each known for their unique health-promoting properties. These categories included polyphenols (in this context, specifically referring to non-flavonoid compounds), flavonoids, iridoids, terpenoids, carboxylic acids and vitamins were also detected. It is also very interesting that no amino acids were detected, while the iridoids a fairly rare compound category was specific to the O-GTE.

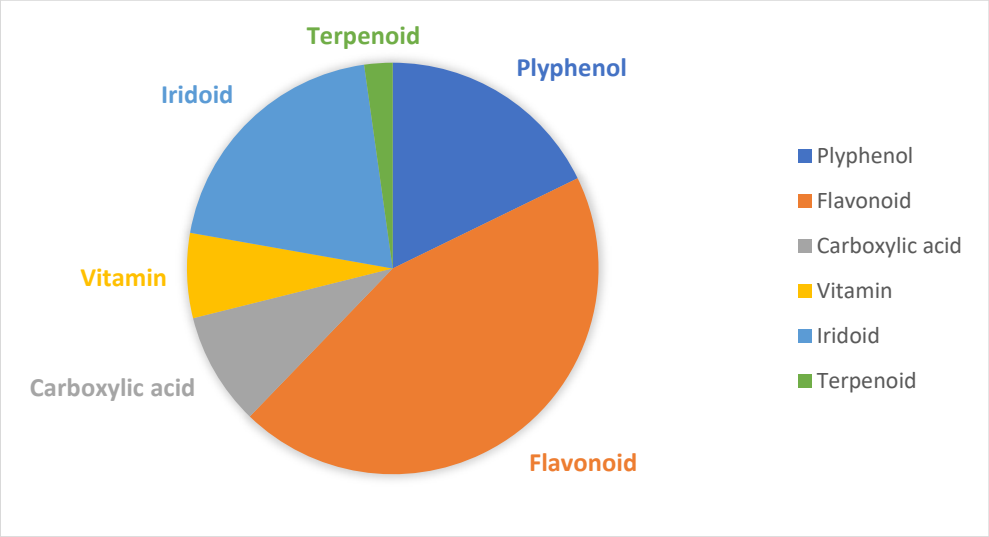

Figure 11. **Phytonutrient profile of O-GTE**

The identification of such a wide range of olive-specific compounds underscores the complexity and richness of O-GTEs in terms of phytonutrient content and most probably the associated biological effects. The presence of diverse phytonutrients like polyphenols and flavonoids, known for their antioxidant properties, along with other biologically active compounds, suggests a broad spectrum of potential health benefits, ranging from anti-inflammatory and anti-oxidative effects to contributions in metabolic and cardiovascular health.

4.1.3. The sweet almond-specific GTE phytonutrient profile

In the sweet almond (SA) SA-GTE, a higher number of phytoconstituents were detected, totaling 103 after the UHPLC–ESI-MS qualitative analysis technique (see Materials and Methods). The Figure 7. presents the identified compounds.

Table 6. **The phytonutrient profile of SA-GTE (sweet almond)**

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
1	Flavonoid	Quercetin-3-O-rutinoside-7-O-glucoside	17.59		771.1984
2		Kaempferol-O-(rahymnosyl)hexoside-O-hexoside	19.09		755.2035
3		Taxifolin (Dihydroquercetin)	19.33		303.0505
4		Quercetin-O-(hexosyl)rutinoside	19.82		771.1984
5		Quercetin-O-dihexoside isomer 1	19.96		625.1405
6		Quercetin-O-dihexoside isomer 2	20.2		625.1405
7		Tetrahydroxyflavanone-O-hexoside isomer 1	20.35		449.1084
8		Myricetin-O-hexoside	20.91		479.0826
9		Kaempferol-O-(hexosyl)hexoside	21.47		609.1456
10		Dihydrokaempferol (Aromadendrin, Katuranin)	21.94		287.0556
11		Prunin (Naringenin-7-O-glucoside)	22.38		433.1135
12		Tetrahydroxyflavanone-O-hexoside isomer 2	22.46		449.1084
13		Hyperoside (Quercetin-3-O-galactoside, Hyperin)	22.69		463.0877
14		Isoquercitrin (Hirsutrin, Quercetin-3-O-glucoside)	22.89		463.0877
15		Rutin (Quercetin-3-O-rutinoside)	23.02	611.1612	
16		Reinutrin (Reynoutrin, Quercetin-3-O-xyloside)	23.25		433.0771

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
17		Avicularin (Quercetin-3-O-arabinofuranoside, Fenicularin)	23.5		433.0771
18		Quercetin-3-O-(6''-malonyl)glucoside	23.68		549.0881
19		Tetrahydroxyflavanone-O-hexoside isomer 3	23.83		449.1084
20		Kaempferol-O-hexoside	24.15		447.0927
21		Guaijaverin (Quercetin-3-O-arabinoside)	24.26		433.0771
22		Kaempferitrin (Kaempferol-3,7-di-O-rhamnoside)	24.31		577.1557
23		Quercetin-O-(rhamnosyl)hexoside	24.31		609.1456
24		Quercitrin (Quercetin-3-O-rhamnoside)	24.48	449.1084	
25		Quercetin-O-(acetyl)hexoside isomer 1	24.49	507.1139	
26		Astragalin (Kaempferol-3-O-glucoside)	24.69		447.0927
27		Isorhamnetin-O-hexoside isomer 1	24.75		477.1033
28		Kaempferol-3-O-rutinoside (Nicotiflorin)	24.86		593.1507
29		Eriodictyol	24.94		287.0556
30		Isorhamnetin-O-hexoside isomer 2	24.95		477.1033
31		Isorhamnetin-3-O-rutinoside (Narcissin)	25.21		623.1612
32		Kaempferol-O-(malonyl)glucoside	25.63		533.0931

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
33		Quercetin-O-(acetyl)hexoside isomer 2	25.68	507.1139	
34		Kaempferol-O-(rhamnosyl)hexoside	26.13		593.1507
35		Quercetin-O-(acetyl)hexoside isomer 3	26.29	507.1139	
36		2''-O-Acetylrutin	26.31		651.1561
37		Quercetin-3-O-(4-coumaroyl)glucoside	26.85		609.1244
38		5,7,3',4',5'-Pentahydroxyflavone (Tricetin)	27.02		301.0348
39		Naringenin	27.24		271.0607
40		Trihydroxy-methoxy(iso)flavanone	27.38		301.0712
41		Multiflorin A (Kaempferol-3-O-[(6-O-acetyl)glucosyl-(1→4)rhamnoside])	28.04		635.1612
42		Trihydroxy-methoxyflavone-O-hexoside	28.37		461.1084
43		Tetrahydroxyflavone	29.36		285.0399
44		Isorhamnetin	29.84		315.0505
45		Kaempferol-O-[(acetyl)rhamnosyl)rhamnoside]	29.96		619.1663
46		Pinocembrin (5,7-Dihydroxyflavanone)	32.23		255.0657
47		Dihydroxy-methoxy(iso)flavanone	32.25	287.092	
48		Dihydroxy-methoxy(iso)flavone	33.9		283.0607
49		Trihydroxy-methoxy(iso)flavone	34.56		299.0556
50	Polyphenol	Coumaroylquinic acid isomer 1	11.96		337.0924

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
51		5-O-(4-Coumaroyl)quinic acid	12.6		337.0924
52		Vanilloylglucose isomer 1	13.64		329.0873
53		Vanilloylglucose isomer 2	14.08		329.0873
54		Coumaroylglucose isomer 1	14.18		325.0924
55		Chlorogenic acid (3-O-Caffeoylquinic acid)	14.28	355.1029	
56		3-O-Feruloylquinic acid	14.57		367.1029
57		trans-Melilotoside (trans-Glucosyl-2-hydroxycinnamate)	14.68		325.0924
58		Coumaroylglucose isomer 2	15.11		325.0924
59		Chryptochlorogenic acid (4-O-Caffeoylquinic acid)	15.61	355.1029	
60		3-O-(4-Coumaroyl)quinic acid	15.67		337.0924
61		Vanilloylglucose isomer 3	16.15		329.0873
62		Coumaroylquinic acid isomer 2	16.87		337.0924
63		4-O-(4-Coumaroyl)quinic acid	17.55		337.0924
64		4-Coumaric acid	17.85		163.0395
65		5-O-Feruloylquinic acid	18.01		367.1029
66		4-O-Feruloylquinic acid	18.56		367.1029
67		Coumaroylquinic acid isomer 3	19.19		337.0924
68		Ferulic acid	19.35		193.0501
69		Isoferulic acid	20.41		193.0501
70		Di-O-coumaroylglucose isomer 1	27.01		471.1291
71		Di-O-coumaroylglucose isomer 2	27.38		471.1291
72	Carboxylic acid	Quinic acid	1.24		191.0556
73		Malic acid	1.32		133.0137
74		Citric acid	1.41		191.0192

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
75		Jasmonic acid	27.77		209.1178
76	Amino acid	Lysine	1.09	147.1134	
77		Arginine	1.22	175.1195	
78		γ-Aminobutyric acid	1.24	104.0712	
79		Aspartic acid	1.27	134.0453	
80		Methionine sulfoxide	1.27	166.0538	
81		Proline	1.27	116.0712	
82		Serine	1.27	106.0504	
83		Threonine	1.27	120.0661	
84		Asparagine	1.29	133.0613	
85		Glutamic acid	1.29	148.061	
86		Isoleucine	1.54	132.1025	
87		Leucine	1.9	132.1025	
88		Phenylalanine	3.25	166.0868	
89	Alkaloid	Choline	1.18	104.1075	
90		Chelidonine	20.05	354.1342	
91		Berberine	22.33	336.1236	
92	Carbohydrate	Benzyl-glucoside	10.39		269.1025
93		Benzyl-primeveroside	16.56		401.1448
94	Aldehid	Indole-4-carbaldehyde	19.04	146.0606	
95	Fatty acid	α-Linolenic acid	44.73		277.2168
96		Linoleic acid	45.75		279.2324
97		Palmitoleic acid	46.05		253.2168
98	Terpenoid	Abscisic acid	25.39		263.1283
99	Vitamin	Adenine (B4)	1.3	136.0623	
100		Nicotinic acid (Niacin, B3)	1.4	124.0399	
101		Nicotinamide	1.41	123.0558	

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
102	Others	2-Oxindole	4	134.0606	
103		Eugenol (4-Allyl-2-methoxyphenol)	23.81	165.0916	

The variety of bioactive compounds identified in SA-GTE, as depicted in Figure 12, encompasses diverse categories, each recognized for their distinctive structures and benefits to health. These compounds represent a wide spectrum of naturally occurring compounds, each contributing most likely in its own way to the health-enhancing potential of SA-GTE. It is also interesting that the SA-GTE contains amino and fatty acids.

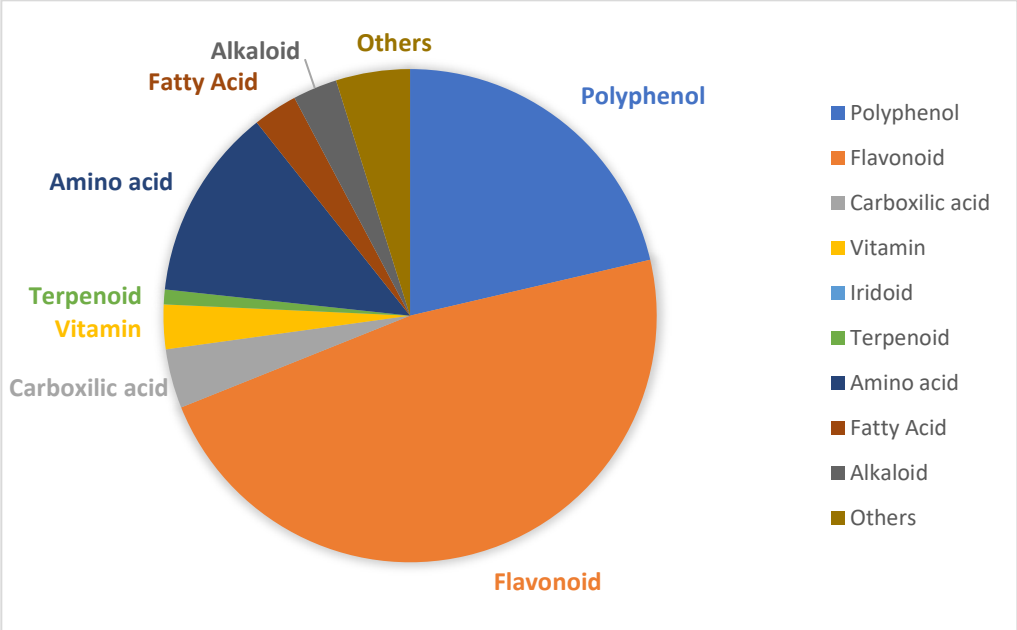

Figure 12. **Phytonutrient profile of SA-GTE**

Above presented data represents the first ever reported phytoconstituent composition of an SA-GTE and it shows certain similarities with the SA seeds that are well known for their macronutrient-rich feature, containing about 50% lipids, 25% proteins, 20% carbohydrates, and many phytonutrients with health-promoting effects, particularly against cardio-metabolic conditions (Barreca D., et al., 2020). In the case of the SA-GTE the flavonoid class, with 49 compounds, appeared to be the most significant based on the number of phytonutrient representatives. Among these flavonoids were flavanols like quercetin, kaempferol, myricetin, narcissin, isorhamnetin, flavones like triacetin, and flavanones such as naringenin and eriodyctiol. The non-flavonoid polyphenols included hydroxycinnamic acids like chlorogenic acid, caffeic acid, coumaric acid, and ferulic acid. It is expected that such a phytonutrient profile would exert some influence on the viability of tested animal species.

4.1.4. The black mulberry-specific GTE phytonutrient profile

The black mulberry (BM-GTE) GTE was found to be particularly rich in its chemical composition, with a remarkable discovery of 111 distinct chemical constituents (Table 7.). This extensive array underscores the complex phytochemical profile of black mulberry, reflecting a diverse blend of natural compounds. Each constituent contributes to the extract's overall potential for health benefits, suggesting a wide range of applications in herbal medicine and nutrition. In BM-GTE, as illustrated in Figure 13, a diverse range of bioactive compounds was identified, covering multiple categories with each known for distinct health-enhancing attributes.

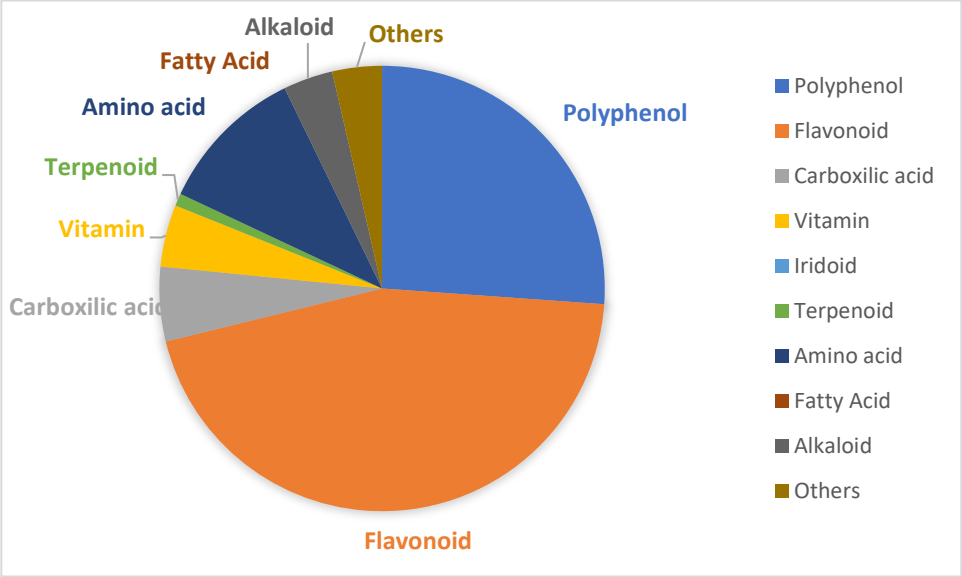

Figure 13. **Phytonutrient profile of BM-GTE**

The BM-GTE phytonutrient profile featured a mix of non-flavonoid and flavonoid polyphenols, followed by amino and carboxylic acids, vitamins, alkaloids, and other compounds. Among the 50 flavonoids detected were flavanols like quercetin, kaempferol, myricetin, narcissin, isorhamnetin, flavones like luteolin, apigenin, triacetin, flavanones like naringenin, eriodyctiol, and isoflavones (Figure 1, Supplementary Materials Table 4). The non-flavonoid polyphenols included hydroxybenzoic acids, hydroxycinnamic acids (chlorogenic acid, caffeic acid, coumaric acid, ferulic acid), and stilbenes (resveratrol).

Table 7. **The phytonutrient profiles of BM-GTE (black mulberry)**

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
1	Flavonoid	Quercetin-di-O-hexoside	17.18		625.1405
2		Quercetin-3-O-rutinoside-7-O-glucoside	17.57		771.1984
3		Quercetin-O-hexoside-O-(malonyl)hexoside	18.19		711.1409
4		Kaempferol-O-(rhamnosyl)hexoside-O-hexoside	19.07		755.2035
5		Taxifolin (Dihydroquercetin)	19.34		303.0505
6		Kaempferol-O-hexoside-O-(malonyl)hexoside	19.8		695.146
7		Quercetin-O-(dihexoside)	20.21		625.1405
8		Isorhamnetin-3-O-sophoroside	21.83		639.1561
9		Dihydrokaempferol (Aromadendrin, Katuranin)	21.94		287.0556
10		Kaempferol-3-O-rhamnoside-7-O-[rhamnosyl-(1→2)-glucoside]	22.1		739.2086
11		Quercetin-3-O-glucuronide	22.73		477.0669
12		Isoquercitrin (Hirsutrin, Quercetin-3-O-glucoside)	22.91		463.0877
13		Rutin (Quercetin-3-O-rutinoside)	23.02	611.1612	
14		Reinutrin (Reynoutrin, Quercetin-3-O-xyloside)	23.23		433.0771
15		Avicularin (Quercetin-3-O-arabinofuranoside, Fenicularin)	23.51		433.0771
16		Quercetin-O-(malonyl)hexoside	23.65		549.0881

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
17		Guaijaverin (rn-3-O-arabinoside)	24.24		433.0771
18		Astragalin (Kaempferol-3-O-glucoside)	24.69		447.0927
19		Kaempferol-3-O-rutinoside (Nicotiflorin)	24.84		593.1507
20		Isorhamnetin-3-O-glucoside	24.93	479.119	
21		Eriodictyol	24.95		287.0556
22		Isorhamnetin-O-hexoside-O-rhamnoside	25.17		623.1612
23		Tetrahydroxyflavanone isomer	25.59		287.0556
24		Kaempferol-O-(malonyl)hexoside	25.61		533.0931
25		Naringenin	27.22		271.0607
26		Luteolin (3',4',5,7-Tetrahydroxyflavone)	27.86		285.0399
27		Methoxy-tetrahydroxy(iso)flavone	28.24		315.0505
28		Norartocarpetin (2',4',5,7-Tetrahydroxyflavone)	28.41		285.0399
29		Kaempferol (3,4',5,7-Tetrahydroxyflavone)	29.34	287.0556	
30		Apigenin	29.68		269.045
31		Chrysoeriol	29.89		299.0556
32		Liquiritigenin (4',7-Dihydroxyflavanone)	29.98		255.0657
33		Sakuranetin (4',5-Dihydroxy-7-methoxyflavanone)	32.04	287.092	
34		Pinocembrin (5,7-Dihydroxyflavanone)	32.24		255.0657
35		Kaempferol-O-(cinnamoyl)hexoside	32.27		577.1346
36		Acacetin (Linarigenin, 5,7-Dihydroxy-4'-methoxyflavone)	33.9	285.0763	

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
37		Dihydroxy-dimethoxy(iso)flavone isomer 1	33.99	315.0869	
38		Dihydroxy-dimethoxy(iso)flavone isomer 2	34.85	315.0869	
39		Dihydroxy-trimethoxyflavone	34.92		343.0818
40		Mornigrol E or Mornigrol F	35.29	439.1757	
41		Kuwanon G (Albanin F, Moracenin B)	36.38		691.2179
42		Kuwanon C (Mulberrin) or Albanin E or Nigrasin H	37.64	423.1808	
43		Apigenin-7,4'-dimethyl ether	38.16	299.092	
44		Albafuran A or Albafuran B	38.33	379.1909	
45		Albafuran A or Albafuran B	38.56	379.1909	
46		Kuwanon E	40		423.1808
47		Kuwanon A or Kuwanon B	40.87	421.1651	
48		Chalcomoracin	40.96		647.2281
49		Kuwanon A or Kuwanon B	41.24	421.1651	
50		Cyclomorusin (Cyclomulberrochromene)	44.56		416.126
51	Polyphenol	Protocatechuic acid (3,4-Dihydroxybenzoic acid)	4.85		153.0188
52		Neochlorogenic acid (5-O-Caffeoylquinic acid)	8.91	355.1029	
53		2,4-Dihydroxybenzoic acid	12.23		153.0188
54		5-O-(4-Coumaroyl)quinic acid	12.59		337.0924
55		Chlorogenic acid (3-O-Caffeoylquinic acid)	14.23	355.1029	
56		1-O-Caffeoylglucose	14.35		341.0873

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
57		Caffeic acid	14.49		179.0344
58		3-O-Feruloylquinic acid	14.57		367.1029
59		trans-Melilotoside (trans-Glucosyl-2-hydroxycinnamate)	14.65		325.0924
60		Cudranin-di-O-glucoside isomer 1	15.31		585.182
61		Chryptochlorogenic acid (4-O-Caffeoylquinic acid)	15.59	355.1029	
62		Coumaroylquinic acid isomer	16.87		337.0924
63		1-O-Caffeoylquinic acid	16.98		353.0873
64		Cudranin-di-O-glucoside isomer 2	17.09		585.182
65		4-O-(4-Coumaroyl)quinic acid	17.54		337.0924
66		12-Hydroxyjasmonic acid-12-O-glucoside or Tuberonic	17.59		387.1655
67		Hydroxycoumarin	17.75		161.0239
68		Cudranin-O-glucoside isomer 1	17.84	407.1342	
69		5-O-Feruloylquinic acid	17.98		367.1029
70		Cudranin-O-glucoside isomer 2	18.33	407.1342	
71		4-O-Feruloylquinic acid	18.51		367.1029
72		Scopoletin	18.55	193.0501	
73		3-O-(4-Coumaroyl)quinic acid	19.17		337.0924
74		Cudranin (Oxyresveratrol, 2,3',4,5'-Tetrahydroxystilbene)	21.33	245.0814	
75		Dicaffeoylquinic acid isomer 1	22.33		515.119
76		Dicaffeoylquinic acid isomer 2	24.18		515.119

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
77		Isoliquiritigenin	24.8		255.0657
78		Ethyl caffeate	25.95	209.0814	
79		Mornigrol D	33.44	395.1859	
80	Carboxylic acid	Quinic acid (metabolite)	1.25		191.0556
81		Citric acid	1.41		191.0192
82		Malic acid	1.44		133.0137
83		12-Hydroxyjasmonic acid	18.04		225.1127
84		Tuberonic acid	18.27		225.1127
85		Jasmonic acid	27.76		209.1178
86	Amino acid	Lysine	1.08	147.1134	
87		Arginine	1.24	175.1195	
88		Citrulline	1.3	176.1035	
89		Proline	1.3	116.0712	
90		Threonine	1.31	120.0661	
91		Asparagine	1.32	133.0613	
92		Aspartic acid	1.34	134.0453	
93		Tyrosine	1.42	182.0817	
94		Isoleucine	1.54	132.1025	
95		Leucine	1.81	132.1025	
96		Phenylalanine	3.23	166.0868	

No.	Compound type	Phytonutrient	RT	M [+]	M [-]
97		Tryptophan	8.06	205.0977	
98	Alkaloid	O-Hexosyl-1-deoxynojirimycin	1.08	326.1451	
99		1,4-Dideoxy-1,4-iminoarabinitol	1.24	134.0817	
100		1-Deoxynojirimycin (Moranoline)	1.29	164.0923	
101		Chelidonine	20.05	354.1342	
102	Ester	Ethyl gallate	17.36		197.045
103		Ethyl 4-hydroxycinnamate	28.68		191.0708
104	Terpenoid	Abscisic acid	25.39		263.1283
105	Vitamin	Adenine (B4)	1.32	136.0623	
106		Nicotinic acid (Niacin,B3)	1.41	124.0399	
107		Nicotinamide	1.42	123.0558	
108		Pantothenic acid (B5)	5.41	220.1185	
109		Riboflavin (B2)	18.6	377.1461	
110	Other	Eugenol (4-Allyl-2-methoxyphenol)	23.83	165.0916	
111		Lumichrome	23.9	243.0882	

4.1.5. The quantitative features of selected flavonoids among the studied GTEs

Figure 14 illustrates the results of a thorough examination of the phytonutrient content of the studied GTEs, which revealed a wide range of polyphenolic components. Due to this variability, a particular set of well-known antioxidant and anti-inflammatory phytonutrients was chosen for a more thorough quantitative study, as shown in Tables S2 and S3.

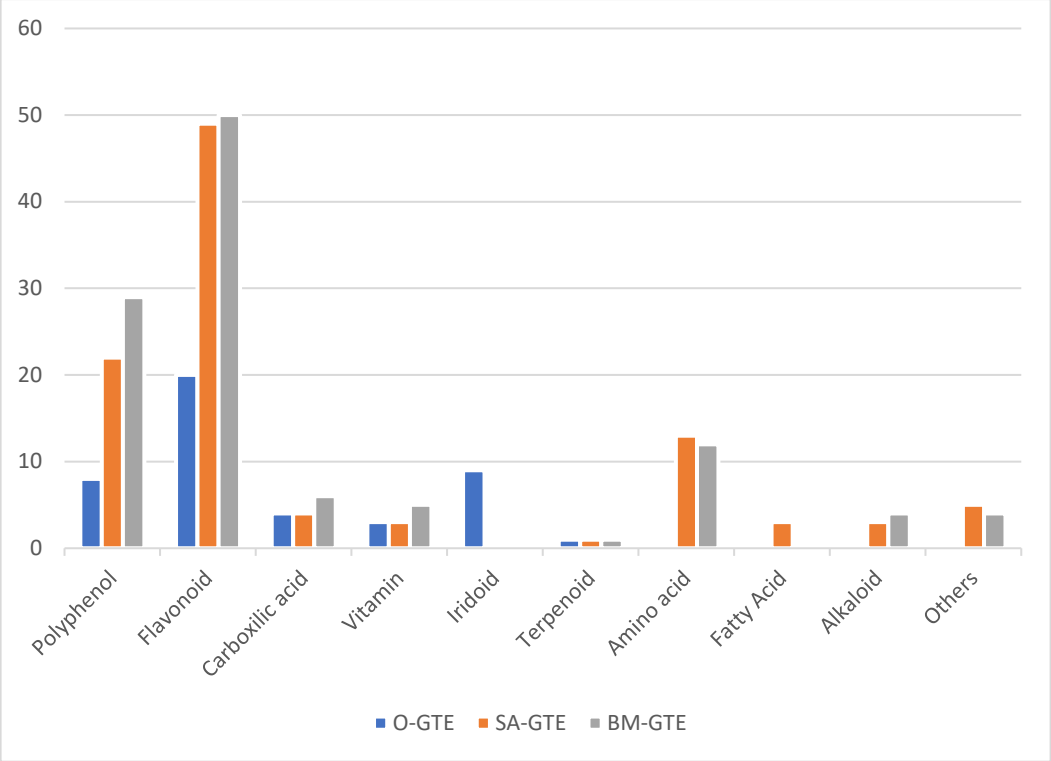

Figure 14. **A comparison of Phytonutrient profiles of GTEs**

For this quantitative evaluation in the GTEs, a variety of polyphenols, including flavonoids and non-flavonoids, were selected. More specifically, caffeic and chlorogenic acids—two non-flavonoid compounds—were chosen. These acids have been researched for possible health advantages, including anti-inflammatory effects, and are well-known for their strong antioxidant qualities.

Ten further flavonoid compounds were found and identified for quantification in addition to these. The flavonoids apigenin, chrysin, hyperoside, kaempferol, luteolin, luteolin-7-O-glucoside, naringenin, quercetin, rutoside (or rutin), and vitexin are known for their various

biological activities. These activities include potential roles in neuroprotection, cardiovascular health, and cancer prevention in addition to their antioxidant and anti-inflammatory properties.

The broad spectrum of polyphenolic chemicals found in GTEs indicates their potential as abundant natural sources of anti-inflammatory and antioxidant substances. Particularly intriguing are the flavonoids' numerous positive impacts for human health. For example, quercetin's antioxidant potential and its ability to alter the body's inflammatory pathways are the subjects of much research.

Additionally, Table 8 provides a thorough quantitative study of these particular polyphenols in the O-GTE. This research is significant as it provides information on the concentrations of these bioactive compounds in the extract, assisting in understanding the O-GTE's possible therapeutic benefits and efficacy.

Table 8. **The quantitative polyphenol profile of GTEs**

Name of Selected Standard and Separated Compound	Standards		O-GTE			SA-GTE			BM-GTE		
	Retention Time (min)	Main MS Transition	Retention Time (min)	Main MS Transition	Content (mg/mL)	Retention Time (min)	Main MS Transition	Content (mg/mL)	Retention Time (min)	Main MS Transition	Content (mg/mL)
Caffeic acid	13.8	179.0 > 135.0				13.5	179.0 > 135.0	0.825 ± 0.0211			
Chlorogenic acid	11.9	353.0 > 191.0	11.9	353.0 > 191.0	0.265 ± 0.0052	11.9	353.0 > 191.0	1.390 ± 0.0417	12	353.0 > 191.0	3.539 ± 0.0125
Apigenin	28.1	269.0 > 117.0	28.1	269.0 > 117.0	0.055 ± 0.0041	28.2	269.0 > 117.0	0.017 ± 0.0005	28.2	269.0 > 117.0	0.103 ± 0.0051
Chrysin	29.7	253.0 > 143.0	29.7	253.0 > 143.0	0.109 ± 0.0054	29.7	253.0 > 143.0	0.103 ± 0.0051	29.7	253.0 > 143.0	0.093 ± 0.0004
Hyperoside	20.3	463.1 > 300.0	20.4	463.1 > 300.0	0.202 ± 0.0115	20.2	463.1 > 300.0	1.967 ± 0.0621	20.2	463.1 > 300.0	0.162 ± 0.0415
Kaempferol	27.9	285.0 > 187.0				27.9	285.0 > 187.0	0.032 ± 0.0011			
Luteolin	26.8	287.0 > 153.0	26.7	287.0 > 153.0	0.049 ± 0.0026				26.8	287.0 > 153.0	0.017 ± 0.0009
Luteolin-7-*O*-glucoside	19.9	447.0 > 284.9	19.7	447.0 > 284.9	1.777 ± 0.0217				19.8	447.0 > 284.9	0.072 ± 0.0042
Naringenin	26.2	271.0 > 119.0	26.2	271.0 > 119.0	0.032 ± 0.0017	26.2	271.0 > 119.0	0.011 ± 0.0007	26.3	271.0 > 119.0	0.046 ± 0.0025
Quercetin	25.4	300.9 > 151.0	25.4	300.9 > 151.0	0.052 ± 0.0029	25.4	300.9 > 151.0	0.201 ± 0.0092			
Rutoside	20.2	609.0 > 300.0	20.2	609.0 > 300.0	0.416 ± 0.0231	20.2	609.0 > 300.0	5.506 ± 0.1174	20.2	609.0 > 300.0	1.367 ± 0.0583
Vitexin	18.4	431.0 > 311.0	18.4	431.0 > 311.0	0.034 ± 0.0015						

The results are presented as mean ± SD.

With all factors considered, this study clarifies the complex phytochemical composition of the studied GTEs and emphasizes the significance of these bioactive substances in enhancing the potential health benefits of the extracts. The conclusions of this analysis may direct future studies and applications in the fields of herbal medicine, nutraceuticals, and dietary supplements, where there is a rising need for naturally occurring substances that promote health.

The analysis of the polyphenol content in various GTEs revealed distinct profiles, with flavonoids like luteolin-7-O-glucoside being particularly prominent in some extracts, notably outweighing other polyphenols. This was followed, in a descending order of concentration, by rutoside, hyperoside, chrysin, quercetin, apigenin, luteolin, vitexin, and naringenin. In terms of non-flavonoid content, chlorogenic acid was present, while caffeic acid was notably absent from the O-GTE, as confirmed through UPLC–ESI-MS chromatogram analysis.

The detailed quantitative analysis of selected polyphenols in the SA-GTE, shown in Table 8, indicated a dominance of flavonoids like rutoside and hyperoside, comprising about 90% of the assessed polyphenols. Non-flavonoids such as chlorogenic and caffeic acid were also detected but in smaller quantities. Other flavonoids like chrysin, kaempherol, naringenin, and apigenin were present at lower levels. Notably, flavonoids such as luteolin, luteolin-7-O-glucosid, and apigenin were not detected in the SA-GTE in both types of analyses conducted using HPLC–MS determinations.

For the BM-GTE, the quantitative analysis, presented in Table 8 and detailed in the supplementary materials, suggested that chlorogenic acid and rutoside might be the most abundant polyphenols, while others such as hyperoside, apigenin, and chrysin were present in lesser quantities. Certain polyphenols like luteolin-7-O-glucoside, naringenin, and luteolin were found in minimal amounts.

In summary, each GTE displayed a unique polyphenol profile, although some similarities were noted across the different extracts. Additionally, the selected polyphenols in these GTEs, including olive, sweet almond, and black mulberry, are expected to promote some kind of antidiabetic and anti-inflammatory properties. This shared feature points to the potential therapeutic applications of these extracts in managing diabetes and/or inflammation.

4.1.6. The total polyphenol and flavonoid content of the analyzed GTEs

The key players in the area of phytonutrients, polyphenols and flavonoids, are widely recognized for their numerous health benefits. This study examined the Total Polyphenol Content (TPC) and Total Flavonoid Content (TFC) of different Gemmotherapy Extracts (GTEs) in recognition of their significance. The analysis's findings revealed that, among the GTEs investigated, the SA-GTE stood out for exhibiting a significantly elevated polyphenol content as shown in Table 9. In fact, compared to other extracts like BM-GTE and O-GTE, it has been shown to have noticeably greater levels of polyphenols. Based on the TPC analysis, SA-GTE had a polyphenol content that was around twice as high as that of BM-GTE and O-GTE, respectively.

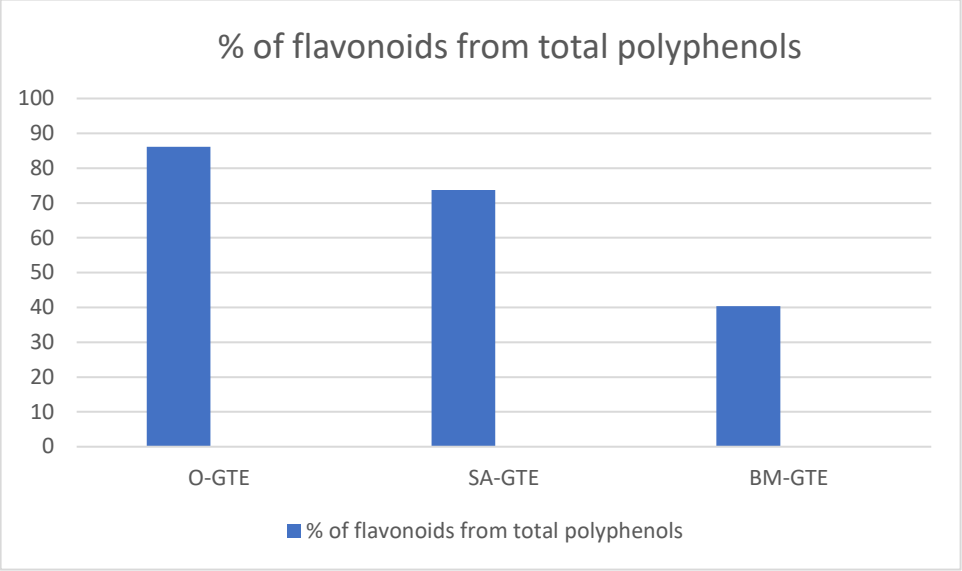

Figure 14**. The TPC and TFC of the assessed GTEs.**

This discovery is important given that it shows that SA-GTE has the potential to be an effective source of phytonutrients that improve health. Due to their potent antioxidant qualities, polyphenols are essential in the fight against oxidative stress, which is connected to a number of chronic illnesses. The elevated concentrations of polyphenols in SA-GTE indicate its potential efficacy in fostering well-being and mitigating ailments. Moreover, this elevated concentration of polyphenols in SA-GTE may enhance its therapeutic efficacy. Polyphenols have been studied for their potential benefits in reducing inflammation, protecting against heart disease, and even exhibiting anti-cancer properties. The substantial

presence of these compounds in SA-GTE thus opens avenues for its use in nutritional and medicinal applications, possibly offering more significant health benefits than other extracts with lower polyphenol content. The discovery of such a high polyphenol content in SA-GTE underscores the importance of plant-based compounds in health and wellness. It also encourages further exploration into the specific health benefits of this extract and its potential applications in dietary supplements, herbal remedies, and natural therapies.

Similarly, when evaluating the Total Flavonoid Content (TFC), measured in (mg RE/mL), the SA-GTE showcased a significantly higher flavonoid concentration compared to the other extracts. Specifically, the flavonoid content in SA-GTE was found to be 2.5 times greater than that of O-GTE and 3.6 times more than BM-GTE. Furthermore, when examining the proportion of flavonoids relative to the total polyphenols in each extract, O-GTE and SA-GTE exhibited impressive percentages of 86% and 73.7%, respectively. This high flavonoid content underscores the potential of both O-GTE and SA-GTE as exceptional sources of these beneficial compounds. On the other hand, flavonoids in BM-GTE account for approximately 40% of its polyphenolic content, suggesting a significant presence of non-flavonoid polyphenols in this extract.

The comparative analysis of the TPC and TFC data reveals no similarities between O-GTE and SA-GTE, with both showing a high concentration of flavonoids. In contrast, BM-GTE presents a more diversified polyphenolic profile, with a balanced mix of flavonoid and non-flavonoid compounds. Overall, these findings highlight the substantial value of the studied GTEs as sources of flavonoids and polyphenols, contributing to their potential health-promoting effects.

Upon analyzing the phytonutrient profile of the GTEs, it was evident that they possess a diverse array of polyphenolic compounds, as illustrated in figure 1. Given the known antioxidant and anti-inflammatory properties of these compounds, we focused on quantifying a select group of well-recognized polyphenols, as detailed in tables 1 and 2. This selection included two non-flavonoid polyphenols, namely caffeic and chlorogenic acids, and an array of ten flavonoids: apigenin, chrysin, hyperoside, kaempferol, luteolin, luteolin-7-O-glucoside, naringenin, quercetin, rutoside (or rutin), and vitexin. These specific polyphenols were chosen for their well-documented health benefits, particularly in combating oxidative

stress and inflammation, which are key factors in various chronic diseases. The quantitative assessment of these polyphenols in the GTEs not only provides insight into their potential therapeutic value but also helps in understanding the specific contributions of each compound to the overall efficacy of the extracts.

The detailed quantitative analysis of these selected polyphenols within the Olive GTE (O-GTE) is presented in table 8. This comprehensive approach enables a thorough evaluation of the polyphenolic content in O-GTE, facilitating a better understanding of its potential as a natural therapeutic agent. The analysis of the polyphenolic content (Table 9) in the O-GTE underscores a distinct dominance of certain flavonoids, particularly luteolin-7-O-glucoside, which significantly surpasses the concentration of other polyphenols. This is followed by other flavonoids in descending order of concentration, including rutoside, hyperoside, chrysin, quercetin, apigenin, luteolin, vitexin, and naringenin. Among the non-flavonoids, chlorogenic acid is present, while the absence of caffeic acid in O-GTE was confirmed through standard authentication methods and UPLC–ESI-MS chromatogram analysis.

For the SSA-GTE, detailed quantitative analysis, as presented in table 5, highlights a different polyphenolic profile. In the case of SA-GTE, flavonoids like rutoside and hyperoside are the most prevalent, accounting for approximately 90% of the flavonoid content. While non-flavonoids such as chlorogenic and caffeic acid are also present, they are found in smaller quantities. Other flavonoids like chrysin, kaempferol, naringenin, and apigenin are detected in lower concentrations. Notably, flavonoids like luteolin, luteolin-7-O-glucosid, and apigenin were not detectable in the SA-GTE, as confirmed by HPLC–MS determinations.

The Black Mulberry GTE (BM-GTE) exhibits a polyphenolic composition where chlorogenic acid and rutoside appear to be the most abundant. This is shown in the quantitative analysis presented in Table 5. Other polyphenols, such as hyperoside, apigenin, and chrysin, are present but to a lesser extent. Polyphenols like luteolin-7-O-glucoside, naringenin, and luteolin are found in trace amounts in the BM-GTE.

Overall, the analysis of the polyphenols reveals distinct profiles unique to each GTE, although certain similarities are also evident across the different extracts. Furthermore, a common characteristic of the evaluated olive, sweet almond, and black mulberry GTEs would be their presumptive antidiabetic and anti-inflammatory properties

Table 9. **The quantitative polyphenol profile of GTEs**

Name of Selected Standard and Separated Compound	Standards		O-GTE			SA-GTE			BM-GTE		
	Retention Time (min)	Main MS Transition	Retention Time (min)	Main MS Transition	Content (mg/mL)	Retention Time (min)	Main MS Transition	Content (mg/mL)	Retention Time (min)	Main MS Transition	Content (mg/mL)
Caffeic acid	13.8	179.0 > 135.0				13.5	179.0 > 135.0	0.825 ± 0.0211			
Chlorogenic acid	11.9	353.0 > 191.0	11.9	353.0 > 191.0	0.265 ± 0.0052	11.9	353.0 > 191.0	1.390 ± 0.0417	12.0	353.0 > 191.0	3.539 ± 0.0125
Apigenin	28.1	269.0 > 117.0	28.1	269.0 > 117.0	0.055 ± 0.0041	28.2	269.0 > 117.0	0.017 ± 0.0005	28.2	269.0 > 117.0	0.103 ± 0.0051
Chrysin	29.7	253.0 > 143.0	29.7	253.0 > 143.0	0.109 ± 0.0054	29.7	253.0 > 143.0	0.103 ± 0.0051	29.7	253.0 > 143.0	0.093 ± 0.0004
Hyperoside	20.3	463.1 > 300.0	20.4	463.1 > 300.0	0.202 ± 0.0115	20.2	463.1 > 300.0	1.967 ± 0.0621	20.2	463.1 > 300.0	0.162 ± 0.0415
Kaempferol	27.9	285.0 > 187.0				27.9	285.0 > 187.0	0.032 ± 0.0011			
Luteolin	26.8	287.0 > 153.0	26.7	287.0 > 153.0	0.049 ± 0.0026				26.8	287.0 > 153.0	0.017 ± 0.0009

Name of Selected Standard and Separated Compound	Standards		O-GTE			SA-GTE			BM-GTE		
	Retention Time (min)	Main MS Transition	Retention Time (min)	Main MS Transition	Content (mg/mL)	Retention Time (min)	Main MS Transition	Content (mg/mL)	Retention Time (min)	Main MS Transition	Content (mg/mL)
Luteolin-7-*O*-glucoside	19.9	447.0 > 284.9	19.7	447.0 > 284.9	1.777 ± 0.0217				19.8	447.0 > 284.9	0.072 ± 0.0042
Naringenin	26.2	271.0 > 119.0	26.2	271.0 > 119.0	0.032 ± 0.0017	26.2	271.0 > 119.0	0.011 ± 0.0007	26.3	271.0 > 119.0	0.046 ± 0.0025
Quercetin	25.4	300.9 > 151.0	25.4	300.9 > 151.0	0.052 ± 0.0029	25.4	300.9 > 151.0	0.201 ± 0.0092			
Rutoside	20.2	609.0 > 300.0	20.2	609.0 > 300.0	0.416 ± 0.0231	20.2	609.0 > 300.0	5.506 ± 0.1174	20.2	609.0 > 300.0	1.367 ± 0.0583
Vitexin	18.4	431.0 > 311.0	18.4	431.0 > 311.0	0.034 ± 0.0015						

The results are presented as mean ± SD.

4.1.7. The antioxidant capacity of the analyzed GTEs

The antioxidant properties of plant-derived polyphenols are well recognized for their role in safeguarding against oxidative stress-induced inflammation and related diseases (Arulselvan et al., 2016). Consequently, evaluating the antioxidant capacity of specific GTEs could illuminate their capabilities in terms of scavenging free radicals or inhibiting their formation, thereby contributing to their health benefits (Maleki et al., 2019). To determine this antioxidant potential, several established methods are available (Pisoschi et al., 2011). In our study, we employed the DPPH, FRAP, and xanthine oxidase inhibition assays as detailed in the Materials and Methods section, with the results presented in figure 15.

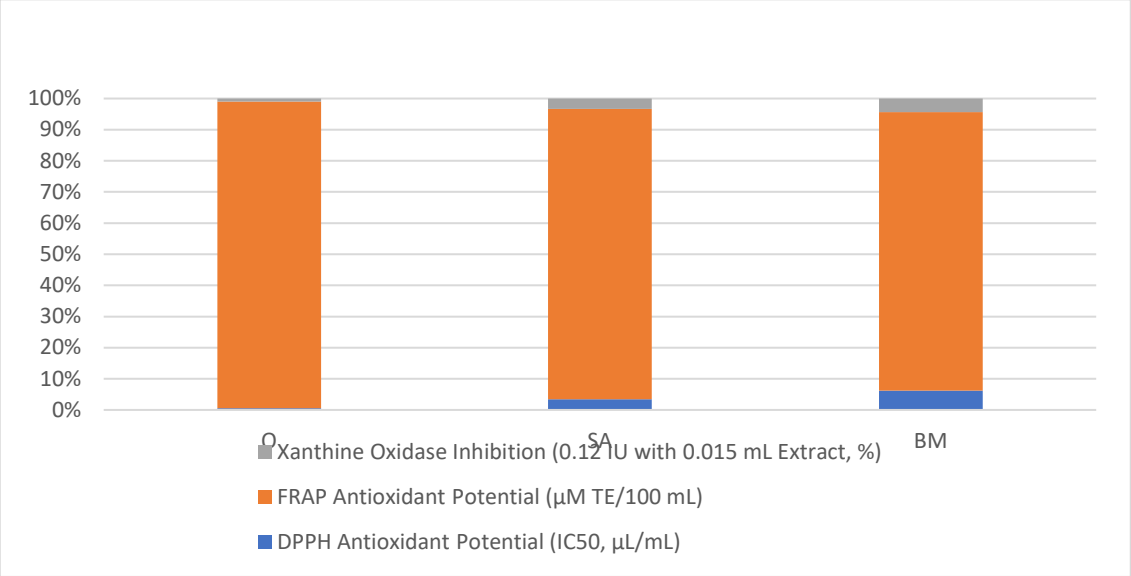

Figure 15. **Evaluation of GTE-specific antioxidant potential.**

The DPPH assay measures the ability of an antioxidant to act as a hydrogen radical scavenger. The degree of reduction in DPPH indicates the antioxidant capacity of the GTE. The half-maximal inhibitory concentration (IC50) is particularly telling, as it indicates the amount of GTE required to reduce the DPPH activity by 50%. In this context, the O-GTE demonstrated approximately twice the efficiency as an antioxidant compared to SA-GTE. When compared with the BM-GTE, the difference in antioxidant efficiency of O-GTE is roughly threefold. This finding suggests that O-GTE may possess a stronger ability to neutralize free radicals compared to the other examined GTEs. Furthermore, in the context of the DPPH assay, BM-GTE emerged as the least effective antioxidant when compared to both SA-GTE and O-GTE. These observations are critical

in understanding the varying capacities of different GTEs to combat oxidative stress. By elucidating the specific antioxidant strengths of each extract, we can better appreciate their potential applications in promoting health and preventing oxidative stress-related conditions. This comparative analysis also highlights the necessity of tailoring antioxidant therapies and supplements based on the unique properties of each GTE, maximizing their potential benefits in health and wellness regimens.

The FRAP assay measures an extract's ability to convert ferric ions into ferrous ions, demonstrating the reducing power of antioxidants in the GTEs. In this assay, the O-GTE showed a remarkably higher antioxidant capacity compared to both the SA-GTE and BM-GTE. Interestingly, the antioxidant potentials of SA-GTE and BM-GTE appeared to be quite similar in this context.

The xanthine oxidase enzyme plays a crucial role in converting hypoxanthine to xanthine and subsequently to uric acid, a process that generates free radicals and can lead to hyperuricemia, a medical condition linked with various diseases (Chen et al., 2016). Our findings suggest that all three GTEs exhibit a reduced inhibition of xanthine oxidase activity. This implies a lesser antioxidant effect in terms of managing uric acid levels or preventing diseases associated with hyperuricemia.

These results are significant in understanding the specific antioxidant actions of different GTEs. While the O-GTE demonstrates superior reducing power in the FRAP assay, indicating a strong potential in combating oxidative stress, all three GTEs show limited effectiveness in inhibiting xanthine oxidase. This insight is crucial for considering the use of GTEs in therapeutic interventions targeting conditions related to oxidative stress and hyperuricemia. By comprehensively understanding the antioxidant capabilities of each GTE, more effective and targeted health strategies can be developed.

4.2.1. The antimicrobial potential of the analyzed GTEs

The antimicrobial potential of any GTE must be analyzed to gain some information about their potential interference with different microbiomes. One can imagine a scenario where a GTE would exert an anti-inflammatory effect that could be or not associated with antibacterial properties. Therefore, the antimicrobial effects could limit the applicability of any GTE especially when their overall positive

physiological outcome is analyzed. In an ideal situation the GTE would exert strong anti-inflammatory properties without significantly affecting the microbiomes. In our antimicrobial study we have focused on bacterial species that are implicated in foodborne bacterial-fungi infestations.

4.2.2. The agar-diffusion method revealed antimicrobial activity of GTEs

When this method was applied to the GTEs investigated, the results showed varying levels of antimicrobial activity. The O-GTE demonstrated effectiveness against 5 microbial strains, whereas SA-GTE was effective for 4 strains, and the black BM-GTE showed efficacy against only 2 microbial species, as detailed in table 10. It is noteworthy that the O-GTE was particularly effective against Gram-negative bacteria, though the extent of its effectiveness varied across a concentration range of 50-100%.

Table 10. **The GTEs specific minimal antimicrobial concentration (%) revealed by agar diffusion method**

Studied microorganisms	***Olea europaea***	***Prunus amygdalus***	***Morus nigra***
Gram-negative bacteria			
B. cereus	50	70	70
S. aureus	50	nd	nd
E. faecalis	100	30	nd
L. monocytogenes	100	nd	nd
Gram-negative bacteria			
P. vulgaris	50	40	80
P. aeruginosa	nd	nd	nd
E. coli	nd	nd	nd
S. enterica	nd	nd	nd

Studied microorganisms	***Olea europaea***	***Prunus amygdalus***	***Morus nigra***
Yeast			
S. cerevisiae	nd	60	nd
Molds			
A. niger	nd	nd	nd
A. flavus	nd	nd	nd
A. ochraceus	nd	nd	nd
P. citrinum	nd	nd	nd
P. expansum	nd	nd	nd

nd – non-detectable

The O-GTE exhibited the most potent antimicrobial effect among the tested extracts. Specifically, the O-GTE successfully inhibited the growth of 5 out of the 14 examined strains. Notably, a concentration of 50% O-GTE demonstrated effectiveness *against Bacillus cereus (B. cereus), S. aureus (Staphylococcus aureus),* and *P. vulgaris (Proteus vulgaris)*. The concentrated GTE at 100% concentration displayed inhibition against *Enterococcus faecalis (E. faecalis*) and *Listeria monocytogenes* (*L. monocytogenes*). However, it showed no effectiveness against the remaining Gram-negative strains, with no growth inhibition observed even with concentrated solutions. Additionally, the olive GTE did not impact the growth of budding yeast (*Saccharomyces cerevisiae*).

Following closely the O-GTE in effectiveness is the SA-GTE demonstrating efficacy against 4 tested microorganism species. The sweet almond extract exhibited inhibition for *B. cereus, E. faecalis, P. vulgaris*, and even *S. cerevisiae*. Notably, the SA-GTE displayed effectiveness against *E. faecalis* at a dilution of 30%, against *P. vulgaris* at 40%, and against *B. cereus* at 70%. The minimum effective concentration for inhibiting *S. cerevisiae* growth was determined to be 60%.

Lastly, it is crucial to note that in our research employing the agar diffusion method, none of the investigated GTEs demonstrated inhibition of the growth of *Escherichia coli (E.*

coli), Salmonella enterica subsp. enterica (S. enterica subsp. enterica), Pseudomonas aeruginosa (S. aeruginosa), and *P. vulgaris*. When considering the collective results of olive, sweet almond, and black mulberry GTEs, there were no observed antimicrobial effects on the tested mold species, as evidenced by the agar diffusion assay.

4.2.3. The assessment of minimal inhibitory concentration (MIC)of the studied GTEs

To enhance the understanding of the antimicrobial capabilities of the GTEs it has been determined the minimum inhibitory concentration (MIC), (see Materiel and Methods). The MIC represents the lowest concentration of a specific GTE that induces a reduction in the cell number of the tested microbe by inhibiting bacterial growth. The outcomes of the MIC assay are detailed in figure 16, presenting the examined GTEs along with their associated MIC values in alignment with the tested microorganisms.

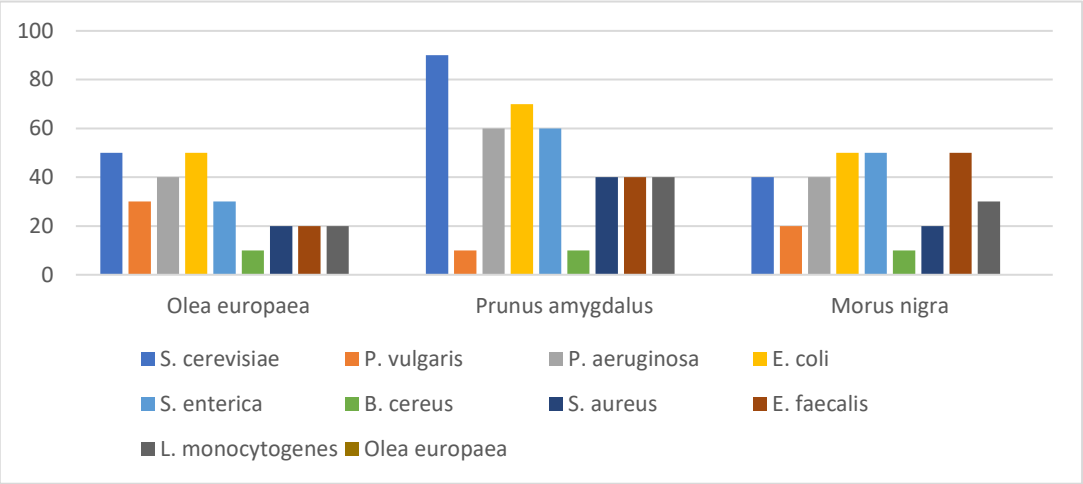

Figure 16. The MIC percentage corresponding to the GTE

In the MIC assay, each GTE exhibited inhibitory effects on all tested microorganisms, each at distinct concentrations.

For the O-GTE, even a 10% extract concentration demonstrated inhibitory effects on the *B. cereus* microbe, while *S. aureus* and *E. faecalis* exhibited MIC values of 20%. *E. coli* and *S. cerevisiae* were the most resistant, requiring O-GTE concentrations above 50% to inhibit reproduction.

In the case of the SA-GTE, the *B. cereus* and *P. vulgaris* were notably sensitive to the 10% GTE, *while S. cerevisiae* proved the most resistant with a MIC value of 90%. Gram-positive bacteria were inhibited at medium concentrations (40%), whereas Gram-negative

bacteria exhibited a wider range of growth inhibition across concentrations from 10% to 70%.

The BM- GTE, at a 10% concentration, already inhibited *B. cereus*, while the 20% concentration was effective against *S. aureus* and *P. vulgaris*. The highest concentration required for Gram-positive bacteria was 50% for *E. faecalis*, and for Gram-negative bacteria, it was also 50% for *E. coli* and *S. enterica*. *S. cerevisiae* was more sensitive, with a 40% concentration sufficient to inhibit growth.

To summarize, the assessed GTEs displayed antibacterial effectiveness against numerous types of bacteria known to cause major human infections, including *S. aureus, L. monocytogenes, E. coli,* and *S. enterica*, as proven by the observed MIC values.

4.2.4. The assessment of minimum bactericidal concentration (MBC) of the studied GTEs

The Minimum Bactericidal Concentration (MBC) test reveals the minimum concentration of an antimicrobial agent, in this case of a given GTE concentration that leads to microbial death. The outcomes of the MBC test (see Materiel and Methods) can be found in Table 14.

The examined GTEs exhibited varying MBC values depending on their concentrations and the specific microorganism species under evaluation.

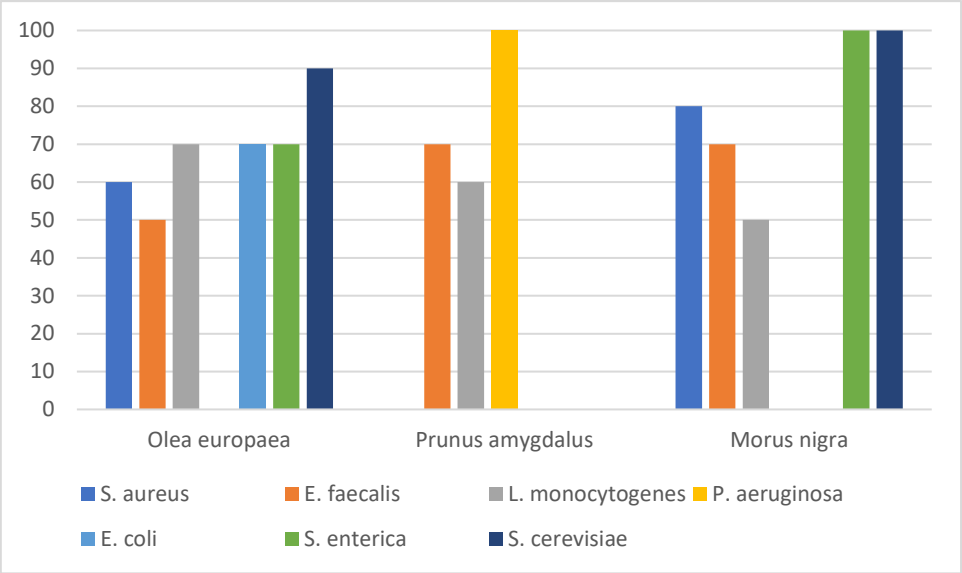

Figure 17. The MBC percentage corresponding to GTEs

The O-GTE showed effectiveness against six strains of both bacteria and yeast. The BM-GTE showed a similar effect to the O-GTE except for *E. coli*, against which it did not have any effect. For the O-GTE, the lowest MBC concentration was approximately 50% for *E. faecalis*, followed by 60% for *S. aureus*, and 70% for *L. monocytogenes*.

It is worth mentioning that the only GTE that showed bactericidal effect against *P. aeruginosa,* was the SA-GTE but did not have any constricted effect on the yeast tested.

4.2.5. Revealing the antimicrobial efficacy of some GTE mixtures

Having seen the antimicrobial properties of individual olive, sweet almond, and black mulberry GTEs it was decided to analyze if their mixtures could elicit similar effects specific to individual extracts. Also, we were curious if some synergistic, additive, or inhibitory type of antimicrobial interactions might emerge that could affect the integrity of microbiota, and therefore would limit the putative therapeutic application(s) of the studied GTE combinations. We can imagine a scenario when two GTEs might possess antidiabetic properties but when combined they would negatively affect the microbiota of the studied organism. Such an outcome would be an important constricting factor in knowing the implications of microbiota concerning diabetes. Therefore, various combinations of GTEs were tested, and their antimicrobial activity was assessed through the agar diffusion method, with the measurement of inhibition zone diameters. The most relevant outcomes of these tests with 1:1 GTE mixtures are detailed in Table 11.

Table 11. **Synergistic antimicrobial interaction of GTEs**

Extract mixture	***B. cereus***	***S. aureus***
O+SM/GTEs	11.13±0.36+	16.91±1.02++
BM+SM/GTEs	8.67±0.1++	nt
O+BM/GTEs	-	nt

Note: (nt) - not tested; (-) no inhibition zone; O – olive GTE; SA – sweet almond GTE; BM – black mulberry GTE; (++) synergistic antimicrobial effect; (+) basic antimicrobial effect.

The results revealed three distinct outcomes for the combined GTEs and their antimicrobial efficacy, determined by the presence and/or diameter of the inhibition zones formed against the tested bacterial strains. It can be noted that most mixed GTEs showed inferior performance compared to their individual counterparts, leading to reduced inhibition zone diameters.

For *B. cereus* the 1:1 blend of olive and almond Gemmotherapy Tree Extracts (GTEs) displayed an inhibition zone measuring 11.13±0.36 mm. This is comparable to the 9.68±0.6 mm observed with 50% olive GTE, but notably higher than the absence of any antimicrobial effect from 50% almond GTE. This suggests that almond GTE did not exert any inhibitory effect on olive GTE.

In the case of *S. aureus*, the mixture of olive and almond Gemmotherapy Extracts (GTEs) resulted in a diameter of 16.91±1.02 mm, significantly surpassing the 9.24±0.15 mm diameter produced by 50% olive GTE, with no inhibition zone observed for 50% almond GTE. Considering the significance of the combined effect of the olive-almond GTE mixture, it could be perceived as another synergistic interaction between the two GTEs. A similar synergistic interaction was identified in the GTE mixture of black mulberry and almond.

4.2.6. The Drosophila melanogaster-based assessment of nutritional potential associated with GTEs

From the very beginning of the project, it has been predicted that the GTEs might feature some nutritive features and to answer this question the macronutrient content of them has been analyzed using standardized and internationally validated methodology (see Material and Methods). The obtained results are listed in the Table 12.

Table 12. **Macronutrient content of GTEs**

NUTRIENTS (w/w%)	Analysed GTEs		
	O	SA	BM
Total protein	1.11	0.86	1.05
Total carbohydrate[1]	0.72	0.61	0.18
Total carbohydrate[2]	0.57	0.39	0.10

The results are the mean of three experimental replicates. Total carbohydrate was determined by the ([1]) phenol–sulfuric acid method and ([2]) by the Luff–Schoorl method.

The obtained data are indicating that all three GTEs are containing proteins, though the O and BM GTEs would feature a grater protein content as compared to the SA. Interestingly the O-GTE also excels with regards to carbohydrate content and is followed by the SA-GTE, while the BM-GTE showed the lowest carbohydrate content among all the analyzed extracts.

These experiments are suggesting that the analyzed GTEs would possess some kind of nutritive properties that are due to macronutrients, and the identified phytonutrients would represent micronutrients that could initiate certain physiological effect that might have health-promoting effects. Therefore, to evaluate the physiological effects associated with the GTEs, it was applied a methodology that also pays attention to the overall nutrient intake. In this respect the studied GTEs were assessed over threes dietary conditions and their viability has been determined (for details see Material and Methods).

4.3.1. The zero-diet specific nutritional values of GTEs

Drosophila melanogaster renowned for its versatility as a model system, is particularly adept at elucidating the genomic and nutritional dependencies of various life-related parameters like hematopoiesis (Banerjee et al., 2019), obesity and diabetes (Chatterjee and Perrimon, 2021), neurodegenerative diseases (Nitta and Sugie, 2022; Bolus et al.,

2020) and immunomodulation (Pratomo et al., 2022). However, the overall nutritional dependence of these phenomena has been only partially addressed, though the eggs production and fertility being an energetically expensive process that would require a massive nutrient load, and therefore, the reproduction only happens when there is a surplus of nutrients. Quite interestingly, it was shown that the *Drosophila Makorin RING finger protein 1 gene (Mkrn1)* functions in the metabolic regulator of oogenesis suggesting that many life related phenomena are regulated based on genetic and nutritional cues (Jeong et al., 2019). Furthermore, again in *Drosophila melanogaster*, the Insulin receptor/dTor signaling in macrophages is controlling the production of the PDGF/VEGF family growth factor Pvf2 that induces the transcription of the sterol biosynthesis *Halloween* genes in the prothoracic gland via its receptor Pvr (PDGF- and VEGF-receptor related). In response to a starvation, the low Pvf2 signal delays steroid biosynthesis until it becomes Pvr-independent, thereby prolonging larval growth before pupariation (Juarez-Carreño and Geissmann, 2023). In this respect the *Inr/dTor/Pvf2* genes behave as a nutritional status checkpoint in the case of macrophages that controls the developmental timing of larval – pupal transition, and ultimately regulates the size of the pupae and adult flies. The research mentioned above clearly demonstrates that the nutritional status does influence the genetic control of some vital phenomena, and together with the macro/micronutrient content of GTEs would plead for studying the nutritional relevance of GTEs.

To question the nutritional effects of the GTEs, it was applied a special dietary condition called the zero-nutrient (0N) media that would contain at different concentrations only the GTE supplied macro- and micronutrients. On such a culture media then staged 0-2 hr old embryos were loaded and let to develop into 1st instar larvae that would be of the same age. Therefore, such an experimental setup would allow to examine the development of individuals that were of the same age and same genotype by monitoring survival and development of fruit fly larvae and pupae. Consequently, observing the transition of individuals to the 3rd instar larval stage (measuring larval survival rate) and to the pupal stage (assessing adult hatching rate), including the duration of these developmental stages, serves as a valuable measure for the nutritional efficacy of the diets tested.

In experiments involving the 0N diet supplemented with specific GTEs, the essential macro- and micronutrients were sourced from the plant materials used to produce the respective GTEs. We also varied the concentrations of individual GTEs in the 0N diet to

determine their potential. Consequently, observing the transition of individuals to the 3rd instar larval stage (measuring larval survival rate) and to the pupal stage (assessing adult hatching rate), including the duration of these developmental stages, serves as a valuable measure for the nutritional efficacy of the diets tested nutritive effects over a range of concentrations. The results of these experiments are depicted on figure 18 A and B.

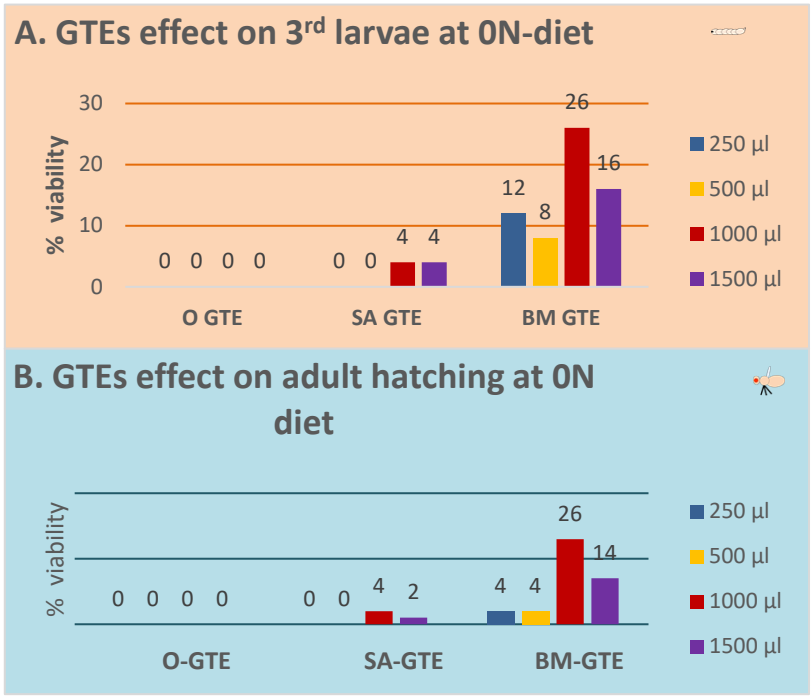

Figure 18. **Evaluation of fruit fly viability in the 0N diet. Viability assessment of the larvae (A) and pupae (B) at the applied concentrations of GTEs (source: Aleya et al., 2023)**

It was observed that the O-GTE did not facilitate the development of larvae or pupae within the 0N dietary setup. In contrast, both the SA-GTE and BM-GTE showed varying degrees of effectiveness in supporting larval and adult viability. Specifically, SA-GTE was found to promote viability at relatively lower rates of approximately 2–4%, whereas BM-GTE significantly enhanced the survival of larvae and adults by up to 26% within the range of concentrations applied.

These observations underscore the differential impacts of various GTEs on the development and survival of *Drosophila melanogaster*, providing insights into their potential nutritional benefits and limitations. The mentioned study highlights the importance of understanding the specific nutritional contributions of different plant extracts in model organisms, which can have broader implications for their use in dietary supplements and health research.

In order to get further insights into the nutritional effectiveness of SA-GTE and BM-GTE, there were determined the body sizes and the ATP content of the imaginal bodies (see Materials and Methods). Notably, the body sizes and ATP content of the SA- and BM-GTE-supported w^{m4h} genotype, particularly in newly hatched adult female and male individuals (maximum age of 1 day) raised on the 0M diet, it was seen significant disparities compared to those reared on the normal media (NM) diet (see figure 19). In *Drosophila melanogaster*, there exists a distinct sexual dimorphism, characterized by females typically being larger in size compared to their male counterparts.

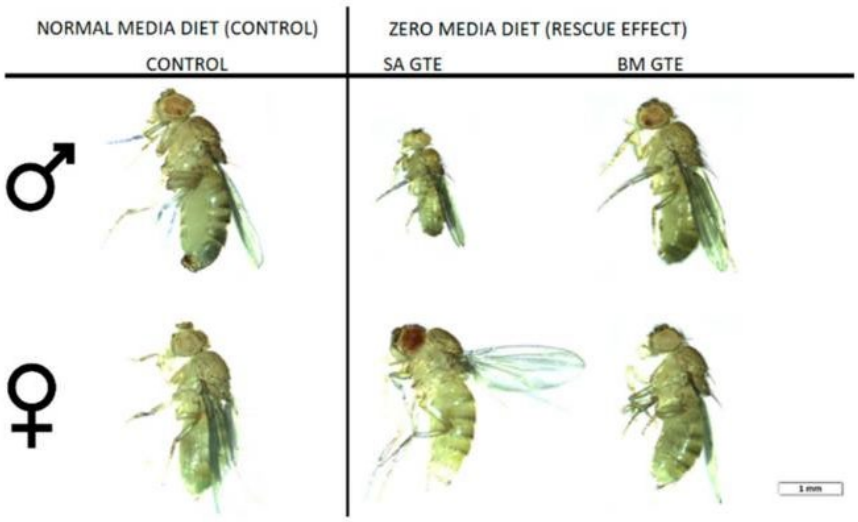

Figure 19. **Images of w^{m4h} newly hatched adults raised at NM (control) and 0M dietary conditions (source: Aleya et al., 2023)**

This difference in physical attributes, such as body size, along with variations in ATP content, highlights the potential influence of diet, particularly the role of GTE supplementation, on the developmental aspects of *Drosophila melanogaster*. The comparison of individuals nurtured on different diets offers valuable insights into how specific nutritional components can affect the growth and energy levels of these organisms. Such findings are crucial in understanding the broader implications of dietary choices and supplementations on the physiological development of model organisms, which can have applications in nutritional science and health research.

In terms of body length, both the SA- and BM-GTEs were found to produce similar sizes in female fruit flies, with a close resemblance in their ATP content as well. However, the male individuals exhibited a greater degree of variability in both size and ATP content. This variability could be attributed to the limited number of individuals assessed, but it might also suggest that chemical composition differences between the two GTEs could differentially impact the growth and ATP production capabilities in fruit flies (see figure 20).

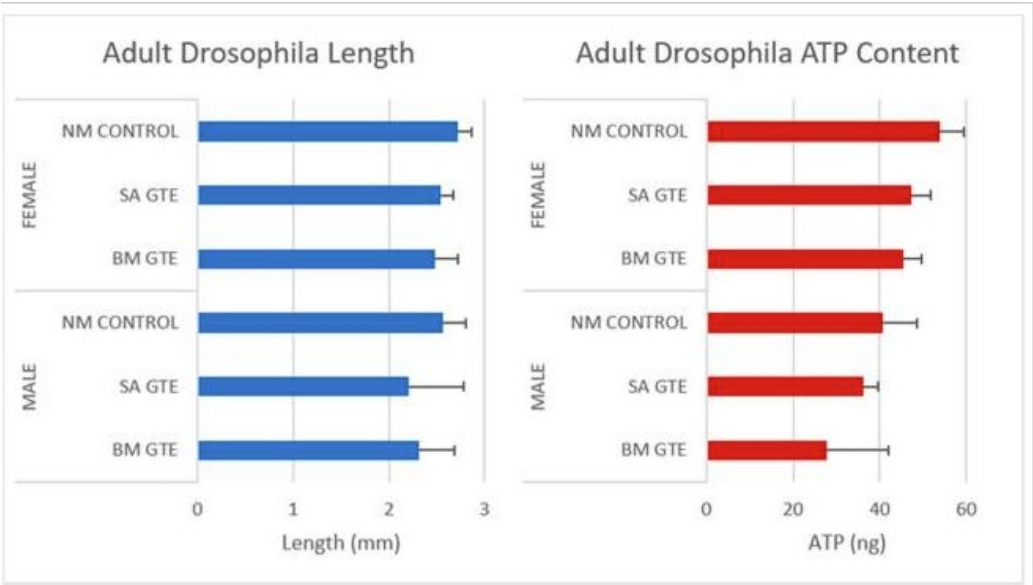

Figure 20. **Assessment of wm4h newly hatched adults for their body length (blue) and ATP content (red), raised in NM (control) and 0 M dietary conditions. (source: Aleya et al., 2023)**

Collectively, the observed concentration-dependent viability enhancement with SA- and BM-GTEs suggests that, apart from the identified macronutrients, other components in the plant extracts, such as free amino acids, may play a role in the observed nutritive effects. This is evident in both the SA and BM extracts. Nevertheless, the nutritive effects specific to SA- and BM-GTEs differ in their potency, with BM-GTE appearing more effective than SA-GTE in meeting the nutritional demands of *Drosophila melanogaster*-specific development.

These findings point to the potential of specific plant extracts in not only providing fundamental macronutrients but also in offering additional nutritional components that contribute to the overall development and health of the organisms. The difference in the

effectiveness of SA- and BM-GTEs highlights the importance of understanding the unique chemical compositions of various plant extracts and their specific impacts on growth and development in model organisms.

4.3.2. The normal versus high-sugar diet specific nutritional values of GTEs

It is important to pinpoint that extensive research has been carried out with regards to the nutritional requirements of *Drosophila melanogaster* that is revealed the critical aspects of diet during the entire life cycle; for instance, the absence of just one essential amino acid like arginine or isoleucine can reduce lifespan by 30–70% (Piper M.D., et al., 2014). Furthermore, for *Drosophila melanogaster*, it has been observed that diets high in sugar (HS) or a carbohydrate caloric overload can lead to developmental delays throughout their lifecycle (Eickelberg et al., 2022; Mattila and Hietakangas, 2017).

Consequently, to examine the impact of our specific HS diet, which contains 1.5 M sucrose, on the survival of 3rd instar larvae and the hatching of adults, we also monitored the developmental progression of the w^{m4h} strain at a constant temperature of 25°C. This assessment aimed to provide a comprehensive understanding of how a high-sugar nutritional intake might influences both the viability and growth stages of this particular fruit fly strain that otherwise would be suitable to study the position effect variegation dependent *w+* gene expression (see figure 21).

Our observation distinctly demonstrates that the HS diet used in our study causes a developmental delay of approximately three days in both the onset and completion of the third larval stage, as well as in the hatching of adults.

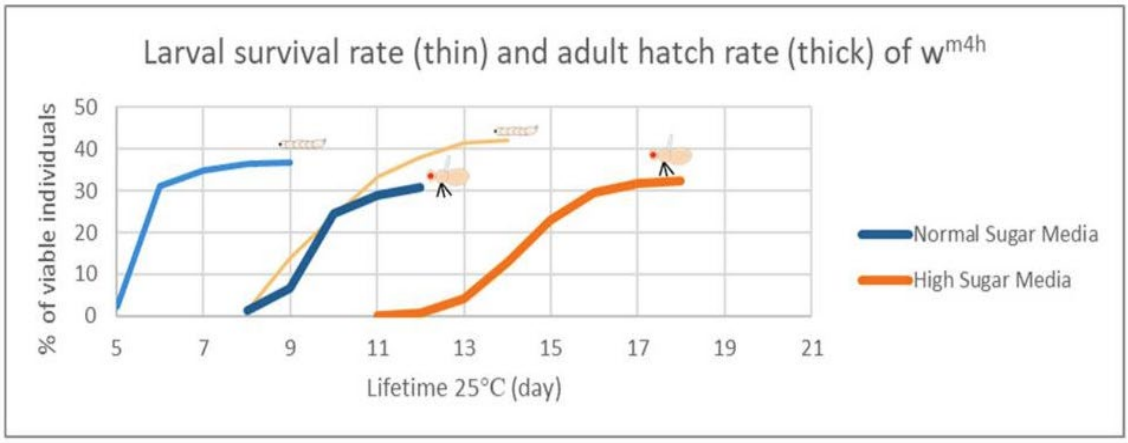

Figure 21. **Developmental timing of fruit fly lifecycle on NS versus HS diet.**

The viability assessment of the larvae and pupae ( ) respectively adults ( ) in the context of the duration of development in NM- and HS-dietary conditions. The blue curves are for normal sugar media respectively the orange one for high sugar media. (Source: Aleya et al., 2023).

Intriguingly, despite this delay induced by the HS diet, there was a marginal increase in the viability of the 3rd larval stage (Figure 22). Furthermore, the rate of adult hatching under the HS dietary condition remained largely comparable to that observed under the normal media (NM) or control diet, as shown in Figure 23. This suggests that while the HS diet impacts developmental timing, it does not significantly affect the overall survival rate from larvae to adulthood.

The findings from these experiments indicate that the high-sugar (HS) diet used in this study does not have a significant lethal impact on the *Drosophila melanogaster* strain being tested. It appears that there may be compensatory mechanisms in place that allow the fruit flies to adapt to the increased carbohydrate intake, thereby overcoming the developmental delays induced by the HS diet. This resilience suggests an inherent ability of the fruit flies to adjust to variations in dietary sugar levels without a corresponding decrease in survival rates.

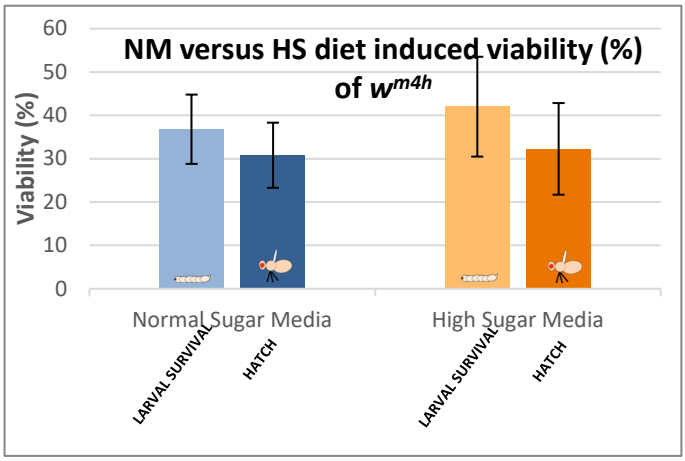

Figure 22. **Comparison of larval and adult viability in NM- and HS-dietary conditions. (Source: Aleya et al., 2023).**

4.3.3. The normal diet specific viability assessment of GTEs at 25°C and 28°C

Subsequently, we evaluated the effects of GTEs on viability when combined with both NM- and HS-diets at a constant temperature of 25°C (for details see Materials and

Methods). It is important to note that the HS dietary condition is known to induce a diabetic-like state in fruit flies (Vatashchuk M.V. et al., 2022). Therefore, if the HS diet is combined with GTEs, and it would be able to overcome the HS diet-specific outcomes by reducing the developmental delay or by increasing the survival rate then a health-promoting prediction can be made regarding the assessed GTE(s).

The specific results for the Olive GTE (O-GTE) are presented in Figures 23 A1, A2, B1,B2. Each of these Figures comprises two graphs: graph (A) displays the pupariation time of the third larval stage, or what we term the third larval survival, while graph (B) shows the time taken for adults to hatch from the pupae, referred to here as adult survival. In experiments conducted with NM diet supplemented with increasing concentrations of O-GTE, it was observed a noticeable enhancement in both larval and adult survival rates, improving from around 50% to approximately 80% (as shown in Figures 23 A1, A2, B1, B2). This increase in viability was accompanied by a reduction in the duration of larval and pupal developmental stages. Such findings suggest that O-GTE has a significant strengthening effect on the viability of fruit flies under normal dietary conditions, potentially by increasing the nutritive effects posed by the NM diet.

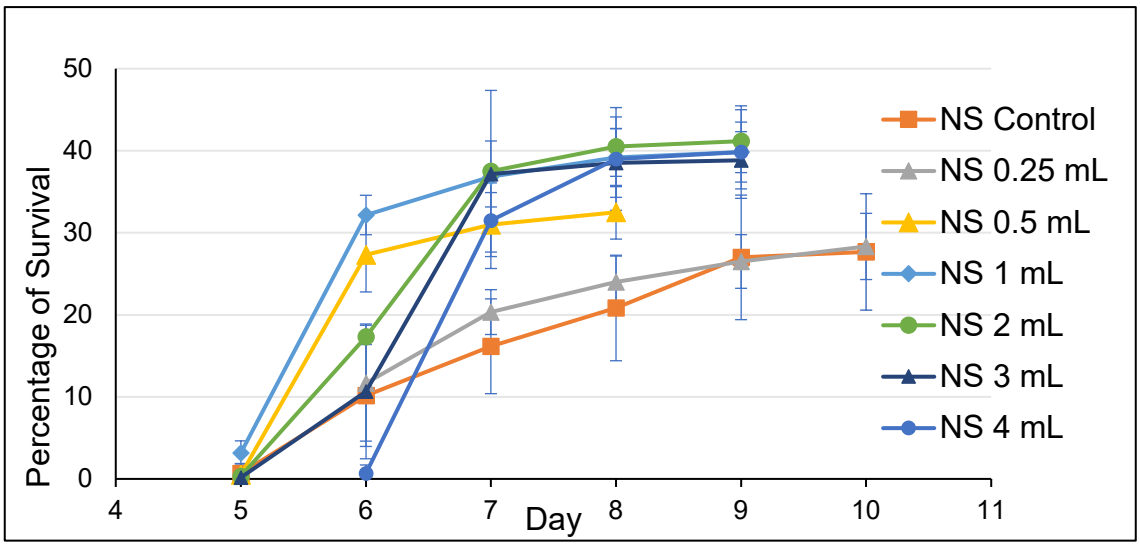

Figure 23 A1. **3rd Larvae viability on O-GTE, NM diet at 25°C (Source: Aleya et al., 2023)**

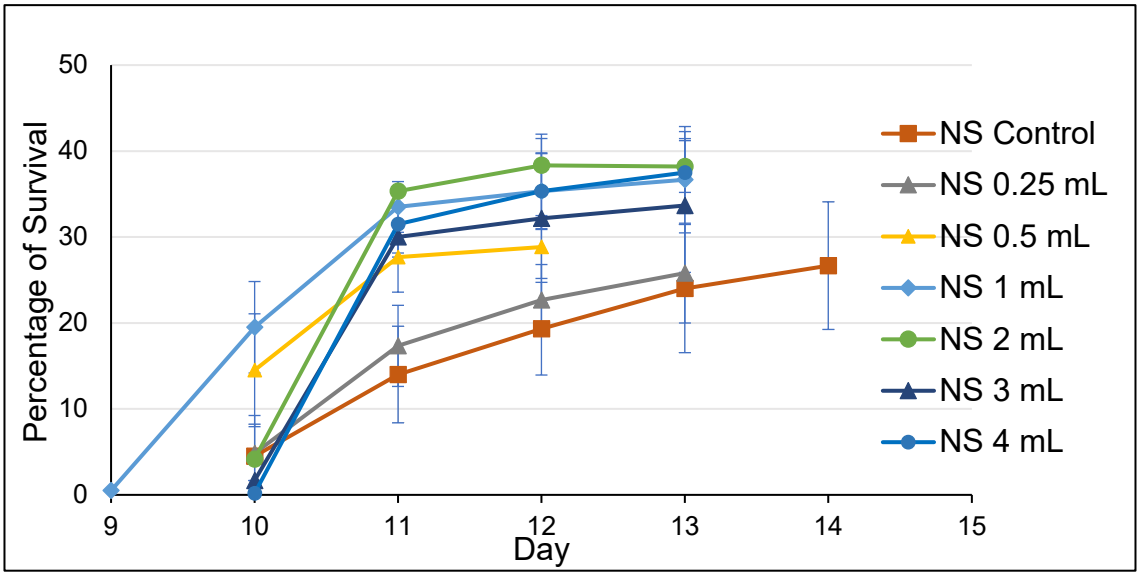

Figure 23 B1. **Hatched flies viability on O-GTE, NM diet at 25°C (Source: Aleya et al., 2023)**

Further to the above-mentioned experiments that were conducted at 25°C, these types of nutritional assessments had also been performed at 28°C, presuming that the raised temperature might increase the metabolic rate, and if so then the GTE might interfere with it. The fruit fly is an ectotherm species, meaning that its body temperature is close to ambient temperature, and therefore, a temperature raise is expected to yield a metabolic rate increment (Goda et al., 1988).

When a NM diet was applied in combination with the O-GTE at 28°C, a similar larval viability pattern emerged as for 25°C. The O-GTE enhanced the survival of larvae at the concentration of 2 mL and 3 mL concentrations, while the 1 mL O-GTE concentration had no obvious effect regarding larval survival (Figure 23 A2.)

However, in when the adult survival rate was determined at NM diet and 28°C temperature, it was noticed the drop of percentage of survival of adults at the concentration of 2 mL O-GTE as compared to the control (Figure 23 B2). However, the 3 mL O-GTE concentration almost recapitulated the larval specific survival rate. Again the 1 mL concentration of O-GTE at 28°C had no effect on the adult survival as it would parallel the control specific effect.

The fact that the 2 mL O-GTE at 28°C had a kind of restrictive effect in case of adult versus larval survival it suggests the existence of a putative restrictive event triggered through the O-GTE that cannot be explained on the existing current knowledge.

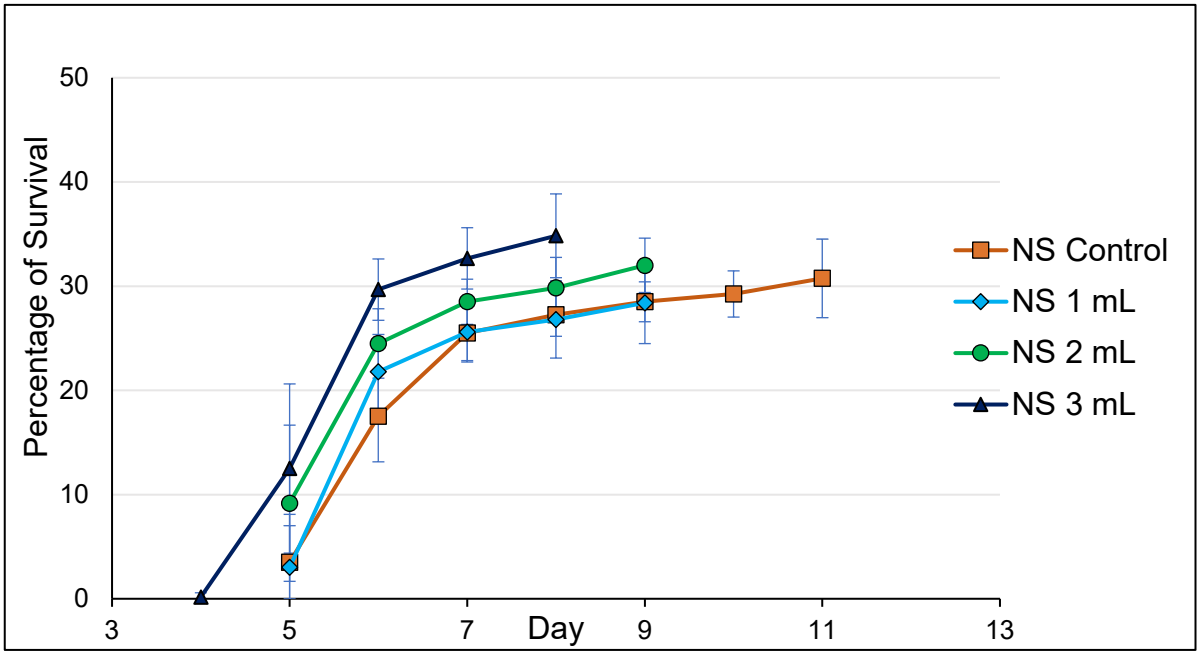

Figure 23 A2. **3rd Larvae viability on O-GTE, Normal diet at 28°C**

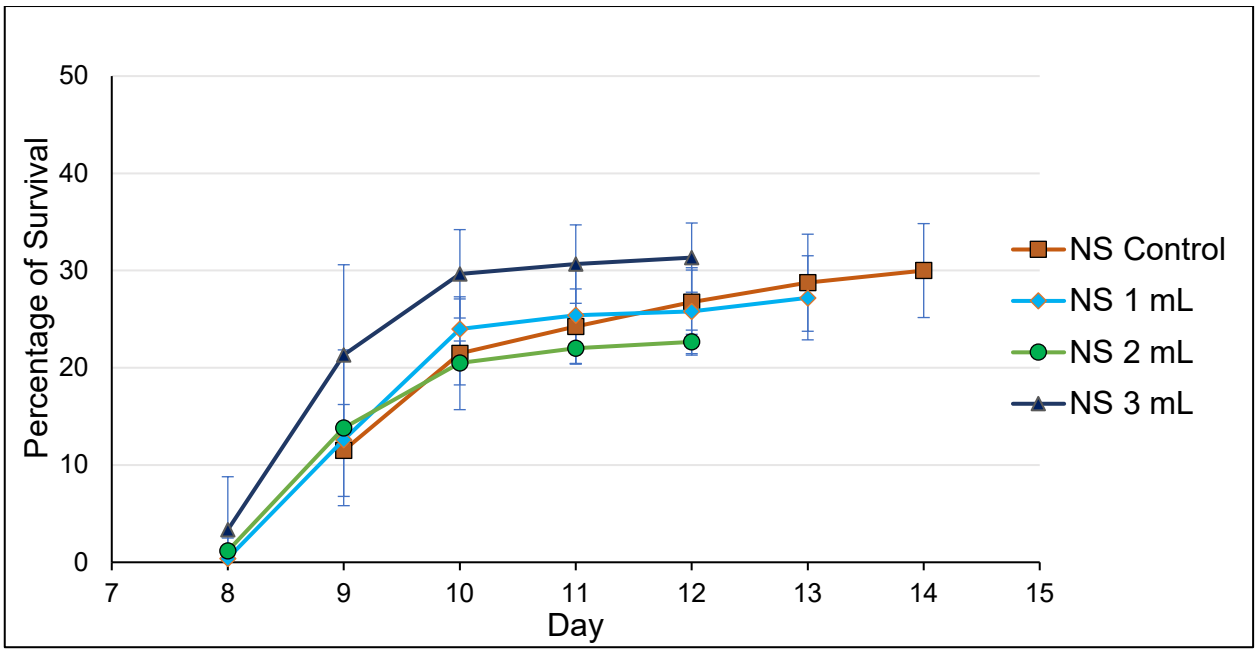

Figure 23 B2. **Hatched flies viability with O-GTE at normal diet at 28°C**

This research sheds light on the potential of O-GTE to not only enhance survival rates in *Drosophila melanogaster* but also to expedite their development through crucial life

stages. The implications of these findings are significant for understanding the role of specific plant extracts in influencing the growth and development of model organisms, particularly under varying dietary conditions.

To explore the potential viability-enhancing properties of the SA-GTE, it was applied the already described experimental protocol used for O-GTE. For both 25°C and 28°C temperatures and under the NM diet, the SA-GTE increased the viability of w^{m4h} fruit flies during both the 3rd larval and adult stages. However, unlike the O-GTE, no significant concentration-dependent impact was observed among the different concentrations of SA-GTE tested though some viability increment could be detected (see Figures 24 A1, A2, B1, B2). This finding suggests that while SA-GTE does have a positive effect on fruit fly viability at both the larval and pupal stages, however, its efficacy does not vary markedly with concentration change, and therefore would differ from the concentration-dependent response noted in the case of the O-GTE. It is rather interesting that at 25°C and NM diet, the rates of viability for 3rd larvae and emerged adults increased almost 7%, while certain developmental delays were also apparent. These kinds of developmental shifts were more pronounced from 2 to 4 mL SA-GTE concentrations, suggesting that it might contain some extract compound(s) that might interfere with the so-called normal larval and pupal development.

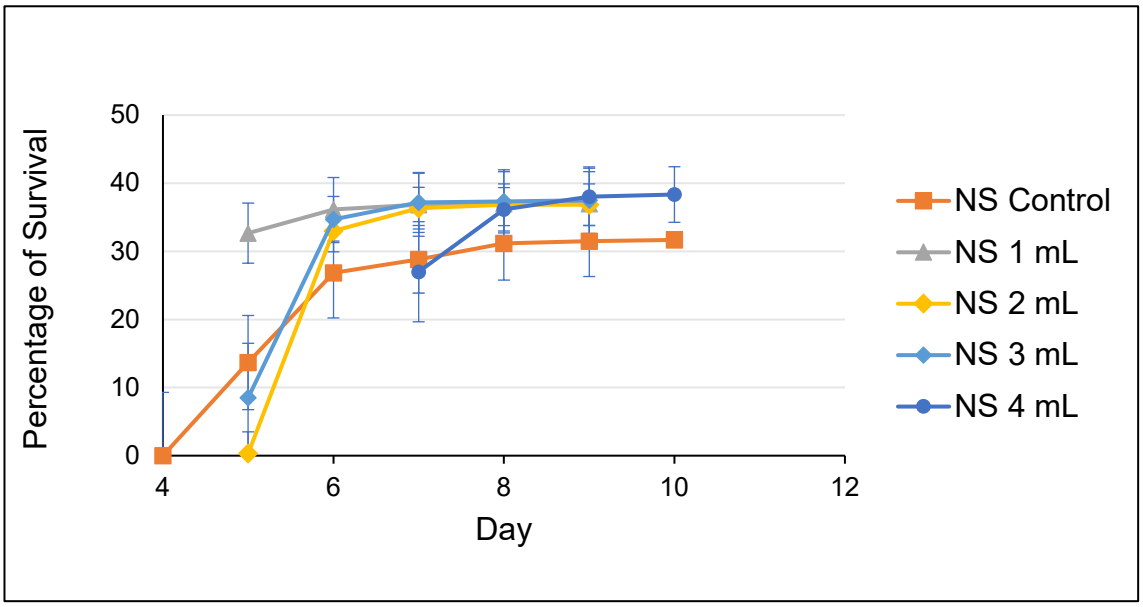

Figure 24 A1. **3rd Larvae viability on SA-GTE, Normal diet at 25°C (Source: Aleya et al., 2023)**

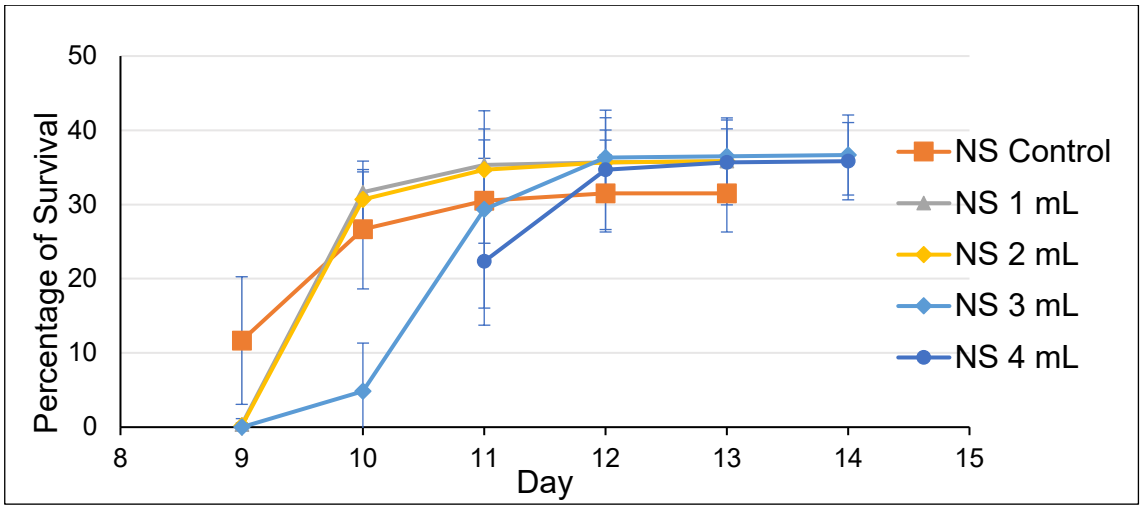

Figure 24 B1**. Hatched flies viability on SA-GTE, Normal diet at 25°C (Source: Aleya et al., 2023)**

Quite remarkably, this kind of developmental setback is overtaken, and the viability will increase steadily.

The above-described SA-GTE experiments on NM diet, were also performed at 28°C, and some kind of viability increase was observable, but the increment was not us pronounced as at 25°C, and no developmental delay was detected (Figure 24 A2 and B2).

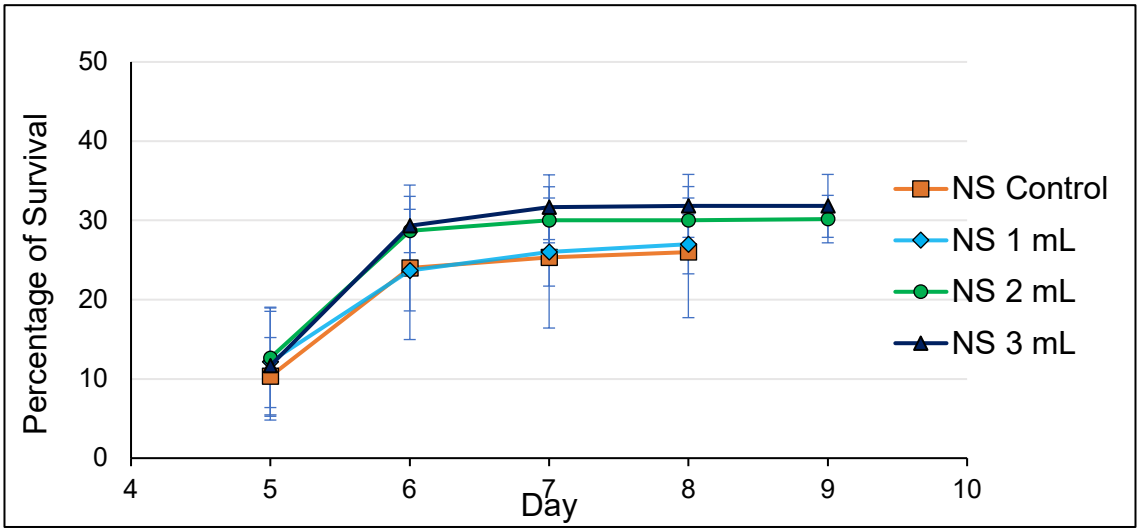

Figure 24 A2. **3rd Larvae viability on SA-GTE, Normal diet at 28°C**

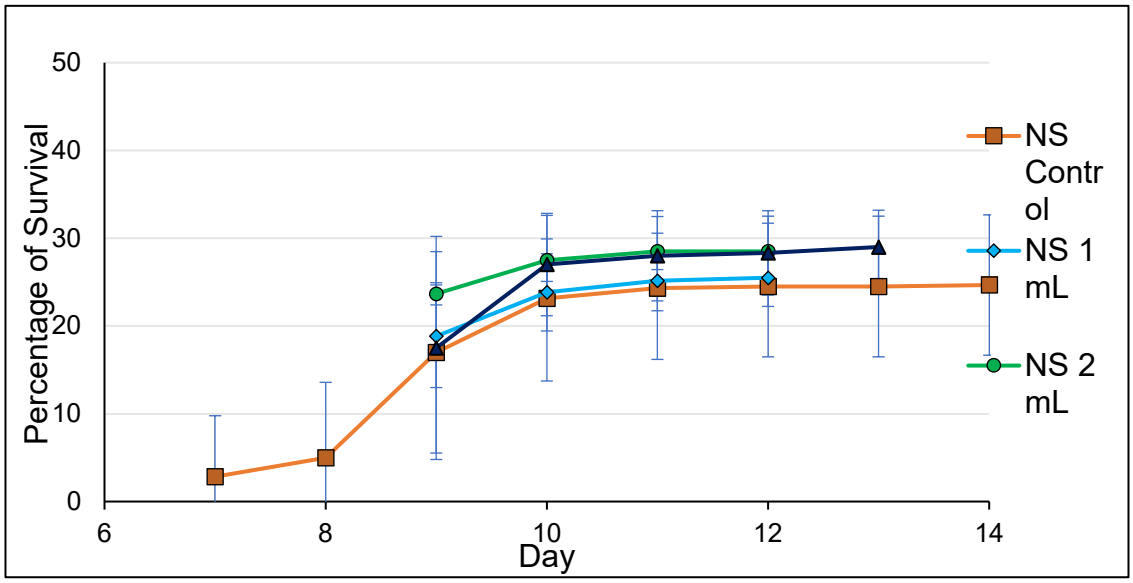

Figure 24 B2. **Hatched flies viability on SA-GTE Normal diet at 28°C**

These kinds of experiments suggest that the SA-GTE could compensate for the 28°C temperature associated limited viability rate.

After examining the effects of O-GTE and SA-GTE, it was investigated in a similar modality the impact of BM-GTE on fruit fly viability. Noteworthy, all three GTEs were analyzed through identical methodology, and this would mean that there the applied methodology would allow to directly compare the experimental data. Under the NM diet and at 25°C, it was observed that the lowest concentration of BM-GTE significantly reduced the survival rate of both larvae and newly hatched adults compared to the control group, as shown on figures 25 A1, B1, A2 and B2.

Interestingly, at higher concentrations ranging between 2-3 mL of BM-GTE concentrations, a significant increase of the survival rate of both larvae and newly emerged adults was observed (see figure 25 A1 and B1). These observations are suggesting that certain phytonutrient(s) present in BM-GTE might need to reach a critical concentration threshold to effectively enhance viability. This finding implies that the relationship between BM-GTE concentration and fruit fly viability is not linear and that optimal concentrations might exist for maximizing the presumptive health benefits of the GTE.

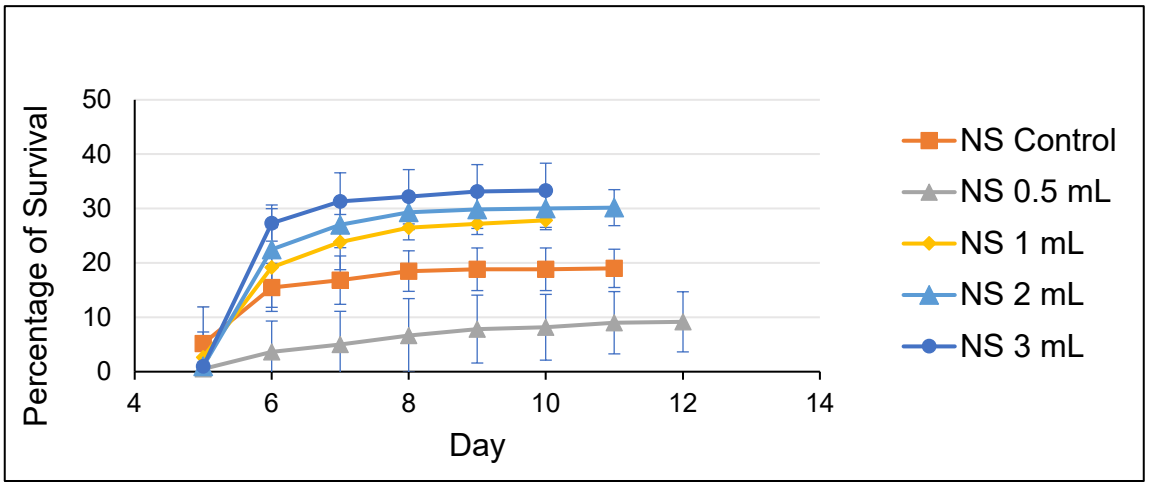

Figure 25 A1. **3rd Larvae viability on BM-GTE, NM diet at 25°C (Source: Aleya et al., 2023)**

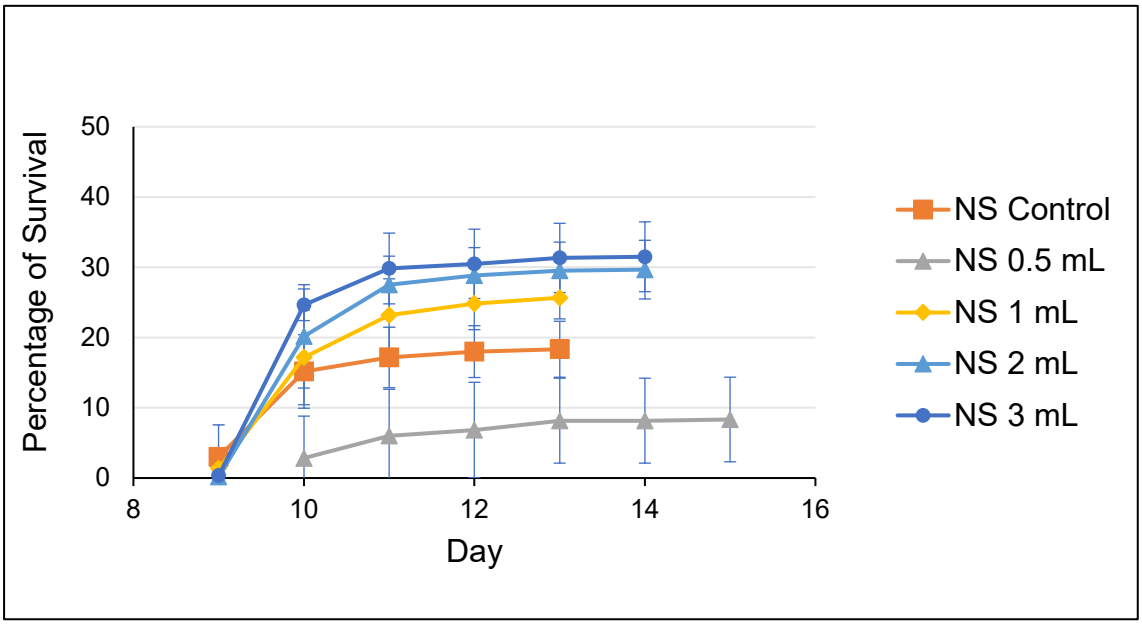

Figure 25 B1. **Hatched flies viability on BM-GTE, Normal diet at 25°C (Source: Aleya et al., 2023)**

The BM-GTE experiments were also conducted at NM diet and 28°C to analyze if the ambient temperature rise induced increased metabolic rate would also influence the BM-GTE associated viability rates (see Figure 25 A2 and B2).

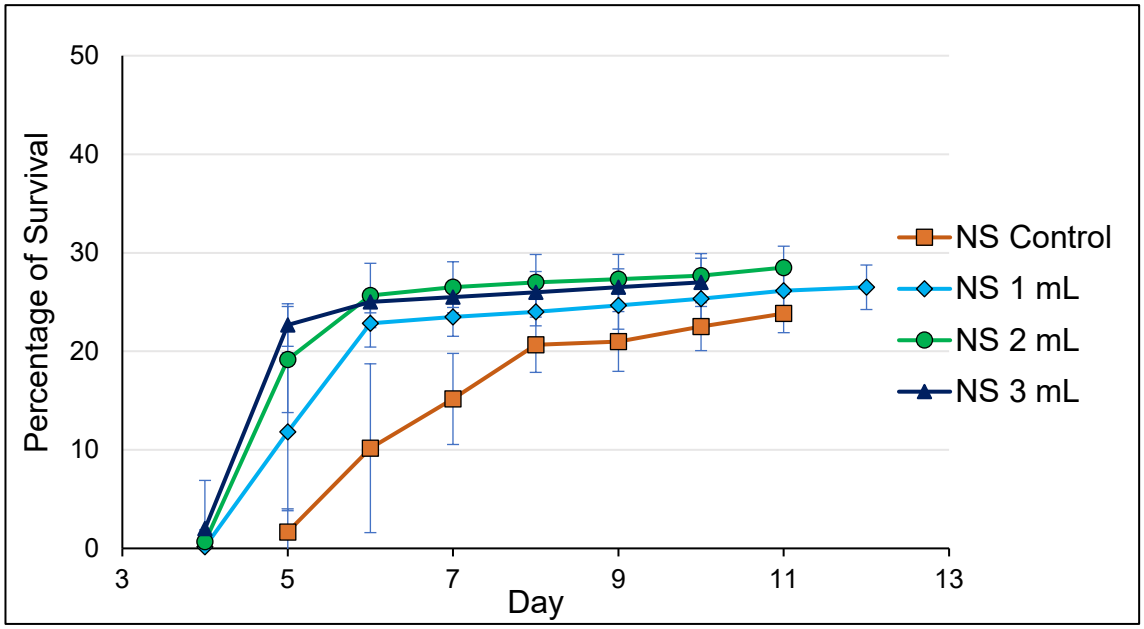

Figure 25 A2. **3rd Larvae viability on BM-GTE, Normal diet at 28°C**

As it can be observed on the above-mentioned Figures, the increased metabolic rate specific survival rate of 3rd larvae and emerged adults yield lower values than at normal 25°C specific conditions. Certain concentration dependency was visible for both larvae and adults, while the viability rise showed a sight increasing tendency.

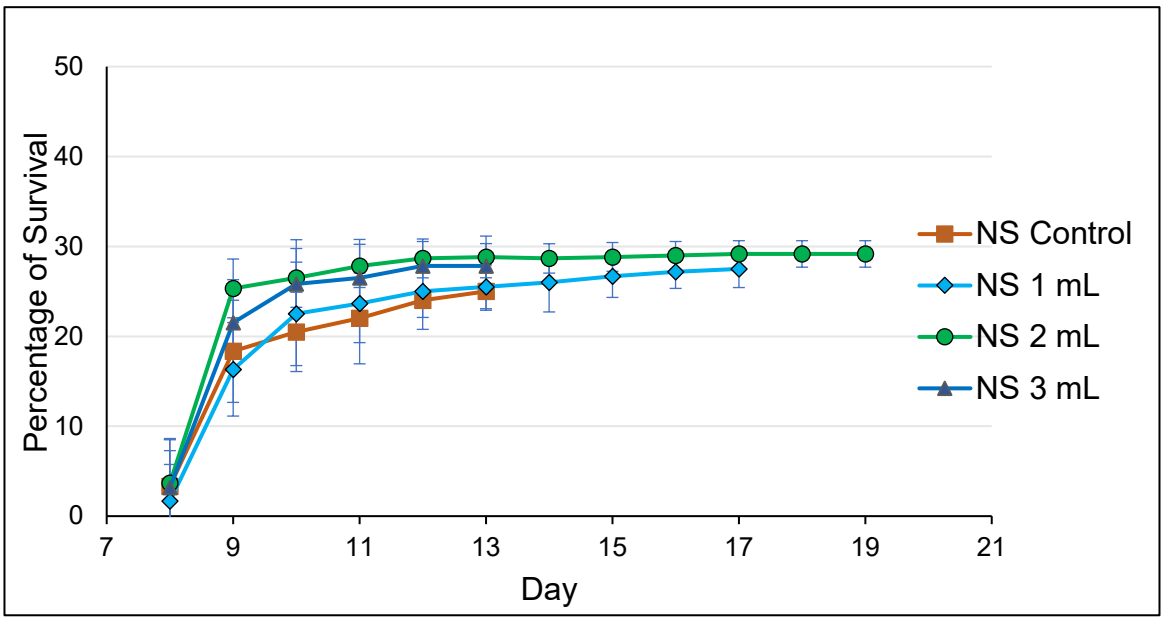

Figure 25 B2. **Hatched flies viability with BM-GTE, Normal diet at 28°C**

4.3.4. The high-sugar diet specific viability assessment of GTEs at 25°C and 28°C

Next were experiments to assess the GTEs associated viability by replacing the NM diet with a carbohydrate overload type of nutrition, the so-called HS (high sugar) diet. As described in paragraph 4.3.2. (*The normal versus high-sugar diet specific viability of Drosophila melanogaster*), the applied HS diet would induce a developmental delay during the larval period of life cycle. This type of developmental shift is due to a diabetes like condition that has been demonstrated by others (Musselman et al., 2011), and is going to affect expression of at about several hundreds of genes that belong to more than ten different functional clusters (unpublished results). In the following, it is described all the experiments that were conducted to study the concentration-dependent viability affecting the effect of the given GTEs.

When the NM diet was substituted with the HS diet, the O-GTE was able to increase the viability of both 3rd instar larvae and hatched adults to about 60% at lower concentrations. Yet, at higher levels of O-GTE, there seemed to be an increase in developmental delay without a corresponding improvement in viability (as illustrated in figures 26 A1, B1, A2 and B2). Notably, a concentration of 0.5 mL of O-GTE was almost equivalent to the control in its effects, while 1–2 mL of O-GTE was able to enhance viability without causing developmental delays. Conversely, 3–4 mL of O-GTE induced a significant delay (approximately 2 days) in both larval and pupal stages, though the viability eventually aligned with control levels.

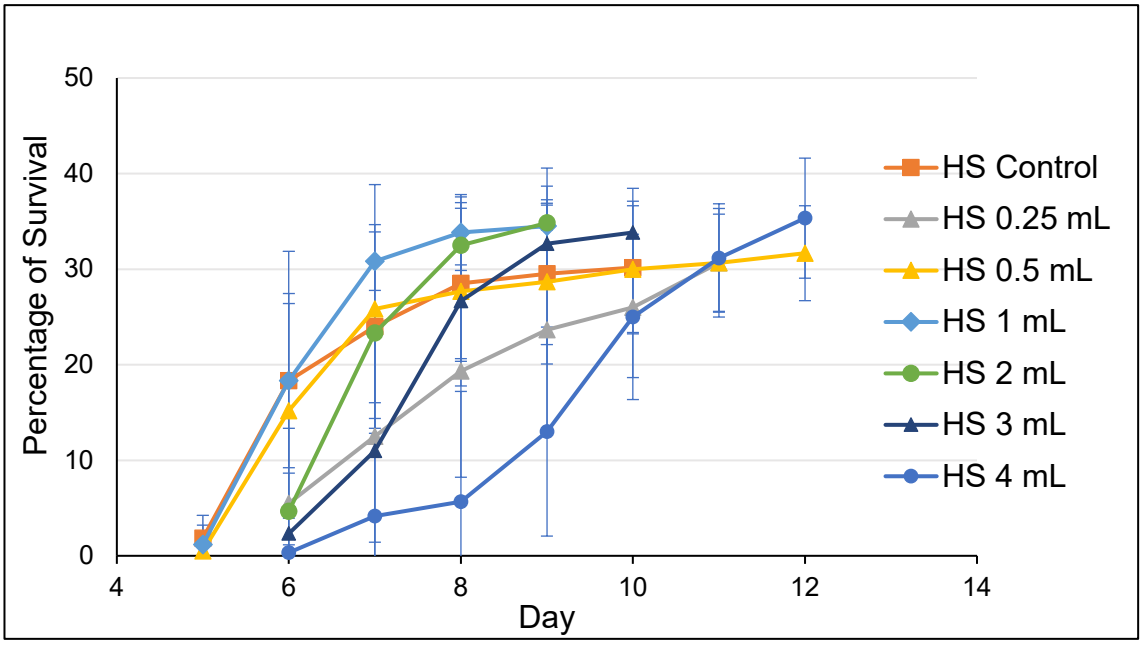

Figure 26 A1. **3rd Larval viability on O-GTE, HS diet at 25°C (Source: Aleya et al., 2023)**

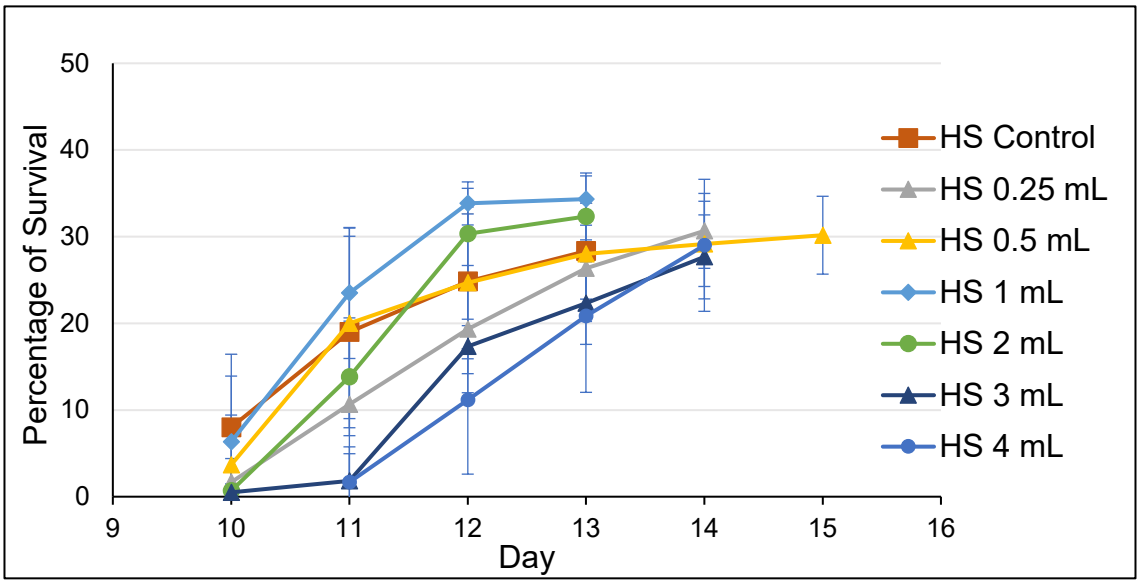

Figure 26 B1. **Hatched flies viability on O-GTE, HS diet at 25°C (Source: Aleya et al., 2023)**

Collectively, these results indicate that O-GTE can boost viability not just under NM but also HS dietary conditions, suggesting that the O-GTE must contain key phytonutrients that support normal development and can potentially mitigate some of the restrictive effects associated with the HS diet. Therefore, at 25°C, the viability boosting effect of O-GTE seems to be less efficient at HS than in the NM diet.

However, at 28°C, all concentrations of O-GTE seemed to reduce the survival rate for both 3rd instar larvae and emerging adults (see Figure 26 A2 and B2).

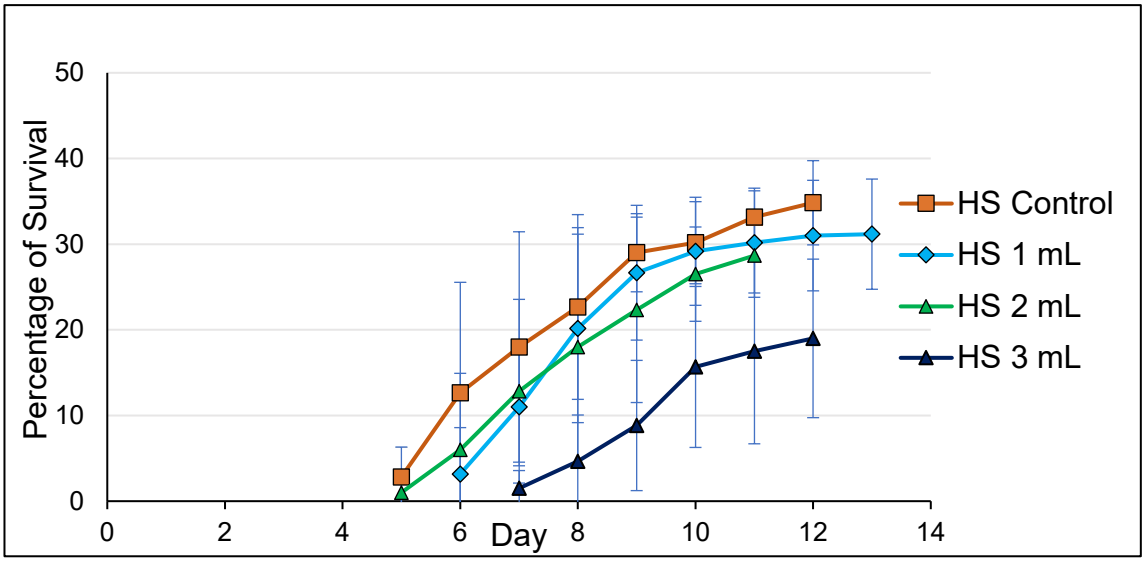

Figure 26 A2. **3rd Larvae viability on O-GTE, HS diet at 28°C**

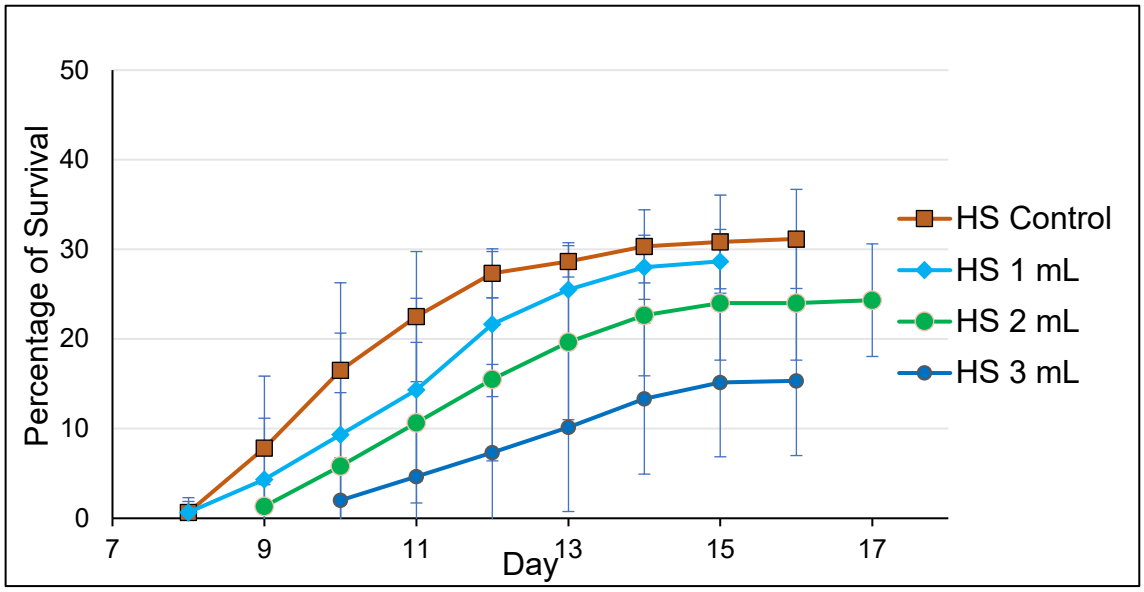

Figure 26 B2. **Hatched flies viability on O-GTE, HS diet at 28°C**

The worst outcome was seen for the 3 mL O-GTE concentration where the survival rate hardly reached 20% of the treated individuals, and all together the concentration dependency of the reducing effect was clearly visible at 28°C. This outcome strongly suggests that the increased temperature induced higher metabolic rate cannot be counteracted by the applied O-GTE.

In the context of the HS diet, the experiments with SA-GTE revealed a concentration-dependent antagonistic effect at 25°C. At lower concentrations like 1 and 2 mL), the SA-GTE appeared to facilitate a more rapid development, while higher concentrations (3 to 4 mL) of SA-GTE tended to prolong the larval and pupal stages (see Figures 27 A1,A2).

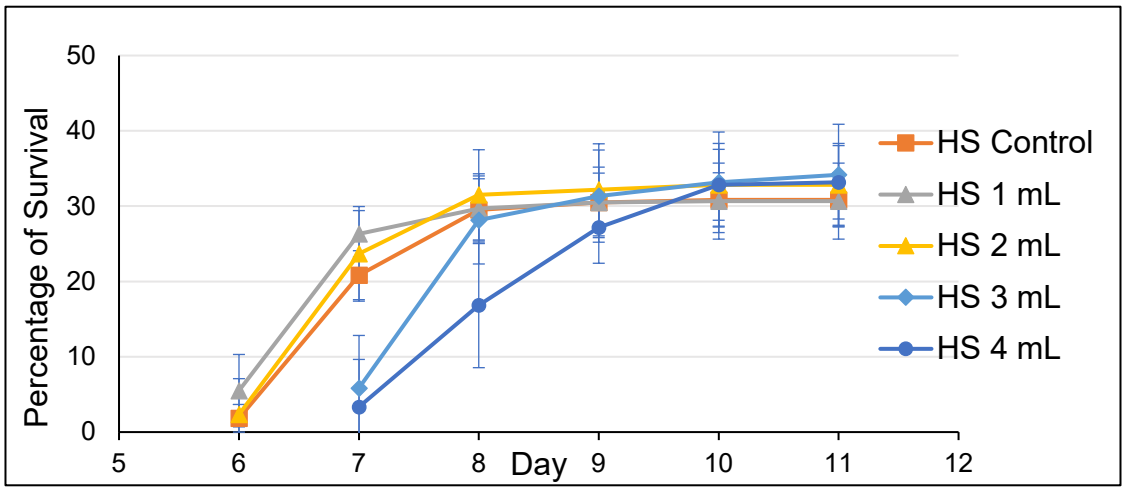

Figure 27 A1. 3rd Larvae viability with SA-GTE, on HS diet at 25°C (Source: Aleya et al., 2023)

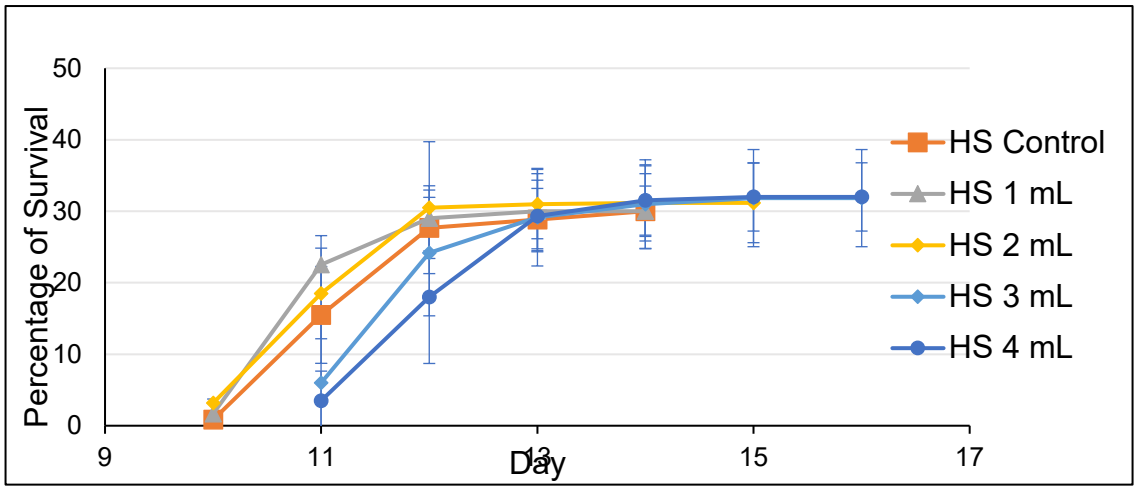

Figure 27 B1. Hatched flies viability on SA-GTE, HS diet at 25°C (Source: Aleya et al., 2023)

However, neither of these concentrations adversely affected the viability since all the observed SA-GTE concentrations would yield survival rates eventually aligning with those of the control group. Consequently, at 25C and HS dietary conditions, the SA-GTE did not significantly enhance the viability rate of 3rd larvae and emerged adults.

Similar kind of experiments were carried out at 28°C and HS dietary conditions. It turned out that at 1mL and 2mL concentration of SA-GTE could increase both survival rates (see Figures 27 A2, B2), while at 3mL a developmental shift was apparent, but the viability rate would look similar to the control.

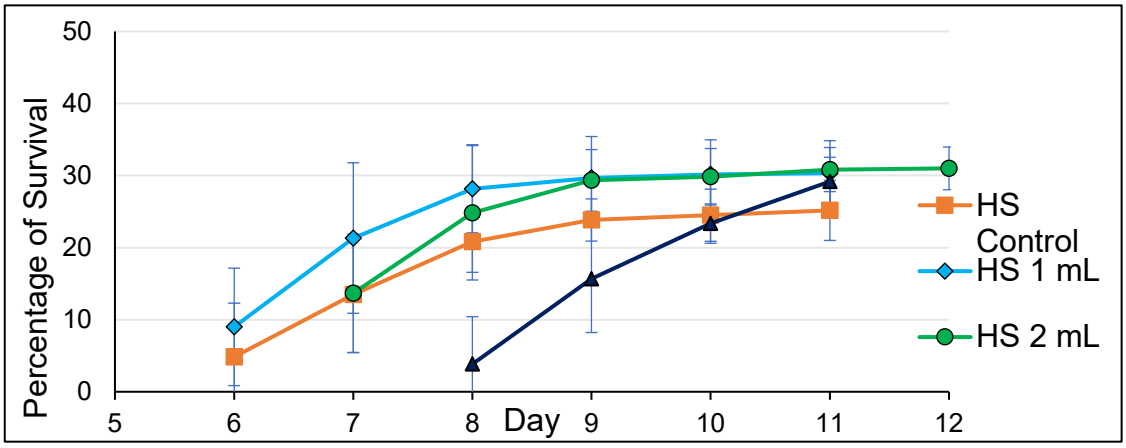

Figure 27 A2. **3rd Larvae viability at SA-GTE on HS diet at 28°C**

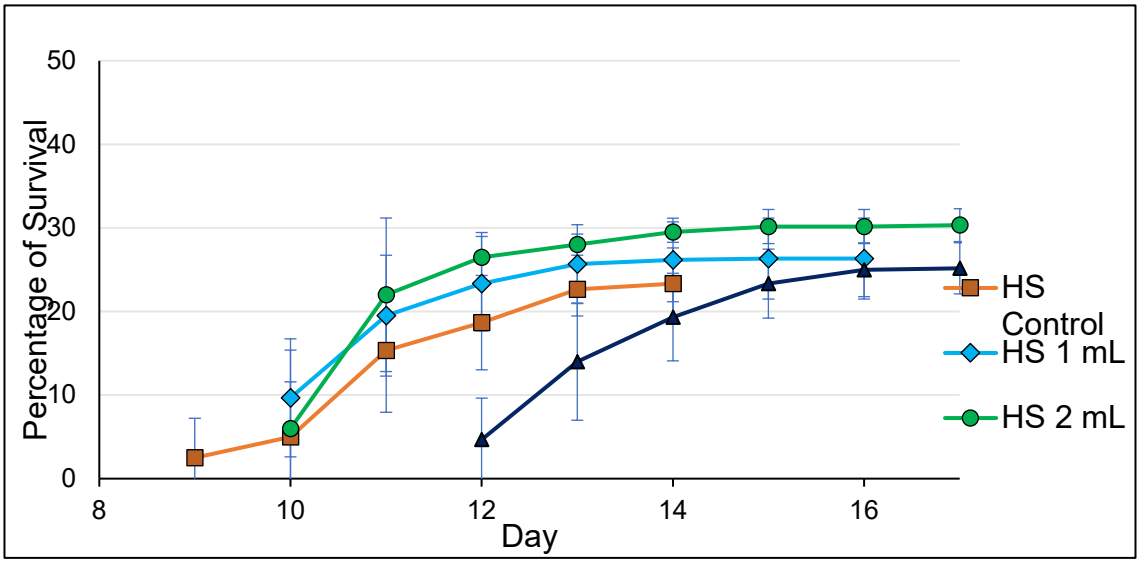

Figure 27 B2. **Hatched flies viability on SA-GTE, HS diet at 28°C**

Therefore, at 28°C and HS diet it was observed a kind of SA-GTE specific effect that could overcome the increased metabolic rate induced viability reduction.

The HS diet and 25°C experiments for the BM-GTE at the 2-3 mL concentrations resulted in augmented viability rates compared to controls (see Figure 28 A1 and B1).

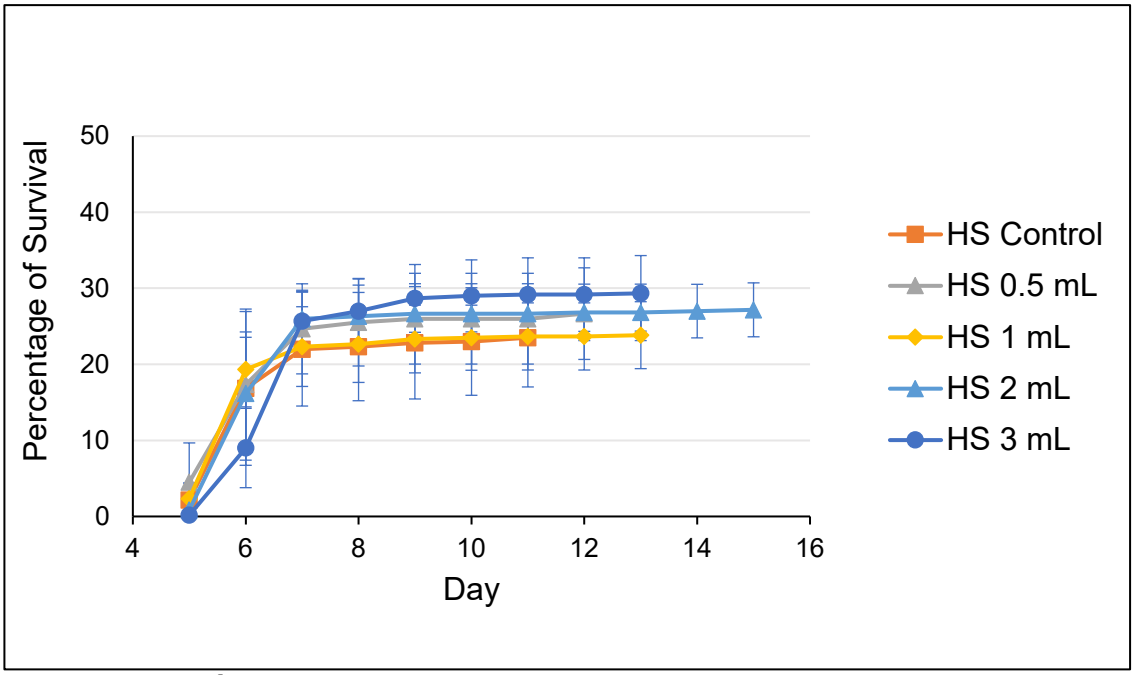

Figure 28 A1. **3rd Larvae viability on BM-GT, HS diet at 25°C (Source: Aleya et al., 2023)**

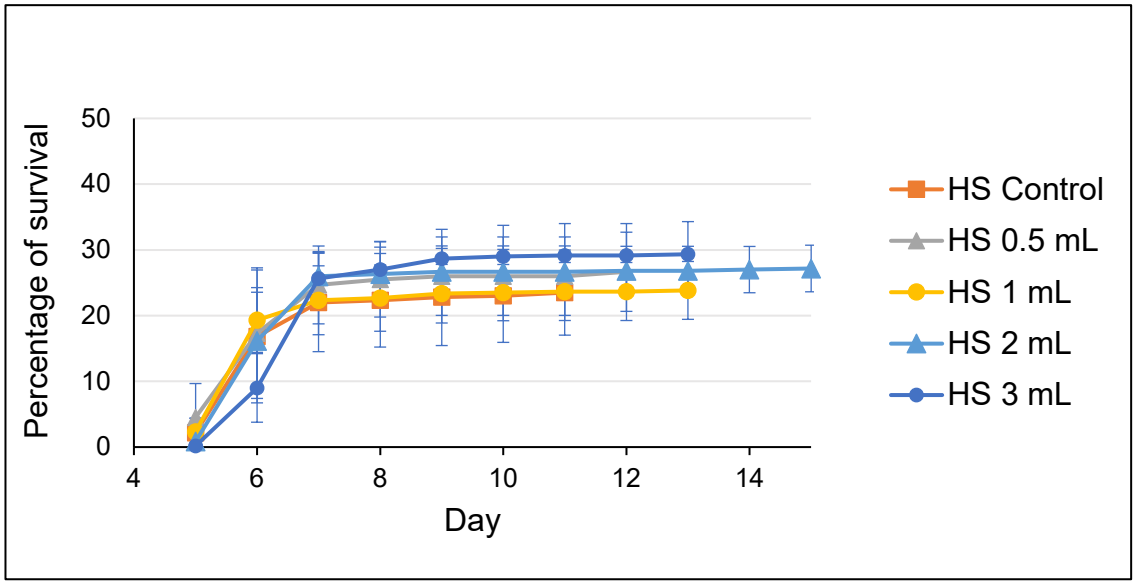

Figure 28 B1. **Hatched flies viability on BM-GTE, HS diet at 25°C (Source: Aleya et al., 2023)**

However, the 1 mL concentration of BM-GTE resulted in a viability rate that was almost identical with the controls, suggesting that the concentrations of putative bioactive compounds would not reach the critical threshold to elicit a HS counteracting effect. The increased viability observed with the BM-GTE supplementation under the HS diet can be

interpreted as indirect evidence of its potential to counteract the diabetic-like condition induced by the HS diet in *Drosophila melanogaster*.

Furthermore, the BM-GTE associated viability effect was also checked at 28°C under HS dietary condition. The results are presented on Figures 28 A2 and B2.

Eventually all the applied concentrations of BM-GTE would yield similar 3rd larval specific survival rates though the highest concentration (3 mL) seemed to delay the larval development. This delay was even more visible if the emerged adults were assessed and consequently, the newly emerged flies number seemed to slightly decrease (see Figure 28 B2). This observation can also indicate that the pupal development might become more affected through the rate limiting action of some BM-GTE specific bioactive phytonutrient that would compromise the faithful completion of metamorphosis. Taken together the emerged data are suggesting that the HS diet and the temperature induced increased metabolic rate would result in a survival rate that cannot be significantly improved by the BM-GTE.

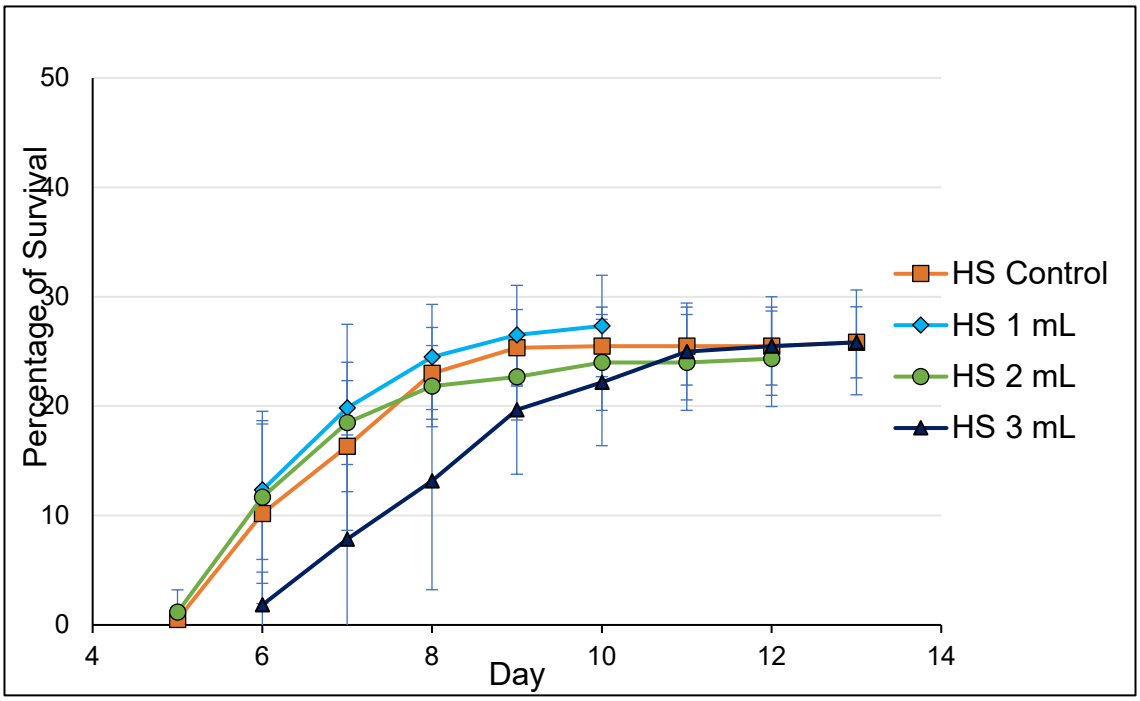

Figure 28 A2. **3rd Larvae viability on BM-GTE, HS diet at 28°C**

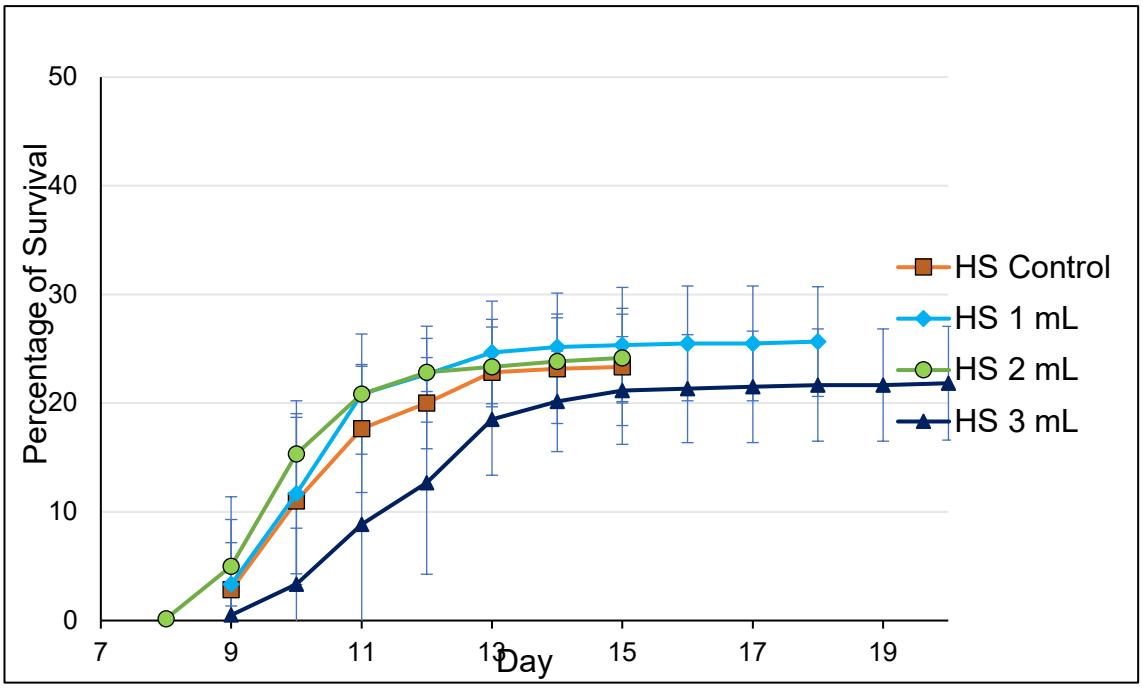

Figure 28 B2**. Hatched flies viability on BM-GTE, HS diet at 28°C**

Collectively, the experiments involving various concentrations of GTEs under both NM and HS dietary conditions highlight specific concentration-dependent effects in enhancing and/or rescuing the viability of larval and adult fruit flies of the wm4h genotype. These findings underscore the potential of GTEs in providing protective or supportive benefits to fruit flies, particularly in the context of dietary challenges such as those imposed by HS diet. The variability in response depending on the type of GTE and the applied concentrations further emphasizes the complexity of these extracts generated interactions with biological systems and their potential applications in addressing nutritional and metabolic challenges.

4.4.1. The carp (Cyprinus carpio) fish larvae-based study of the GTEs associated nutritive potential

The *Drosophila*-based experiments brought about important information regarding the implication of the studied GTEs concerning certain nutritional cues. However, *Drosophila melanogaster* is a Holometabolous insect species, and it seems also logical to study any GTE-associated nutritive properties using evolutionary higher positioned species and compelling a novel translational model system. Such a model system should be based on different animal species and is meant to produce more in-depth

and applicable results that directly benefit animal husbandry and/or human health-related clinical studies that should be fully compliable with ethical, moral, and economical considerations. Considering the GTEs, a proper translational model should provide enough scientific evidence(s) to support their applicability for animal feeding and the safe nutrition of humans. Based on the above mentioned considerate, the carp (*Cyprinus carpio)* has been chosen to include in a translational model. The carp is an omnivorous species, so its herbivorous diet would include aquatic plants, but it would also choose insects, crustaceans, and mollusks.

4.4.2. Assessment of viability as a nutritive indicator

When the nutritional potential is evaluated using *in vivo* methodology, the presumptive food or feed must be administered to individuals of human or animal species. A proper food and/or feed should cover the energetic needs of the tested population but equally important would be to cover the micronutrient and phytonutrient requirements without any toxicity to assure the proper and efficient functioning of the human and animal organisms. It is foreseeable that if a macro- or micronutrient will have a rate-limiting effect, then its absence must affect the quality of life and ultimately the viability of the species tested. The viability-affecting effect of a food/feed should be even more relevant if it is related to certain life cycle-specific stages. It has been demonstrated that suboptimal nutrition could compromise the life cycle by delaying development and the fitness of adults but the interorgan and intercellular signaling pathways coordinating such events are weakly understood. The search for and use of a more nutritionally sensitive in vivo model system, such as fish larvae, to study the nutritional properties of GTEs was motivated by this inconvenience.

4.4.3. Assessment of fish larvae specific growth and ATP content

Animal nutrition fundamentally involves providing organisms with essential nutrients to efficiently produce ATP and meet the energy demands of various life processes. Given that the GTEs we studied have shown a differential impact on the viability of fruit flies, starting from the larval stages when food intake begins, we were motivated to further investigate these effects using a carp fish larval model. Following successful propagation

in carp, embryogenesis proceeds and its completion is followed by the eventual hatching of larvae (see figure 29.C).

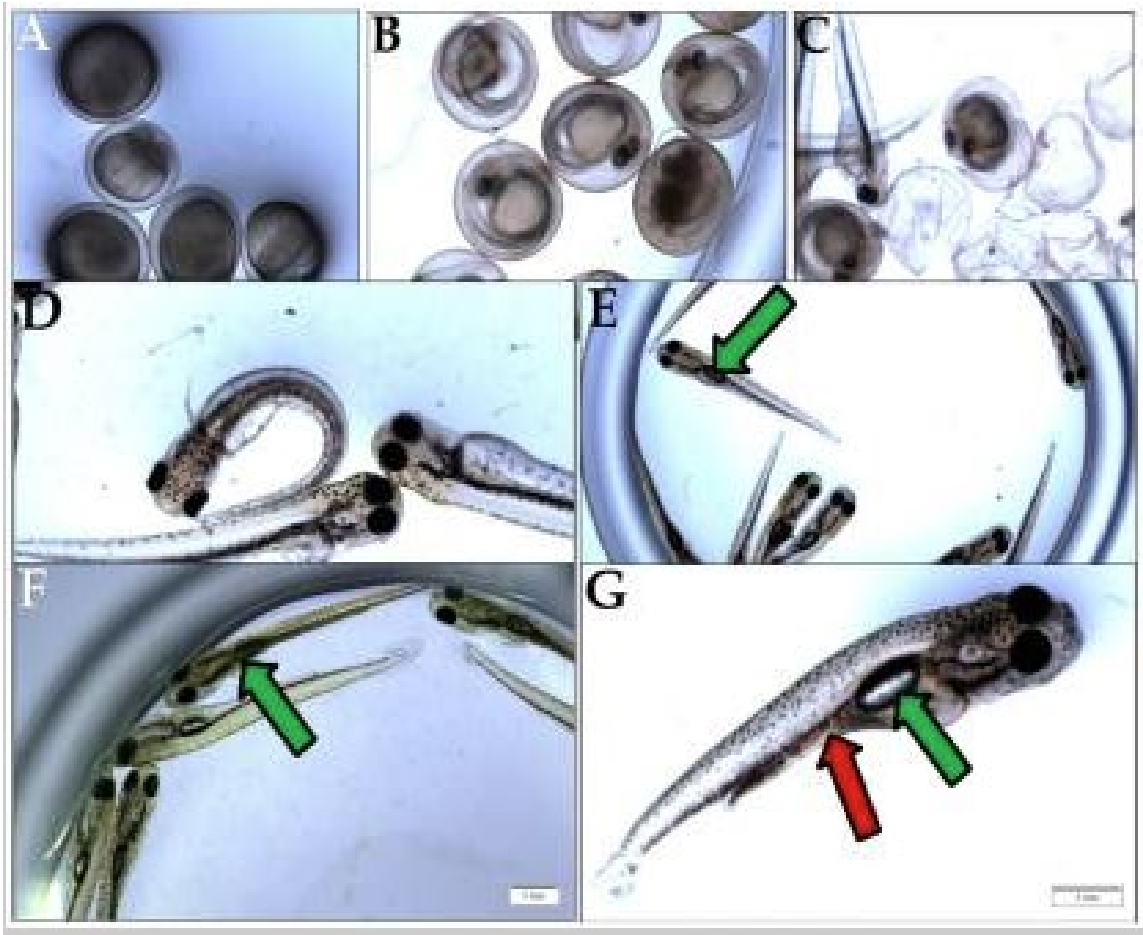

Figure 29. Assessment of carp larvae viability. (A) Fertilized eggs during embryogenesis; (B) Eggs that completed embryogenesis with visible larvae; (C) Larvae at the time of hatching; (D) Non-feeding larvae at day 1 after hatching, also called pre-feeding larvae; (E) Feeding larvae, day 3 after hatching (green arrow indicates the swim bladder); (F) Feeding larvae at day 7 after hatching, fed with GTE; (G) Feeding larva at day 7, fed with brine shrimp (red arrow). (Source: Aleya et al., 2023).

Initially, the newly hatched carp larvae do not feed themselves. However, typically on the second or third day after hatching, depending on the ambient temperature, they begin to feed on live brine shrimp (Artemia salina), which is considered the most appropriate food under artificial rearing conditions.

The feeding phase commencing from the third day is the most critical period for carp fish larval development. Any nutritional deficiencies or disturbances during this transitional period could result in growth retardation and possibly larval mortality. Notably, three days

after hatching, the carp fish larvae undertake another developmentally crucial step by filling in their swim bladder with air and commencing feeding (see on figure 29 E). The importance of this developmental milestone is underlined by the fact that from this point on, proper nutrition becomes essential for their survival and growth. At this point, the metabolism of the carp larvae begins to actively produce ATP, using the nutrients from the consumed feed and the oxygen from the air.

The undertaken study will allow the comparison of the nutritional efficacy of brine shrimp (the control feed) with that of the GTEs under investigation. This comparison focused on evaluating the whole-body specific ATP production and body length growth per larva (as detailed in Section 2—Materials and Methods). The obtained data were used to compel Figure 30 and Table 13). The collected data revealed that, for the stages involving fertilized eggs and non-feeding larvae, there were no significant differences between the individuals fed with brine shrimp and those receiving GTEs. However, interesting observations were made during the feeding larval stage, which starts 3 days after hatching. While the body sizes of the larvae were similar, there were notable differences in ATP yields between the groups. The O-GTE diet produced the greatest increase in ATP synthesis, whereas the ATP levels associated with the Artemia diet were significantly reduced compared to the O-GTE specific yield. The BM and SA GTEs generated significantly less ATP as compared to the control or O-GTE on the 3rd day post-hatching larvae. These findings would suggest that the initial stages of larval development could be significantly influenced by the type of nutrition provided, and the ATP synthesis during this critical feeding phase does vary depending on the dietary source. This variation in ATP yield could have implications for the overall energy metabolism and growth potential of the larvae, highlighting the importance of understanding the nutritional characteristics and effects of different feeding options in aquaculture practices.

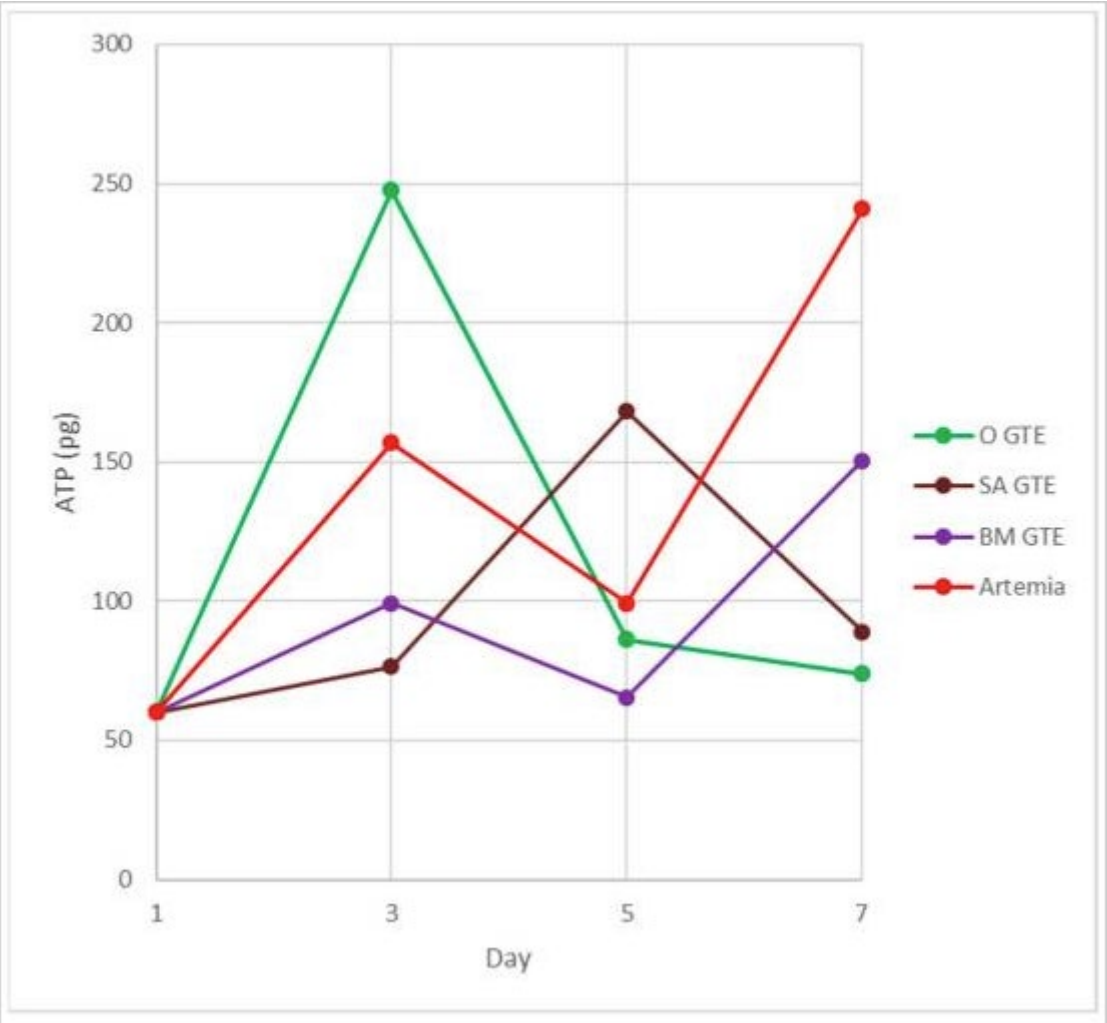

Figure 30. **Fish feed-induced ATP synthesis in carp larvae. The colors indicate the fish feed: green—O-GTE; red—control, i.e., brine shrimp (Artemia salina); brown—SA-GTE; purple—BM-GTE. (Source: Aleya et al., 2023)**

Interestingly, the O-GTE's ATP yield was over three times higher than that of the SA-GTE, approximately 2.5 times more than the BM-GTE, and 1.5 times greater compared to the brine shrimp (see Figure 31). These observations would also suggest that O-GTE may have a greater potential to induce ATP synthesis that might be due to its phytonutrient profile (see paragraph 4.1.2 and Table 5). Among the O-GTE phytocomponents, the hydroxytyrosol could be particularly interesting since its implication in regulating mitochondrial activity has been demonstrated.

Table 13. **The nutritional effect of GTEs on carp larvae model**

Feed	**Eggs (Day 0)**		**Non-Feeding Larvae (Day 1)**		**Feeding Larvae (Day 3)**		**Feeding Larvae (Day 5)**	**Feeding Larvae (Day 7)**	
	Length (mm)	**ATP (ng)**	**Length (mm)**	**ATP (pg)**	**Length (mm)**	**ATP (pg)**	**ATP (pg)**	**Length (mm)**	**ATP (pg)**
O-GTE	1.95 ± 0.05	77.29	5.84 ± 0.10	51.71	7.21 ± 0.09	247.67	86.19	7.77 ± 0.16	74.02
SA-GTE						76.56	168.07		88.98
BM-GTE						99.37	65.403	8.74 ± 0.09	150.58
Artemia						156.92	99.372		240.83

Note: Artemia represents the brine shrimp type of control food.

Next on the 5th day after larval hatching, the ATP levels were also assessed, and both the O-GTE and the Artemia control related ATP yields were relevantly decreasing. The BM-GTE also featured a reduced ATP amount, while the SA-GTE relatively doubled the generated ATP production. Moreover, the length of larvae did not seem to change by the 5th day of larval development (not shown on figure 29).

At the 7th days after hatching timepoint, the larvae fed with all three types of GTEs exhibited similar body lengths, which were slightly shorter compared to the control group. Moreover, by day 7, there was a substantial increase in ATP concentration in the larvae fed with brine shrimp, and a somewhat lesser yet rising ATP content was observed in larvae fed with BM-GTE. However, this was only 60% of the ATP level found in the control group. In contrast, the ATP concentration in larvae fed with O-GTE and SA-GTE visibly decreased by day 7. Remarkably, by day 9 post-hatching, only the larvae fed with brine shrimp were still alive.

These results demonstrate that initially, on day 3 post-hatching, the O-GTE displayed superior ATP-producing capabilities compared to the control brine shrimp-fed larvae. However, by day 5, an increase in ATP was only detected in larvae fed with SA-GTE. By

day 7, the rise in ATP content was evident solely in larvae fed with BM-GTE, and the day 9 post-hatching timepoint was not reached by any of the fish larvae fed by GTEs. These observations are indicating that the assessed GTEs did feature limited nutritional potential since they were able to support ATP systems to a limited extent. However, no immediate toxic effect was revealed for any of the GTEs analyzed, having in mind that the 3 to 7 days carp fish larval period seemed to be a metabolically active period. It is also interesting to note that from the 5th day, the carp larvae require sustained amounts of food to meet the ever-increasing energy requirements to support their physiological activities. The O-GTE specific ATP synthesis boosting effect at the onset of larval development is an important discovery that should be further analyzed and combined with sustainable macronutrient sources to obtain a more proficient fish larval feed.

5. CONCLUSIONS, RECOMMENDATIONS

The presented research data are indicating that the evaluation of GTEs specific phytonutrient profiles and nutritive potential brings about an unprecedented but important discovery. Based on the contained phytonutrients, the GTEs are expected to generate important health-promoting mechanism(s). However, through their nutritive properties they would also promote the synthesis of ATP that could further increase the efficiency of GTE based healing processes. This kind of perspective makes it even more exciting to carry out studies that will provide additional encouraging and, above all, more direct evidence in support of this novel hypothesis to explain the possible health benefits of certain GTEs.

5.1. The relevance of comparative studies regarding GTEs specific biological activities

The comprehensive analysis of GTEs involved both qualitative and quantitative chemical assessments to gain a deeper understanding of the composition of these plant extracts. Figure 1 illustrates that the predominant chemical compounds found in the GTEs span various categories, including polyphenols (encompassing both flavonoids and non-flavonoids), coumarins, iridoids, alkaloids, amino and carboxylic acids, lignans, terpenoids, and vitamins. Using Ultra-High-Performance Liquid Chromatography–Electrospray Ionization-Mass Spectrometry (UPLC–ESI-MS), the study identified 45 unique phytoconstituents in olive GTE, 103 in sweet almond GTE, and 111 in black mulberry GTE, many of which are being reported for the first time in this research.

Further quantitative analysis of the polyphenols present in these GTEs has provided intriguing insights and allowed for predictions regarding potential health benefits. In the case of O-GTE, the analysis revealed three polyphenols with notable concentrations (as shown in Figure 31 of the Annex). Among these, luteolin-7-O-glucoside was found in increased quantities, suggesting that O-GTE could have significant anti-inflammatory and anti-proliferative effects. These findings are supported by existing research indicating the therapeutic potential of luteolin-7-O-glucoside in reducing inflammation and inhibiting cell proliferation, offering promising avenues for its use in health-promoting applications (Ho H. et al., 2021; De Stefano et al., 2021).

This detailed characterization of the polyphenol profiles in GTEs not only enhances our understanding of the complex chemical composition of these extracts but also sheds light on their potential therapeutic uses. The identification of specific phytoconstituents and their predicted health benefits could pave the way for further research and development of GTE-based treatments and supplements, particularly in the realms of anti-inflammatory and anti-cancer therapies.

In addition to luteolin-7-O-glucoside, the Olive GTE (O-GTE) contains rutoside as its second-most abundant flavonoid. Research conducted by others has demonstrated that rutoside not only exhibits anti-inflammatory properties but also shows potential in managing diabetes (Ghorbani, 2017). This suggests that the presence of rutoside in O-GTE could contribute significantly to its therapeutic benefits, particularly in anti-inflammatory and antidiabetic applications.

Furthermore, chlorogenic acid, identified as the third-most abundant compound in O-GTE, is known to play a role in enhancing its hypoglycemic, hypolipidemic, and anti-inflammatory properties (Miao and Xiang, 2020; Yan et al., 2020). The presence of chlorogenic acid adds to the multifaceted nature of O-GTE, suggesting a broad spectrum of potential health benefits. These findings align with existing research indicating that chlorogenic acid can help regulate blood sugar and lipid levels, while also reducing inflammation, thereby supporting the overall health-promoting potential of O-GTE.

Turning to the SA-GTE, its distribution of quantified polyphenols is detailed in Figure 32 in the Annex. Notably, SA-GTE, characterized by a higher content of rutoside and hyperoside, is expected to exhibit significant anti-diabetic and anti-inflammatory effects. This anticipation is grounded in previous studies, which have highlighted the therapeutic efficacy of these compounds in managing diabetic conditions and reducing inflammation (Habtemariam and Belai, 2018).

The presence of these specific polyphenols in SA-GTE underlines its potential as a valuable natural remedy, particularly in addressing chronic conditions like diabetes and inflammation-related disorders. The varied composition of SM-GTE, rich in bioactive

compounds, points to its potential as a multifunctional therapeutic agent, offering a natural alternative for managing various health conditions.

Rutoside and hyperoside, both derivatives of quercetin present in SA-GTE, are critical constituents in the context of metabolic regulation and anti-inflammatory processes (Hosseini et al., 2021). Even though quercetin is found in relatively small quantities in SA-GTE, its presence, along with rutoside and hyperoside, may contribute to the extract's potential in managing metabolic disorders and reducing inflammation. These compounds are understood to be actively involved in metabolic pathways, possibly influencing insulin sensitivity and glucose metabolism, as well as mitigating inflammatory responses. Additionally, the significant presence of chlorogenic and caffeic acids in SA-GTE is expected to enhance its antioxidant, anti-inflammatory, and metabolic regulatory effects (Muhammad Abdul Kadar, 2021). These acids, known for their health-promoting properties, are thought to contribute to the overall therapeutic potential of SA-GTE as indicated by previous research (Muhammad Abdul Kadar, 2021). Chlorogenic acid has been associated with antioxidant activities, possibly reducing oxidative stress and inflammation, while caffeic acid is known for its role in protecting cells and tissues from inflammatory damage.

Regarding BM-GTE, quantitative analysis has revealed high concentrations of chlorogenic acid and rutoside (as illustrated in Figure 33 of the Annex). These compounds have demonstrated efficacy in animal models, particularly in protecting mice against hypoxic conditions, indicating their potential role in cellular protection and adaptation to stress (Masoomzadeh et al., 2021). Furthermore, leaf extracts of white mulberry (*Morus alba*) closely related to BM and rich in chlorogenic acid and rutoside, have been shown to exert anti-diabetic effects in type 2 diabetic rats (Hunyadi et al., 2012). This suggests that BM-GTE, with its similar composition, could also elicit benefits for managing diabetes and the related metabolic disorders. However, research has indicated that rutoside and chlorogenic acid may exhibit a slight synergism in inhibiting thyroid peroxidase, an enzyme critical for thyroid hormone synthesis (Habza-Kowalska, 2019). Therefore, while these components of BM-GTE have demonstrated beneficial effects, their impact on thyroid function warrants careful consideration, especially in therapeutic contexts.

Chrysin and apigenin, two notable flavonoids present in the BM-GTE, have been identified for their health-promoting properties. Research has highlighted that the combination of these flavonoids exhibiting anti-inflammatory (Zhang, 2021) and antioxidant (Ożarowski and Karpiński, 2021) effects, contributing to the potential therapeutic potential of BM-GTE. These properties suggest their usefulness in mitigating oxidative stress and reducing inflammation, which are key factors in various chronic diseases. However, it's important to note that studies have also revealed a contrasting aspect of chrysin, showing it to exhibit cytotoxicity at relatively low concentrations (2 μM), (Tsuji and Walle, 2008). This finding underscores the need for careful consideration and balance in the use of these compounds, especially in therapeutic dosages and formulations.

In our comprehensive analysis of the studied GTEs, it has been observed that all three of them displayed significant rutoside content. However, each GTE has its unique composition that distinguishes it from the others. O-GTE is characterized by higher amounts of luteolin-7-O-glucoside, while SA-GTE has a more substantial content of hyperoside, and BM-GTE is notable for its chlorogenic acid content. These specific polyphenol combinations in each GTE could serve as quantitative markers, helping to differentiate and identify the extracts based on their unique phytochemical profiles.

Regarding their potential health benefits, these GTEs exhibit similarities in their anti-inflammatory and anti-hyperglycemic effects. The presence of polyphenols like rutoside, luteolin-7-O-glucoside, hyperoside, and chlorogenic acid in these extracts suggests that they could be beneficial in managing conditions related to inflammation and blood sugar regulation. Such similarities in their pharmacological profiles suggest that, despite their different composition, they may have overlapped therapeutic potential, particularly in the treatment of inflammatory and metabolic conditions.

Overall, the diverse range of polyphenols present in these GTEs highlights their potential as natural remedies with multiple possible health benefits. The specific polyphenolic content of each extract not only defines its unique identity but also contributes to its therapeutic efficacy, offering promising avenues for natural health product development and alternative therapies for various health conditions.

5.2. The olive GTE associated biological effects

In the case of the O-GTE, a detailed analysis revealed a particularly rich diversity in certain compound categories. Notably, flavonoids and iridoids constituted the most abundant types of compounds found within this extract. These two classes of compounds are known for their anti-inflammatory and antioxidant properties (Aisyah Jaafar et al., 2024; Han Tao et al., 2022). On the other hand, the presence of non-flavonoid polyphenols, carboxylic acids, and vitamins was also detected in the O-GTE, though these components were represented in smaller numbers compared to the flavonoids and iridoids. Non-flavonoid polyphenols, for instance, are recognized for their antioxidant capabilities, while coumarins have been studied for their potential pharmacological properties, including anti-tumor and anti-coagulant effects (Cezar Miguel et al., 2023). Carboxylic acids play various roles in metabolic processes, and vitamins are essential for numerous physiological functions.

The variation in the concentration of these different classes of compounds highlights the complex and multifaceted nature of the O-GTE. While flavonoids and iridoids dominate the profile, the contribution of the other compounds, though less in quantity, adds to the overall potential therapeutic value of the extract. Each category of compounds brings its unique properties and benefits, contributing to the holistic health-promoting potential of the O-GTE.

Even more interesting characteristics are to be expected when comparing O-GTE with olive oils. The polyphenol content of the olive oils has been the subject of extensive research. Of the eight phenolics previously identified in olive oils – tyrosyl, hydroxytyrosyl (HT), oleuropein, pinoresinol, caffeic, ferulic, vanillic, and p-coumaric acid (Bayram B. et al.,2012) – our analysis of the O-GTE confirmed only the presence of HT and oleuropein. Studies have shown that HT can stimulate the biosynthesis of mitochondria and increase the expression of electron transport chain complexes in certain adipocytes, including ATP synthase. Additionally, HT activates the 5′AMP-activated protein kinase (AMPK), enhancing fatty acid oxidation (Hao et al.,2010), which may explain its role in preventing diabetes mellitus (Hao et al.,2010). Oleuropein, on the other hand, is known for conferring stability to olive oils and has anti-inflammatory and anti-

cancer properties (Maha et al., 2013, Loredana et al., 2023, Gorzynik-Debicka et al., 2018).

Our O-GTE analysis also detected other polyphenols such as chlorogenic acid and verbascoside, known for their antioxidant, anti-inflammatory, neuroprotective properties, antineoplastic activities and beneficial effects on carbohydrate and lipid metabolism (Atcharaporn et al., 2023, Yi Zhao et al., 2023, Alipieva et al., 2014). The O-GTE contained coumarins like esculetin, scopoletin, and dihydroxycoumarin, each studied for their antioxidant, anti-inflammatory, anticancer, antidiabetic, and cardiovascular protective effects (Cezar Miguel et al., 2023, Yasser Fakri Mustafa, 2023, Zhang, 2022). Kynurenic acid, a carboxylic acid identified in the O-GTE, is a significant metabolite of the kynurenine pathway with anti-neuroinflammatory and anti-cardiomyopathy features, crucial for maintaining normal brain and cardiac function (Parasram et al., 2018). The 12-hydroxyjasmonic acid, another carboxylic acid found, is a plant hormone involved in defense against pathogens (Aleya et al., 2023). Ginkgolic acid, also present in the O-GTE, has been shown to inhibit SUMOylation in *vitro* and *in vivo* conditions, suggesting its potential anti-cancer and anti-neurodegenerative effects (Brackett et al., 2020).

The iridoids in the O-GTE, such as oleoside, neonuzhenide, nuzhenide, oleuropein, and lingstoride, are expected to offer a range of health benefits, including neuroprotective, hepatoprotective, anti-inflammatory, antitumor, hypoglycemic, and hypolipidemic effects (Wang et al., 2020).

The vitamins identified in the O-GTE, particularly nicotinic acid (B3) and nicotinamide adenine, are vital for the coenzyme nicotinamide adenine dinucleotide (NAD+), the reduced form of NADH, and flavin adenine dinucleotide (FAD), all implicated in ATP synthesis, redox potential, and inflammation regulation (Amjad et al., 2021).

It is possible that the O-GTE specific ATP boosting effect seen in the case of the 3rd day post-hatched carp fish larvae could be attributed to the HT and nicotinic acid that could facilitate the mitochondrial ATP synthesis (Figure 31). Similarly, the viability increment observed for *Drosophila* 3rd larvae and hatched adults could be attributed to the above-mentioned olive specific phytoconstituents.

In the O-GTE, the flavonoids category includes about 20 phytonutrients such as quercetin, taxifolin, katuranin, luteolin, isoquercetin, rutin, cosmosiin, ligstroside, naringenin, apigenin, isorhamnetin, chrysoeriol, and pinocembrin. A comparison between young and

mature olive leaf extracts showed differences in the presence of oleuropein and flavonoid aglycones, with the young leaves excelling in oleuropein and glucosylated forms of luteolin being more prevalent in the mature leaves (Laguerre M., et al., 2009). Our study on the young shoots, denoted as O-GTE, corroborates these observations, confirming the presence of oleuropein and various quercetin derivatives, along with six glycosylated forms of luteolin. Two lignans and a terpenoid were also identified, known for their antioxidant, antitumor, anti-coronary heart disease, and estrogenic/antiestrogenic properties (Hu Y., et al., 2021, Cör D., et al., 2018). This data suggests the phytonutrient profile of O-GTE is highly complex, comprising various compounds with potential health-promoting effects.

Having seen the multiple health benefits associated with olive we were also curios about the antimicrobial activity of the studied O-GTE. Our experiments demonstrated that the O-GTE was an effective antibiotic that had a notable bacteriostatic impact on *B. cereus, S. aureus*, and *E. faecalis* in order of preference, while for *S. aureus* and *E. faecalis* had the most significant bactericidal property (Héjja et al., 2024). *S. aureus* is an opportunistic bacterium that can cause a variety of acute or chronic illnesses as well as infections that are challenging to treat in hospitals and other public settings. While *E. faecalis* has been observed in most healthy persons, it can also result in endocarditis, infections of the urinary system, meningitis, and ultimately sepsis. The growth of S. enterica was also inhibited by the O-GTE. In summary, O-GTE has a selective antimicrobial property for some of the microbial species tested, which may not dramatically affect the gut microbiota, but this type of antimicrobial testing should be expanded.

5.3. The sweet almond GTE associated biological effects

The SA-GTE-specific carboxylic acids, malic and citric acids, are crucial metabolites, while quinic acid's presence is promising due to its role in facilitating tryptophan and nicotinamide synthesis in the gastrointestinal tract, aiding in DNA repair, and exhibiting anti-inflammatory effects (Pero et al., 2009). The SA-GTE also contained essential and non-essential amino acids, and alkaloids like choline, an essential nutrient involved in various physiological processes, chelidonine with pro-apoptotic and anti-metastatic properties (Wiedeman et al., 2018; Herrmann et al., 2018), and berberine, known for reducing inflammation and insulin resistance (Cao et al., 2019). The vitamins in the SA-GTE were identical to those in the O-GTE. The presence of fatty acids such as alpha-

linoleic acid (ALA, omega-3 PUFA), linoleic acid (LA, omega-6 PUFA) and palmitoleic acid (omega-7 MUFA) in SA-GTE is noteworthy, as these have not been identified in other GTEs analysed. LA and ALA have neuroprotective properties (Kim and Song, 2024), while the cis- and trans-isomers of palmitoleic acid have contrasting physiological effects (Tokunaga et al.,2021). Furthermore, 2-oxindole, found in the SA-GTE, and its derivatives possess various pharmacological activities (Khetmalis et al., 2021). The aldehyde indole-4-carbaldehyde, also present, inhibits adipogenesis through AMPK pathway activation (Kang et al., 2017). Eugenol's identification in the SA-GTE raises questions about its toxicity despite its antimicrobial and anti-inflammatory properties (Mohammadi Nejad et al., 2017). Benzyl-glucoside and benzyl-primeveroside, also found in the SA-GTE, have been previously identified in other *Prunus genus* species (Poonam et al., 2011).

5.4. The black mulberry GTE associated biological effects

The presence of albafuran A/B of 2-arylbenzofuran flavonoids, with antifungal properties (Mitsuo et al., 1982), was confirmed for the BM-GTE. Chalcomoracin, another benzofuran found, possesses antibacterial and anti-cancer properties (Zhang S.R., et al., 2020, Kim Y.J., 2012). Other 2-arylbenzofuran flavonoid derivatives detected were mornigrol E and F. Kuwanon A, B, C, E, and G, initially found in white mulberry (*Morus alba*) leaves and known for their anti-inflammatory and anti-cancer properties (Ma et al., 2022; Chan et al.,2016; Zelová et al., 2014) were also present in the BM-GTE. The presence of 12 amino acids in the BM-GTE, including essential ones like lysine, arginine, threonine, isoleucine, leucine, phenylalanine, and tryptophan, and non-essential ones like proline, asparagine, aspartic acid, and tyrosine, was notable. Citrulline's presence is significant due to its role in augmenting arginine bioavailability, nitric oxide production, exercise performance, and recovery (Gonzalez, 2020). Among the carboxylic acids, malic, citric, and quinic acids were reported in the SA-GTE. However, jasmonic, hydroxyjasmonic, and tuberonic acids are specific to the BM-GTE and are odoriferous components of plant-based essential oils. All vitamins seen in the BM-GTE correspond to B2-5 types, and, together with the chelidonine alkaloid, they were also detected in the SA-GTE analysis. The other BM-GTE-specific alkaloids, 1-deoxynojirimycin (moranoline) and O-hexosyl-1-deoxynojirimycin, are alpha-glucosidase inhibitors with

antidiabetic effects (Vichasilp et al., 2011). A combination of 1-deoxynojirimycin and 1,4-dideoxy-1,4-imino-d-arabinitol was proven to inhibit glycogenolysis in the hippocampal region, preserving astrocyte glycogen reserves, which could be beneficial for delaying or preventing depression (Seidel J.L., et al., 2011). Lumichrome's presence in the BM-GTE, thought to arise from riboflavin photolysis, was notable, along with eugenol, which has antimicrobial and anti-inflammatory properties but also raises toxicity concerns (Mohammadi Nejad et al., 2017).

5.5. Antibacterial potential of GTEs

The investigation into the antimicrobial properties of olive GTE reveals that it contains a plethora of phytonutrients, which not only possess anti-inflammatory attributes but also demonstrate potential for therapeutic applications by curtailing microbial proliferation and alleviating inflammation. This dual action contributes significantly to its healing capabilities.

In studying the effectiveness of these extracts, the MIC and MBC analyses are particularly valuable. They help distinguish between bacteriostatic (inhibiting bacterial growth) and bactericidal (killing bacteria) effects. The findings indicate that olive GTE, in particular, shows bactericidal activity at lower concentrations, which is a crucial feature for developing new antibacterial agents. This lower concentration effectiveness helps prevent the development of bacterial resistance, a critical advantage in the ongoing battle against antibiotic-resistant strains.

Almond and black mulberry GTEs, on the other hand, predominantly exhibit bacteriostatic properties. They specifically target L. monocytogenes but interestingly do not display synergistic effects when used in combination. This specificity and lack of interaction suggest that each GTE retains its distinct mechanism of action without enhancement from combination.

The detailed analysis of olive GTE further underscores its robust antimicrobial properties, particularly against B. cereus, S. aureus, and E. faecalis. It demonstrates significant bacteriostatic effects and shows stronger bactericidal action particularly against S. aureus and E. faecalis—both of which are capable of causing severe infections in humans.

Moreover, the effect on S. enterica highlights its potential to combat gastrointestinal pathogens (Cavestri et al., 2022).

The broader implications of these findings emphasize the potential of GTEs as effective antimicrobial agents. Each GTE brings unique properties to the table, and while some are more effective than others, all demonstrate the capacity to inhibit microbial growth effectively. The variance in effectiveness based on concentration also suggests that dosing levels can be optimized for maximum efficacy, furthering their potential use in medical and therapeutic applications.

Thus, this research contributes significantly to the understanding of GTEs' antimicrobial capabilities and sets the stage for future applications in combating infections and disease, potentially reducing reliance on traditional antibiotics and helping manage antibiotic resistance.

5.6. The phytonutrient profiles of GTEs and their viability increasing and/or hypoglycemic effects

The performed experiments comparing the development of fruit fly larvae under NM and HS diets have yield to significant developmental differences. The HS diet induced a developmental delay of approximately three days (as shown in Figures 22 and 21). However, the inclusion of GTEs in these diets revealed interesting concentration-dependent effects on larval viability, although the developmental delay associated with the HS diet remained unchanged.

For instance, O-GTE notably enhanced larval viability in NM diets and, to a lesser extent, in HS diets at mild concentrations (Figure 22). The BM-GTE exhibited a similar trend to O-GTE, with higher concentrations demonstrating a more pronounced rescue effect on viability (Figure 9). Conversely, the SA-GTE showed only a modest increase in viability under NM diets, and no significant differences were observed under HS diets compared to the control (Figure 23). These findings indicate that while olive and black mulberry GTEs could surpass the viability threshold specific to HS diets, SA-GTE did not significantly alter it. Additionally, none of the GTEs compensated for the developmental delays caused by the HS diet. These results suggest that the distinct phytonutrient profiles

of the GTEs could be responsible for the observed variability in viability under different dietary conditions.

The qualitative and quantitative analyses of the GTEs uncovered both similarities and differences in their chemical compositions. Unique compounds such as tyrosol, hydroxytyrosol (HT), oleuropein, pinoresinol, and iridoids were exclusively found in O-GTE. In contrast, fatty acids like omega-3, omega-6, and omega-7 were specific to SA-GTE. The phytonutrient profile of BM-GTE showed similarities to SA-GTE but with the unique presence of certain flavonoids like benzofurans, specific to mulberry. Notably, nicotinic acid and nicotinamide adenine were common across all the studied GTEs, indicating their potential role in supporting ATP synthesis, redox balance, and inflammation regulation.

There have been conducted experiments to analyze the Total Polyphenol Content (TPC) and Total Flavonoid Content (TFC) of GTEs, followed by an assessment of their *in vitro* antioxidant capacities. The results showed that SA-GTE had the highest TPC and TFC, followed by BM-GTE and O-GTE for TPC. However, for TFC, O-GTE slightly surpassed BM-GTE (Figure 14). When evaluating antioxidant capacity using the Ferric Reducing Ability of Plasma (FRAP) method, O-GTE emerged as the most potent, with BM-GTE closely following the reduced antioxidant capacity of SA-GTE (Figure 15). These findings contribute to our understanding of the health-promoting properties of GTEs, highlighting their potential use as dietary supplements and natural health products.

The analysis of GTEs using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay reinforced the findings obtained through the FRAP (Ferric Reducing Ability of Plasma) method, indicating that Olive GTE (O-GTE) possesses the highest in vitro antioxidant capacity. This was followed by Sweet Almond GTE (SA-GTE) and then Black Mulberry GTE (BM-GTE) in decreasing order of antioxidant potential. It's important to note that while the FRAP method relies on an electron transfer-based assay, the DPPH method involves a mixed test related to electron/hydrogen atom transfer, indicating that these two procedures assess antioxidant capacity in different ways (Kotha R.R., et al., 2022). Despite these methodological differences, the in vitro antioxidant capacity assessments of the GTEs revealed a consistent pattern across both methods, with olive GTE leading, sweet almond GTE in the intermediate position, and black mulberry GTE showing the most reduced potential.

Several diverse studies on plant extracts have suggested that the antioxidant capacity measured in vitro may not directly correlate with *in vivo* health-promoting effects. Nevertheless, the TPC and TFC values of the GTEs could provide significant insights into the biological impact of these extracts on consumers. Recent research has highlighted the potential of polyphenols to mimic the effects of caloric restriction (CR), known as caloric restriction-mimetic (CRM) properties (Hofer et al.,2021, Yessenkyzy et al., 2020).

CR triggers a series of biochemical events, such as depletion of cytosolic acetyl coenzyme A (AcCoA), inhibition of acetyltransferases, stimulation of protein deacetylation, and induction of autophagy. These mechanisms are highly specific and evolutionarily conserved across various species, including yeast (*Saccharomyces cerevisiae*), nematodes (*Caenorhabditis elegans*), flies (*Drosophila melanogaster*), rodents (*Mus musculus*), and non-human primates (*Macaca mulatta*), (Kepp et al., 2020). Our unpublished research has shown that in fruit fly larvae subjected to an HS diet, there was a significant increase in the expression of genes like *Acetyl Coenzyme A synthase (AcCoAS)* and *Acetyl-CoA acetyltransferase1 (ACAT1),* which are involved in acetylation processes. Conversely, genes associated with deacetylation, such as *sirtuin1* (an NAD+-dependent deacetylase), *histone deacetylase 4* and *1 (HDAC4* and *1),* and *ATG8a* (a gene inducing autophagosome formation) were downregulated compared to NM-diet type of controls.

These findings suggest that the HS diet likely increases acetylation via elevated *AcCoAS* and *ACAT1* gene activities, while simultaneously reducing deacetylation through the repression of genes like *Sirt1* and *HDAC4* and *1*. Interestingly, the reversal of these gene activities is associated with the beneficial effects of caloric restriction (Barger J.L., et al., 2017), and certain polyphenols have been shown to mimic these effects (Yessenkyzy A., et al., 2020). Polyphenols such as resveratrol, quercetin, hydroxytyrosol, and myricetin, all identified in the studied GTEs, have been found to act like CRMs, influencing mitochondrial biogenesis and mitophagy (Davinelli et al., 2020). However, whether the studied GTEs can induce cellular mechanisms specific to caloric restriction remains an open question for further investigations.

The polyphenol and flavonoid compositions of the studied GTEs - O-GTE, SA-GTE and BM-GTE - have been thoroughly examined both qualitatively and quantitatively. Notably, rutoside emerges as a common flavonoid across all three GTEs. In O-GTE, the most abundant polyphenols are luteolin-7-O-glucoside and rutoside; in SA-GTE, they are

rutoside and hyperoside; and in BM-GTE, chlorogenic acid and rutoside predominate. Rutoside has been documented for its antihyperglycemic effects, operating via an insulin-mimetic mechanism that involves crucial genes in the insulin signaling pathway, such as *phosphoinositol 3 kinase* (*PI3K*), *protein kinase C* (*PKC*), *peroxisome proliferator-activated receptor gamma* (*PPARγ*), and *glucose transporter* (*Glut*), (Ghorbani A., 2017). Intriguingly, a HS diet in fruit flies was found to downregulate genes integral to insulin signaling, including *PI3K21B*, *PKC98C*, *Eip75B*, *Glut1*, and *Glut4EF*.

Rutoside (rutin, quercetin-3-rutinoside) is known for its ability to maintain intracellular NADPH levels by inhibiting aldose reductase, the enzyme catalyzing the first step in the polyol pathway, which is crucial in the conversion of glucose to sorbitol. Excess sorbitol production is a key factor in the development of secondary diabetic complications like retinopathy, nephropathy, and neuropathy (Singh M., et al., 2021). In the context of a HS diet, the expression of certain *Drosophila* genes involved in the polyol pathway, such as *Aldo-keto reductase 1B (Akr1B), CG9436*, *CG10863*, and *CG12766*, was significantly increased. This suggests that the GTEs, particularly through their rutin content, might counteract the adverse effects of sorbitol synthesis. Additionally, the potential increase in the activity of sorbitol dehydrogenases, as indicated by the upregulation of the *Drosophila sorbitol dehydrogenase genes (Sodh1 and 2)*, suggests active sorbitol to fructose conversion and the formation of advanced glycation end products (AGEs). Rutin's multiple -OH groups also indicate its capacity for in vivo free radical scavenging, potentially reducing oxidative stress and inflammation caused by hyperglycemia and neurodegeneration (Enogieru et al., 2018).

The high rutoside content in BM-GTE, and its potential association with increased viability in fruit flies subjected to a HS diet presents a complex scenario involving multiple biochemical pathways. However, the reduced *in vitro* antioxidant potential of BM-GTE suggests that its antihyperglycemic effects, leading to increased viability, might involve cellular mechanisms beyond direct oxidative stress reduction. The combination of chlorogenic acid with rutoside in BM-GTE could enhance the hyperglycemic effect, possibly through Nrf2 signaling pathway activation (Wang D., et al., 2021). Similarly, the combinations of rutoside with luteolin-7-O-glucoside in O-GTE and with hyperoside in SA-GTE might induce different cellular responses to nutritional intake.

The specific polyphenol and flavonoid profiles of the GTEs might trigger a range of interactions within cellular and organismal physiology, including synergistic, additive, and/or inhibitory effects. These interactions could impact various physiological processes, such as caloric restriction mimicry, insulin signaling, carbohydrate and lipid metabolism, polyol and Nrf2 pathways, and immunity. Therefore, a comprehensive understanding of the antidiabetic, anti-inflammatory, and neuroprotective effects of GTEs necessitates a holistic approach, considering dietary factors and the complex interplay of these biochemical pathways.

5.7. The amino acids from GTEs possess nutritive features

The nutritional needs of *Drosophila melanogaster* at different life stages, particularly during the larval stage, have been extensively studied. It is widely recognized that essential amino acids (EAAs) play a critical role in larval growth, particularly under diet manipulation scenarios (Manière et al., 2020; Chönborn et al., 2019). In experiments using holidic media (HM), considered the minimal dietary condition for complete development at 25°C, larval growth progresses linearly with a consistent water content of 85–89% over 7 days. An increase in sucrose content, like our HS diet does not enhance larval growth rate. However, doubling the presence of EAAs in HM boosts both the growth rate and protein content in larvae. Essential amino acids like arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine have been shown to support fruit fly development, egg-laying in adult females, and lifespan on an HM diet (Piper et al., 2014).

Tryptophan, an important EAA, was shown by others to play a pivotal role in protein synthesis, the production of methoxyindoles (serotonin and melatonin), and through the kynurenine pathway it regulated NAD+ synthesis (Badawy, 2017), as well as zinc and redox homeostasis (Garay et al., 2020). It also has implications in aging and lifespan (Oxenkrug et al., 2011). The absence of EAAs like arginine or isoleucine can be detrimental, while glutamate might substitute for other non-essential amino acids.

In assessing the effects of O-GTE, SA-GTE, and BM-GTE on larval and pupal viability under various dietary conditions, specific features emerged. Under a zero-nutrient (0N) diet, the O-GTE did not support larval or pupal development stages, with no visible second or third instar larvae in the media at any concentration (Figure 7 A, B). The SA-

GTE, at higher concentrations, showed minimal larval and pupal viability, indicating a reduced supporting effect over the 0N diet. Remarkably, BM-GTE overcame the nutrient-deficient nature of the 0N diet, with larval and pupal viability reaching about 26% at nearly the highest extract concentration. No significant differences were observed in the body length and ATP content of female flies raised under NM or 0N diets supplemented with either SA- or BM-GTE (Figures 18). However, in males, more variability in body length and ATP content was evident.

These findings, particularly the viability-enhancing effects of SA- and BM-GTE under 0N diet conditions, suggest that these extracts contain nutrients essential for larval development. Given the well-documented importance of amino acids, particularly EAAs, for fruit fly larval and pupal development stages (Manière et al.,2020), it is reasonable to infer their relevance for the studied GTEs. Amino acids in the body of fruit flies are detected by the fat body and certain brain cells, regulating protein synthesis and metabolism. Among EAAs, leucine is known to stimulate insulin release and larval growth in fruit flies (Ziegler et al., 2018, Manière et al., 2016), while tryptophan and phenylalanine are precursors for serotonin and dopamine-like neurotransmitters, respectively. Notably, histidine and valine were absent from all studied GTEs, SA-GTE lacked tryptophan, and methionine was missing from BM-GTE (Table 14 of the Annex). The absence of EAAs in O-GTE explains its inability to support larval viability on the 0N diet. In contrast, the exclusive presence of tryptophan in BM-GTE suggests its involvement in larval/pupal viability. It is also conceivable that factors other than tryptophan, such as individual EAA concentrations, contribute to larval viability on a 0N diet, but these may remain unidentified due to methodological limitations.

The nutritional composition of BM-GTE offers intriguing insights into its ability to support metabolic processes essential for larval and pupal development in *Drosophila melanogaster*. BM-GTE lacks certain glucogenic essential amino acids (EAAs) like histidine, methionine, and valine. However, it contains ketogenic EAAs such as leucine and lysine, and others like isoleucine, phenylalanine, threonine, and tryptophan, which are both glucogenic and ketogenic. This composition suggests that BM-GTE could effectively fuel the Krebs cycle, utilizing pyruvate and acetyl-CoA derived from these amino acids. Additionally, amino acids like threonine and isoleucine could potentially

provide an alternative entry point into the Krebs cycle through succinyl-CoA. This implies that BM-GTE has the capacity to reallocate its amino acid resources effectively to support the energy requirements for larval and pupal development.

Recent research has shown that fruit fly larvae can adapt to low-protein diets by inducing the ATF4 target genes in the fat body, specifically responding to the non-essential amino acid (NEAA) tyrosine (Kosakamoto et al., 2022). This response is crucial for adapting to protein scarcity, as it leads to a reduction in protein synthesis and an increase in food intake. Tyrosine's role in this process is both necessary and sufficient for triggering these adaptive responses. In the context of BM-GTE, the presence of tryptophan, an EAA, along with tyrosine, an NEAA, suggests that these amino acids could be minimally required for the viability of larval and pupal stages in the w^{m4h} *Drosophila melanogaster* strain. This combination might provide just enough nutritional support for the larvae to survive and develop in conditions where protein is limited, such as in the 0N-diet.

However, the exact role of tryptophan in this context remains a topic for further investigation. It is unclear whether tryptophan acts as a limiting EAA that could potentially inhibit protein synthesis in larvae beyond its specific concentration in BM-GTE. Tryptophan's involvement in various metabolic pathways, including protein synthesis and the kynurenine pathway, makes it a critical factor in larval development. Understanding whether and how tryptophan concentration in BM-GTE influences these pathways could provide deeper insights into the nutritional requirements of *Drosophila* larvae and the mechanisms through which BM-GTE supports their growth and development under nutritionally challenging conditions.

5.8. The relevance of translational model systems for GTE assessment

The analysis of physiological effects induced by plant extracts using animal models is a rapidly growing and a highly complex field of research. These models offer invaluable insights into medical science and the development of treatments for human diseases (Robinson N.B., et al., 2019). Addressing the limitations in this area of research requires innovative approaches, and one such a method would be related to the use of translational model systems. The presented study makes use of two distinct species: *Drosophila melanogaster*, a widely used insect model, and *Cyprinus carpio*, commonly known as carp that is omnivorous especially if its larval stage is considered. These two animal

species were selected for their larval developmental stages as this period is a critical life cycle period being highly sensitive to nutritional changes and demands.

Our experimental design involves assessing the effects of GTEs on these two species during their larval stages. The applied setup allows for direct comparison of the biological effects of three different GTEs on larvae of *Drosophila melanogaster*, all sharing the same genotype and age, and subjected to controlled dietary regimes. Moreover, this approach enabled us to investigate the impact of GTEs under various nutritional conditions, namely the zero nutrient (0N), normal nutrient (NM), and high sugar (HS) diets.

The 0N diet, essentially a negative control lacking nutrients, becomes a critical test when supplemented with a GTE. When larval progression, pupariation and subsequent hatching of adult flies occurs with a GTE supplement, this result strongly supports the nutritive value of the plant extract. Additionally, if GTE supplementation enhances the viability of individuals in NM conditions, it directly evidences the extract's role in promoting viability. Conversely, a rescue effect observed in the HS diet context may indicate an efficient adaptive stress response, potentially implicating anti-diabetic and/or anti-inflammatory mechanisms (Table 15 of the Annex).

The evaluation of GTEs derived from Olive, Sweet Almond and Black Mulberry alongside a normal nutrient (NM) diet has illuminated their potential to enhance viability in fruit flies. Notably, the effects of O-GTE and BM-GTE on viability were marked by a distinct concentration dependency, indicating that their beneficial effects increased with higher concentrations. SA-GTE, while also contributing to increased viability, displayed less dependence on concentration.

When these GTEs were tested with a high-sugar (HS) diet, distinct patterns emerged. O-GTE demonstrated a notably variable effect on viability, contingent on the concentration used. In contrast, SA-GTE showed a modest but consistent increase in viability, irrespective of concentration. The most striking observation was with BM-GTE, which exhibited a significant, concentration-dependent increase in viability on the HS diet. It's important to recognize that these categorizations of viability are somewhat subjective and based on our interpretation of the data. Drawing conclusions about the specific regulatory mechanisms at play would be speculative at this stage.

Furthermore, the research extended to *Cyprinus carpio* larvae allowed for a nuanced understanding of these GTEs specific nutritional impact. By measuring larval body length

and ATP content in carp larvae, it was observed that the magnitude of these parameters varied, suggesting that GTEs may provide certain nutritional benefits through their unique nutrient composition. The metabolic regulation of carp larvae must have some specificities compared to fruit fly larvae, although there must also be similarities in the biochemistry of metabolic pathways.

When the 0N diet supplemented with GTEs was used to test their nutritional potential, there was some viability effect for the SA and BM GTEs, while under the NM diet the viability increase was even more pronounced for all three GTEs. The fact that the analyzed GTEs could assist besides fruit fly larval and pupal development together with the fish larval development to variable extent should also be seen as further prof for the GTEs specific nutritive effect. This dual-species approach, using both fruit flies and carp, provides an innovative translational experimental model in which species with different physiological constitutions/parameters can be compared.

Therefore, the similarities associated with GTEs, as revealed by the proposed translational model, would suggest at least two novel directions for future research. On one side the *Drosophila* based NM and HS-diets are indicating the search for viability boosting and antidiabetic effect(s) that would be of a great relevance for diabetic people. In this respect, the combination of O- and BM-GTEs could be of particular interest, as the resulting GTE mixture would be expected to have a viability enhancing effect due to the specific phytonutrient content of olive and black mulberry, if some synergistic or additive effects are generated. The 0N diet highlighted the importance of amino acids, and particularly the dependence of fruit fly embryogenesis on tryptophan, an EAA shown by others to be involved in NAD+ synthesis and functions (Cambronne and Kraus, 2020). On the other hand, the carp larvae experiments suggest the possible relevance of GTEs for the development of novel fish feeds. All three GTEs studied should be combined to competitively support the 3-7 days post-hatch larval transition period and, if necessary, must increase the macronutrient content too.

The fact that the developed translational model offers significant and reproducible biological effects would provide further justification to delve deeper into more sophisticated studies. Such investigations would aim to unravel the genetic, molecular, and cellular underpinnings of the viability-increasing and/or antidiabetic mechanisms induced by the GTEs. This stepwise approach ensures that resources are efficiently

utilized and that the most promising avenues for further research are pursued, ultimately contributing to a better understanding of how plant extracts can influence animal physiology and potentially lead to novel therapeutic strategies for human diseases.

It is also crucial to note that throughout these studies, no toxic or hormetic effects were detected in the organisms exposed to the GTEs. This absence of adverse reactions further underscores the potential of these plant extracts as beneficial supplements in various dietary conditions, particularly in contexts where enhanced viability is desired. However, it remains essential for future research to delve deeper into the underlying mechanisms of these viability-enhancing effects and to explore the full spectrum of potential applications and implications of GTEs in both human and animal health.

6. NEW SCIENTIFIC RESULTS

1. This study stands out for its comparative approach, offering an in-depth examination of O-GTE, SA-GTE, and BM-GTE, each revealing unique phytonutrient profiles and biological effects.
2. A total of 45, 103, and 111 distinct phytonutrients were identified in O-GTE, SA-GTE, and BM-GTE, respectively, highlighting their distinct phytonutrient profiles.
3. The meticulous methodologies employed ensured consistency in experimental conditions, leading to reliable and reproducible results.
4. The specific nutritive properties of GTEs were assessed using *Drosophila melanogaster* and *Cyprinus carpio* larvae. BM-GTE significantly enhanced larval and adult survival by up to 26%, while SA-GTE supported viability at rates of approximately 2–4%, demonstrating a robust model system.
5. The dual-species approach enhanced the reliability of nutritional findings, allowing for a comprehensive comparative analysis.
6. Connections between the unique phytonutrient compositions of GTEs and their biological impacts were explored, showing that SA-GTE exhibited twice the total polyphenol content and 2.5 times the flavonoid content of O-GTE, while BM-GTE had a more balanced polyphenolic profile with both flavonoid and non-flavonoid compounds. (Flavonoids in BM-GTE: Out of the 111 identified phytonutrients, 50 are flavonoids, which make up approximately 45% of the total phytonutrient profile. Non-Flavonoids in BM-GTE: The remaining 55% consist of non-flavonoid polyphenols, amino acids, vitamins, and other types of phytochemicals.)

7. PRACTICAL RESULTS

The GTEs display a rich and varied qualitative and quantitative composition, and this study:

1. Identified several novel phytonutrients in GTEs, potentially supporting their documented physiological effects.
2. Demonstrated that GTEs enhance larval and pupal viability under normal nutrient conditions, with variability linked to phytonutrient diversity.
3. Highlighted the role of essential and non-essential amino acids in GTEs, emphasizing their importance in nutrition.
4. Found that total phenolic and flavonoid content do not directly correlate with viability effects, but specific polyphenols and flavonoids may play rescue roles.
5. Quantified ATP levels, showing that BM-GTE is the most efficient in ATP generation, with O-GTE and SA-GTE also effective under specific dietary conditions.
6. Developed a translational model using fruit fly and carp species to investigate the nutritional significance and health-promoting effects of GTEs.

In summary, this study presents a multifaceted exploration of the complex chemical compositions of GTEs and their physiological effects. It also pioneers the development of a translational model system using fruit fly and carp species, aiming to thoroughly investigate the nutritional significance and health-promoting effects of GTEs. This approach not only enhances our understanding of GTEs but also contributes significantly to the broader field of nutritional science and phytotherapy.

Proposals:

Future research areas might be proposed to further examine the nutritional and physiological effects of GTEs, building on the thorough findings of this study. Here are a few suggested studies:

- Expanded Phytonutrient Profiling and Bioactivity Assessment: To find more phytonutrients and their bioactive characteristics, carry out more thorough chemical analysis of GTEs. Using sophisticated spectroscopic and chromatographic methods may be necessary for this. The primary goals should be to isolate, describe, and ascertain the unique biological activity of new molecules.
- o Design and production of novel GTE-based feeds suitable for fish larvae by optimizing macronutrient content and feed particle size and testing them by the translational model syste.
- Molecular Mechanisms of GTEs in Metabolic Regulation: Examine the metabolic regulation, insulin signaling, and energy balance molecular pathways that are affected by GTEs in model species. Studying gene expression, proteomics, and metabolomics may be necessary to comprehend how GTEs interact with biological processes.
- GTEs in Disease Models: To assess the therapeutic potential of GTEs, animal models of human pathological conditions such as diabetes, obesity, inflammation and neurodegenerative disorders are used. This may shed light on how GTEs may be used to treat or manage certain disorders.
- Long-term Effects and Safety Studies: To evaluate the long-term effects and safety profile of GTEs, conduct long-term feeding experiments in animal models. This would need long-term surveillance for any possible hormetic or hazardous consequences.
- o Cross-species comparative studies: To study the effects of GTEs in other biological systems, the translational model system should be extended to other species, such as mammals (e.g. mice or rats). This could improve understanding of the generalizability of the findings.
- Human Clinical studies: design and carry out clinical studies to assess the safety and effectiveness of GTEs in people based on the results from animal models. This would be essential for converting the advantages shown in model organisms into possible medicinal uses for human health.

- Design and production of GTE-Based Nutraceuticals: Investigate the possibility for producing GTE-based nutraceuticals. This includes not just the extraction and formulation processes, but also the standardization of dosages and the evaluation of pharmacokinetics and bioavailability in human subjects.
- Environmental and Genetic Factors in GTE Efficacy: Examine how genetic differences in plants and environmental factors like soil quality and climate impact the phytonutrient content and effectiveness of GTEs. This might entail researching several cultivars or the circumstances under which the source plants develop.
- Integrative Approaches in Phytotherapy: Examine how GTEs can be used with other therapeutic methods, such as prescription drugs, to see whether there are any additive or synergistic benefits on the treatment of disease.
- Implications for Public Health and Nutritional Policy: Evaluate the implications for public health and nutritional policy based on the findings of these research, especially regarding integrating GTEs into dietary recommendations or treatment plans for certain groups.

By offering a comprehensive knowledge of GTEs—from their biological activity and chemical composition to their applications in both health and illness—these suggested research hope to advance the fields of nutritional science and phytotherapy.

8. SUMMARY

The use of plant extracts in medicine spans the history of mankind, encompassing different cultures and civilizations. This tradition, enriched by the collective knowledge of civilizations such as the Christian, Chinese, Indian and Islamic worlds, has evolved into the holistic approach of phytotherapy. Distinct from orthodox pharmacology, phytotherapy focuses on utilizing whole plants to promote overall health, reflecting a resurgence in interest towards natural, less forceful therapeutic alternatives, especially given the limitations of synthetic drugs like adverse secondary effects or antibiotic resistance.

This study integrates botany, chemistry, pharmacology, developmental biology, biochemistry, microbiology, and clinical sciences to investigate the medicinal properties of plant extracts, leveraging advanced analytical techniques such as NMR spectroscopy, GC-MS, and HPLC for identifying and quantifying bioactive compounds. Among these, polyphenols and flavonoids are of particular interest due to their potential for antidiabetic, anti-inflammatory, antioxidant, and neuroprotective activities, which are crucial in preventing and managing chronic diseases and in cell metabolism processes.

The research specifically targets the biological effects of olive (*Olea europaea*), sweet almond (*Prunus amygdalus*), and black mulberry (*Morus nigra*) species, known for their health-promoting properties. Olive products, for instance, are rich in antioxidants; almonds are appreciated for their lipid-lowering and anti-stress effects; and black mulberries are noted for their antihypertensive, anti-diabetic, and anti-inflammatory properties. The objective is to elucidate the corresponding GTEs nutritive and viability influencing effects, cellular mechanisms, and induction by these plant extracts through a multi-disciplinary approach, assessing their potential antimicrobial activity and contribution to nutrition and metabolism.

The preparation of GTEs involved collecting young olive shoots, sweet almond buds, and black mulberry buds, with a strict quality control process to ensure high-quality extracts. These were prepared using a mixture of 96% ethanol and glycerol at a 1:1 ratio, ensuring the preservation of the plant materials' natural qualities and therapeutic potentials. Quantitative analysis was performed using the Shimadzu Nexera I LC-MS-8045 system, highlighting the meticulous process involved in

analyzing the protein and carbohydrate contents through the Kjeldahl method and the phenol–sulfuric acid method, respectively.

The antimicrobial properties of the GTEs were investigated against a selection of bacteria and fungi, revealing varying degrees of effectiveness. For instance, olive GTE showed significant activity against five microbial strains, particularly effective within a concentration range of 50-100%. The study also explored the antimicrobial effects of GTEs using the agar diffusion method, with the olive GTE demonstrating effectiveness against specific strains at concentrations as low as 50 mg/mL.

Experiments on *Drosophila melanogaster* and carp larvae aimed to assess the GTEs effecting growth, viability, and metabolic health under different dietary conditions. In Drosophila, the HS diet caused a developmental delay, but GTE supplementation showed variable effects on larval viability without altering the delay. Olive-GTE notably improved viability in NM diets and to a lesser extent in HS diets, displaying a concentration-dependent impact. Similarly, Black Mulberry GTE demonstrated a significant increase in survival rates up to 26%, showcasing the nuanced differences in nutritional impacts of various GTEs on fruit fly development and survival.

The qualitative and quantitative chemical analyses revealed unique phytonutrient profiles for each GTE, identifying bioactive compounds such as hydroxytyrosol, oleuropein in O-GTE, and unique fatty acids in SA-GTE. These analyses highlighted the rich diversity of compounds within the GTEs and their potential health benefits, suggesting their utility in health promotion and disease prevention.

The study's findings, particularly on the antimicrobial capabilities and the nutritional effects of GTEs on model organisms, underline the complex interplay between diet, nutritional content, and health. The dual-species approach enhances understanding of the GTEs' s associated nutritive effects, emphasizing the potential of these plant extracts in developing natural health solutions and contributing to nutritional science and phytotherapy fields.

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10. *PUBLICATIONS IN THE FIELD OF RESEARCH*

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Subject: PhD Publication List

Candidate: Amina Alaya
Doctoral School: Doctoral School of Animal Husbandry
MTMT ID: 10065282

List of publications related to the dissertation

Foreign language scientific articles in Hungarian journals (1)

1. **Alaya, A.**, Máthé, A. B., Frecska, E., Máthé, E.: The olive (Olea europaea) and the almond (Prunus amygdalus) related phytonutrients, and the associated health-promoting biological effects, a review.
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Foreign language scientific articles in international journals (3)

2. Héjja, M., Mihok, E., **Alaya, A.**, Jolji, M., György, É., Mészáros, N., Turcuş, V., Oláh, N. K., Máthé, E.: Specific Antimicrobial Activities Revealed by Comparative Evaluation of Selected Gemmotherapy Extracts.
   Antibiotics-Basel. 13 (2), 1-31, 2024. EISSN: 2079-6382.
   DOI: http://dx.doi.org/10.3390/antibiotics13020181
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3. **Alaya, A.**, Mihok, E., Pecsenye, B., Jolji, M., Kertész, A., Bársony, P., Vigh, S., Cziáky, Z., Máthé, A. B., Burtescu, R. F., Oláh, N. K., Neamtu, A. A., Turcuş, V., Máthé, E.: Phytoconstituent Profiles Associated with Relevant Antioxidant Potential and Variable Nutritive Effects of the Olive, Sweet Almond, and Black Mulberry Gemmotherapy Extracts.
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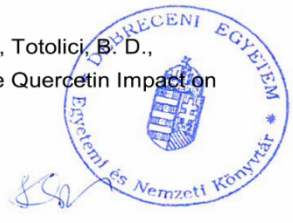

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List of other publications

Foreign language scientific articles in international journals (2)

5. Jolji, M., Pecsenye, B., Mposula, Z., **Alaya, A.**, Kiss, T., Máthé, E.: Development and comparative analysis of protein-polyphenol-fibre bars as nutritional supplements suitable for healthy senior consumers.
   Acta Univ. Sapientiae, Alim. 16 (1), 103-125, 2023. ISSN: 1844-7449.
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6. **Alaya, A.**, Oláh, N. K., Pripon Furtuna, F. R., Burtescu, R. F., Chise, E., Hepcal Cuc, I. M., Hanganu, D., Bota, V., Ivănescu, L. C., Ungureanu, O., Arsene, G. G., Turcuş, V., Máthé, E.: Regulating mechanism of blood pressure by the hawthorn and olive phyto- and gemmo-therapic extracts.
   Stud. Univ. "Vasile Goldiş" Arad, Ser. Ştiinţ. Econ. 32 (1), 37-43, 2022. ISSN: 1584-2339.

Foreign language abstracts (2)

7. Héjja, M., Mihok, E., **Alaya, A.**, Oláh, N. K., György, É., Máthé, E.: Analytical and microbiological examination of gemmotherapy extracts = Gemmoterápiás extraktumok analitikai és mikrobiológiai vizsgálata.
   In: XXIX. Nemzetközi Vegyészkonferencia = 29th International Conference on Chemistry. Ed.: Majdik Kornélia, Erdélyi Magyar Műszaki Tudományos Társaság, Kolozsvár, 1, 2023, (ISSN 2734-7109)

8. **Alaya, A.**, Máthé, E.: Comprehensive evaluation of gemmotherapy extracts generated effects using animal models.
   In: 19th Wellmann International Scientific Conference : Book of abstract. Ed.: Kiss Orsolya, University of Szeged Faculty of Agriculture, Hódmezővásárhely, 16, 2022. ISBN: 9789633068601

Total IF of journals (all publications): 16,4
Total IF of journals (publications related to the dissertation): 16,4

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

24 April, 2024

Statistical Analysis

ANOVA (Analysis of Variance) was used to compare means of three or more samples. A Post-Hoc Test identified specific group differences following ANOVA. The T-Test compared means of two groups, useful in microbial inhibition studies or viability assays. Regression Analysis examined the relationship between dependent and independent variables. The Chi-Square Test determined associations between categorical variables, particularly for microbial growth inhibition data.

11. STATEMENTS

STATEMENT

I wrote this thesis in the framework of the University of Debrecen Doctoral School of Animal Science for the purpose of obtaining a doctoral degree (Ph.D.) at the University of Debrecen.

Debrecen, 2024

Amina Alaya
PhD candidate

STATEMENT

I hereby certify that the doctoral candidate **Amina ALAYA** has carried out his/her work under my/our supervision within the framework of the above-mentioned Doctoral School between 2018.-2024 The candidate has made a decisive contribution to the results of the thesis through his/her independent creative work, and the thesis is the candidate's independent work. I/we recommend that the thesis be accepted.

Debrecen, 2024

.....
supervisor

12. ANNEXES

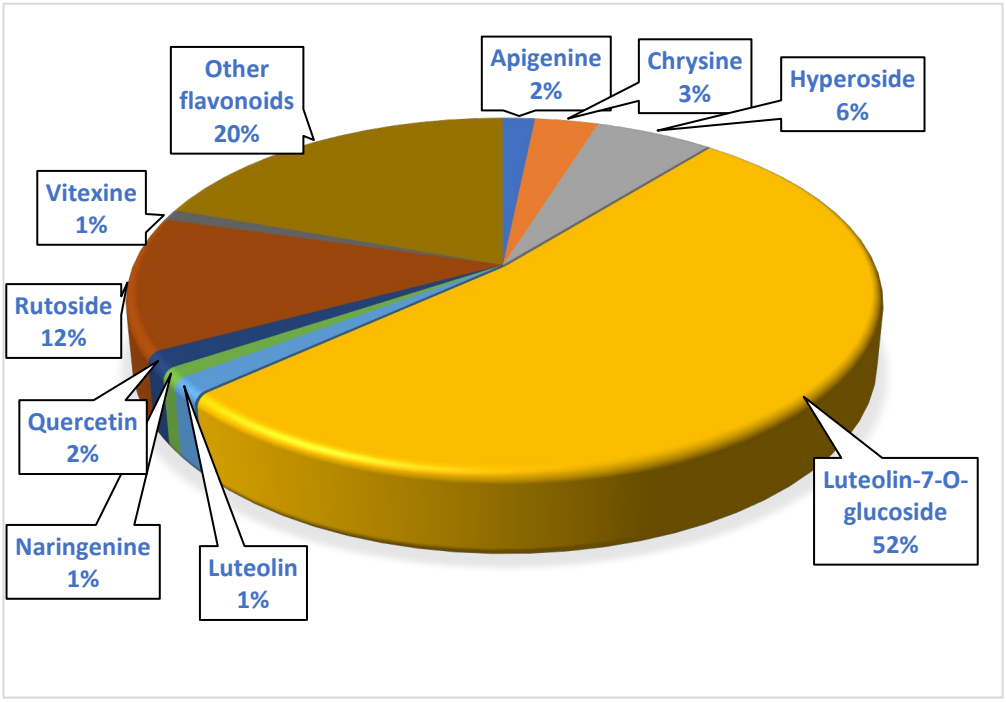

Figure 31. **The distribution of quantitatively assessed selected polyphenols in O-GTE. (Source: Aleya et al., 2023).**

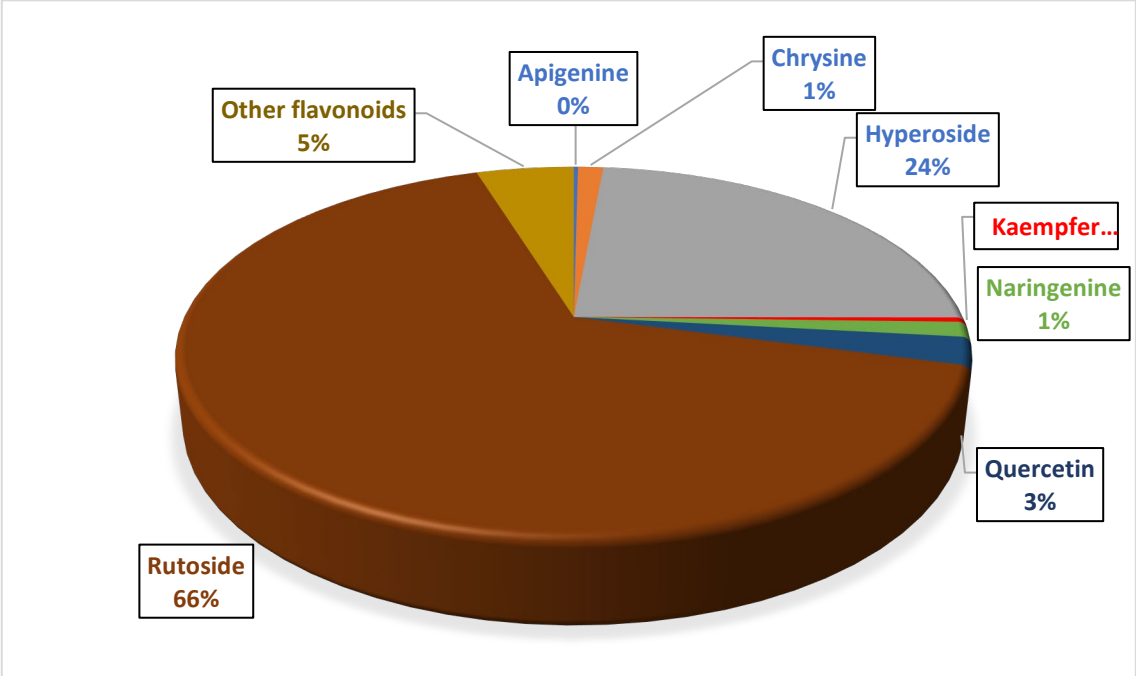

Figure 32. **The quantitatively assessed selected polyphenol distribution in SA-GTE. (Source: Aleya et al., 2023).**

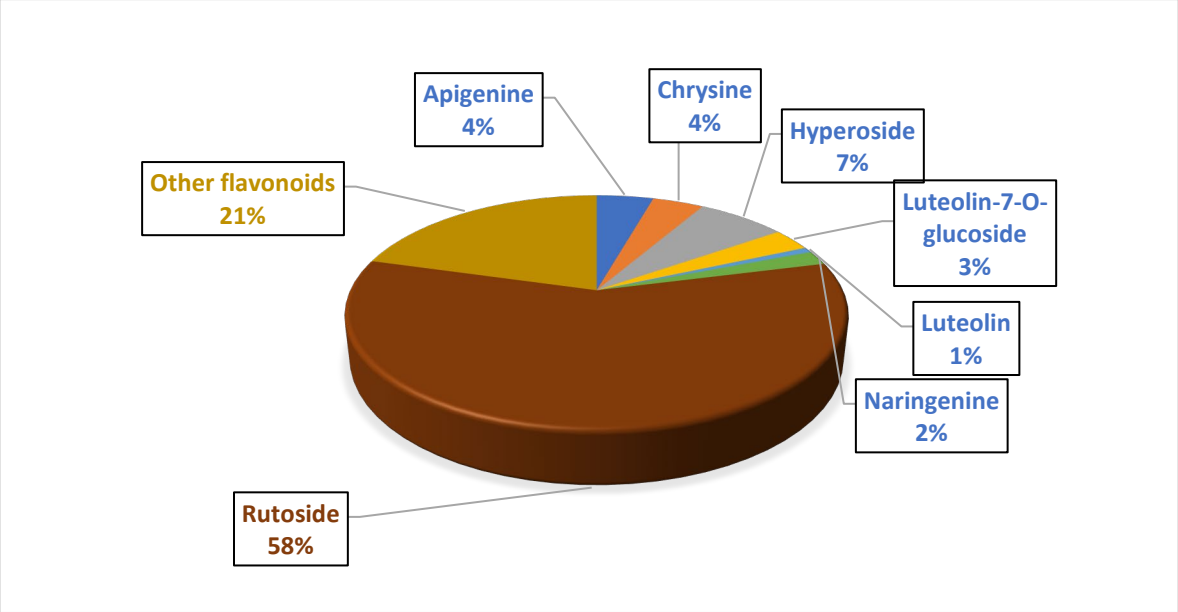

Figure 33. **The quantitatively assessed selected polyphenol distribution in BM-GTE. (Source: Aleya et al., 2023).**

Table 14. **Catabolic classification of amino acids: (*glucogenic*), (*ketogenic*), (*glucogenic/ketogenic*), (*) limiting AA.**

AA		GTE	
		SA	BM
EAA	*Arginine—Arg*	+	+
	Histidine—His	−	−
	Isoleucine—Ile	+	+
	Leucine—Leu	+	+

AA		GTE	
		SA	BM
	*Lysine *—Lys*	+	+
	*Methionine *—Met*	+	−
	Phenylalanine—Phe	+	+
	*Threonine *—Thr*	+	+
	*Tryptophan *—Trp*	−	+
	Valine—Val	−	−
NEAA	Alanine—Ala	−	−
	Asparagine—Asn	+	+
	Aspartate (aspartic acid)—Asp	+	+
	Cysteine—Cys	−	−
	Glutamate (glutamic acid)—Glu	+	−
	Glutamine—Gln	−	−
	Glycine—Gly	−	−
	Proline—Pro	+	+
	Serine—Ser	+	−
	Tyrosine—Tyr	−	+
	Citrulline	−	+
	γ-aminobutyric acid —GABA	+	−

Where the meaning are: (−) absent; (+) present in the corresponding GTEs.

Table 15. **The summary of the assessed GTE-associated viability effects. (↑) increased effect, (↑/=) variable but slightly increased effect, (↑↓) highly variable effect.**

GTE	Diet Type	Relative Viability		
		3rd Larva Prepupa	**Hatched Adult**	**Conc. Dependent Effect**
O	0N	lethal	lethal	None
	NM	↑	↑	strong
	HS	↑↓	↑↓	strong
SA	0N	weak	weak	weak
	NM	↑	↑	weak
	HS	↑/=	↑/=	weak
BM	0N	strong	strong	strong
	NM	↑	↑	strong
	HS	↑	↑	strong

Acknowledgement

I am profoundly grateful for the support and opportunity provided by **the Stipendium Hungaricum** scholarship program. This scholarship has been a pivotal element in my academic journey, and I am deeply thankful for the opportunity it has provided me. I am committed to upholding the values of excellence, innovation, and inclusivity that the Stipendium Hungaricum represents and to contributing to the academic community with the knowledge and skills I have acquired through this opportunity.

I would like to extend my deepest gratitude to the **Doctoral School of Animal Science** in Debrecen for providing me with an outstanding platform for my research endeavors. A special word of thanks goes to **Dr. Komlosi Istvan**, the head of the Doctoral School, whose leadership and vision have greatly contributed to fostering a nurturing and inspiring academic community.

My heartfelt appreciation is reserved for my supervisor, **Dr. Endre Mathe**. His unwavering dedication, insightful feedback, and relentless encouragement have been the cornerstone of my research project. Dr. Mathe's expertise and mentorship have not only guided me through the challenges of my doctoral studies but have also profoundly shaped my growth as a researcher. I am immensely grateful for his patience, knowledge, and the confidence he has placed in me. I also would like to thank to the entire community of the Institute of Nutrition Science and all the coauthors of my papers, since they were outstanding partners and greatly facilitated my research activities and knowledge.

To **my parents and my siblings**, whose love, sacrifices, and belief in my abilities have never wavered, I owe everything. Your endless support and encouragement have been my strength and motivation. Thank you for being my constant source of inspiration and for instilling in me the values of hard work and perseverance.

Lastly, I wish to acknowledge **my friends**, the family I chose in my home away from home. The journey through the PhD program was made memorable and joyful because of your companionship, understanding, and support. You have all been a crucial part of my support system, providing laughter, solace, and motivation during the most challenging times. I am fortunate to have met such wonderful individuals who have enriched my life in countless ways.