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Pharmacological, biochemical and behavioral approaches to the effects of selected essential oils (Lamiaceae) with therapeutic potential for dementia conditions

PHD THESIS SUMMARY

PhD supervisor:

Univ. Prof. skilled dr. LUCIAN HRIȚCU

PhD student:

LUMINIȚA CĂPĂȚÎNĂ

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Content

KEYWORDS:

1. REO - Rosmarinus officinalis essential oil;
2. TEO - Thymus vulgaris essential oil;
3. OEO – essential oil of Origanum vulgare subsp. hirtum
4. AD – Alzheimer's disease;
5. Beta-amyloid (Aβ) deposits;
6. Hyperphosphorylation of tau protein;
7. Neurofibrillary tangles (NFTs);
8. Neurotrophins (NT);
9. Acetylcholinesterase (AChE);
10. Scopolamine – Sco (100 mμ);
11. Muscarinic cholinergic receptors (mAchRs);
12. Amyloid precursor protein (APP);
13. Apolipoprotein E (ApoE);
14. Model of neurodegeneration;
15. Hypocretin / orexin neurons (Hcrt);
16. Medial pallium (MP);
17. Dorsal palaeal division (DP);
18. Ventral pallium (VP);
19. Lateral pallium (LP);
20. Novel tank test (NTT);
21. The teleost telencephalon;
22. Superoxide dismutase (SOD);
23. Malondialdehyde (MDA);
24. Ecotoxicity;
25. Carbonylated proteins;
26. BDNF – neurotrophic factor derived from the brain;
27. Neuropeptide Y (NPY);
28. Creb1 ;
29. The Y maze test;
30. Novel object recognition test (NOR);
31. The T-maze test;
32. Quantitative real-time polymerase chain reaction (RT-qPCR

Introduction

Alzheimer's disease (AD) is one of the most common causes of mental deterioration in the elderly, accounting for approximately 50% - 60% of all dementia cases in people over 65 years of age. In 2010 it was estimated that 36 million people are affected by AD and this figure is expected to increase to 66 million by 2030 (Wimo et al., 2013). Pathological signs of AD consist of the loss of function of cholinergic neurons and decreased levels of acetylcholine (ACh) in the brain of AD patients. The most notable pathological feature of AD is amyloid-beta (Aβ) peptide deposits and intracellular neurofibrillary tangles (NFTs) of hyperphosphorylated tau protein. Scientific research has constantly explored the pathogenesis and subsequently the types of therapeutic interventions, especially at the neurobiological level, and the discovery of neurotrophins (NTs) was a milestone that provided new insight into neurogenesis and neuronal survival (Song, Yu and Tan, 2015).

The zebrafish (Danio rerio) is an important experimental model used in the fields of genetics, neurophysiology and biomedicine. Laboratory studies on zebrafish behavior have focused mainly on growth, feeding, and learning (Kalueff and Cachat, 2011). The organization of the zebrafish brain is similar to that of other vertebrates, despite smaller cerebral hemispheres, optic lobe structure and function, having similarly defined areas, such as the lateral pallium structures in the telencephalon, which appear to be homologous to the mammalian hippocampus (Santana, Rico and Burgos, 2012).

The use of essential oils (EO) and their components is known from traditional medicine and aromatherapy for the treatment of various diseases, being recently intensively studied due to their contribution to naturopathy. Numerous clinical studies using EOs have confirmed their benefits against neurodegenerative disorders, especially AD, mainly on the specific pathological aspects of dementia including Aβ plaques, NFTs, cholinergic hypofunction, oxidative stress and glutamatergic abnormalities, but also on the symptoms of other neurological disorders, such as anxiety, depression, epilepsy and seizures (Ayaz et al., 2017).

Studies in the specialized literature support the fact that the essential oils of Rosmarinus officinalis (REO), Thymus vulgaris (TEO) and Origanum vulgare subsp. hirtum (OEO) counteracts the decline in cognitive performance and enhancement of the anxiety response resulting from scopolamine treatment through a mechanism involving attenuation of brain oxidative stress and regulation of acetylcholinesterase (AChE) activity. They also have

reparative effects on memory and behavioral disorders produced by scopolamine and may have beneficial effects in the treatment of AD (Ozarowski et al., 2013; TOPCU and KUSMAN, 2014; Komaki et al., 2016).

In this PhD thesis we aimed to highlight the effects of these three essential oils (REO, TEO and OEO) selected from the Lamiaceae family, against the typical symptoms of AD disease, using an animal model of dementia represented by the zebra fish (Danio rerio).

The general objective of the doctoral thesis was fulfilled as a result of the achievement of the following specific objectives:

1. In vivo induction of an animal model of dementia represented by the zebrafish (Danio rerio) using scopolamine (100 μM), a muscarinic cholinergic receptor blocker;

2. Evaluation of the effects of the essential oils taken (REO, TEO and OEO) on cognitive performance and anxious and depressive responses using the animal model (zebra fish-Danio rerio) of scopolamine-induced dementia;

3. Evaluation of the antiacetylcholinesterase potential of the studied essential oils (REO, TEO and OEO) from the brain homogenates of the animal model of dementia;

4. Evaluation of the antioxidant potential of the studied essential oils (REO, TEO and OEO) from the brain homogenates of the animal model of dementia;

5. Evaluation of the effects of the studied essential oils (REO, TEO and OEO) on the gene expression of some molecular markers in the brain of the animal model of dementia;

6. Publication of the obtained results in Web of Science indexed journals with a high impact factor, as well as dissemination of publishable experimental results at national and international scientific conferences.

THEORETICAL PART

CHAPTER 1. GENERAL DATA REGARDING THE MECHANISMS INVOLVED IN THE PATHOGENESIS OF ALZHEIMER'S DISEASE

AD is a chronic neurodegenerative disease with a well-defined pathophysiological mechanism, mainly affecting the medial temporal lobe and associated neocortical structures. Regarding AD disease prevalence, literature data estimate that the number of AD patients will reach 82 million globally by 2030, and the number is expected to reach 152 million by 2050, of which the Asia region -Pacific will contribute 71 million cases. The total cost of AD is estimated at $183 billion, which could rise to $1.1 trillion by 2050. Although the most prevalent hypothesis describing AD pathology is that of Aβ, scientists have proposed several other hypotheses, including the cholinergic hypothesis. Decreased cholinergic transmission in AD is responsible for abnormalities in the cognitive and functional domains of patients with this disease. Another hypothesis is that of the neurofibrillary bands, there being a better correlation between the presence of the bands and the state of the disease's evolution; however, the Aβ hypothesis is valid as Tau protein mutations have been observed not to result in amyloid plaque deposition (Saleem and Kannan, 2018).

Neurological plaques and neurofibrillary tangles are the pathological hallmarks of AD and are consequently related to the accumulation of Aβ peptide in brain tissues and cytoskeletal changes arising from hyperphosphorylation of the microtubule-associated protein tau in neurons. Genetic, age-related, and environmental factors contribute to a metabolic shift that favors the amyloidogenic degradation of APP (amyloid precursor protein) over the physiological, secretory pathway (De-Paula et al., 2012).

AD is also defined as a progressive neurodegenerative disorder, with an average duration of approximately 8.5 years between the onset of clinical symptoms and death. Brain regions associated with higher mental functions, particularly the neocortex and hippocampus, are most affected in the characteristic pathology of AD. This includes extracellular Aβ (APP-derived) deposits in senile plaques, intracellular formation of neurofibrillary tangles, and loss of neuronal synapses and pyramidal neurons (Francis et al., 1999).

1.1. History and epidemiology

In 1907, Alois Alzheimer described the case of a 51-year-old woman whose memory was deteriorating relatively quickly, accompanied by psychiatric disorders. The woman studied died four years later. Over time, AD has been divided into two clinical conditions based on age of onset: presenile dementia affecting people under 65 years of age, while senile-like dementia, i.e. people over 65 years of age, has formerly termed senile dementia of the Alzheimer type after pioneering studies by Tomlinson, Roth, and Blessed (Castellani, Rolston, & Smith, 2010). AD is the most common form of dementia, accounting for 50-60% of all cases studied. The prevalence of this disease is less than 1% in people aged 60 to 64 years, but it shows an almost exponential increase with age, so that in people aged 85 years or > 85, the prevalence is between 24% and 33 %, especially in the west.

Apart from aging, which is the most obvious risk factor for the disease, epidemiological studies have suggested several tentative associations: low brain reserve capacity, reduced brain size, low educational and occupational level, low mental ability in youth and mental activity and reduced physical fitness in old age (Blennow, de Leon, & Zetterberg, 2006).

1.2. Clinical presentation and pathology

The clinical presentation has considerable variability based on the brain regions affected. Thus, we can talk about the well-known dysfunctions in areas such as speech, personality and judgment, vision, the association of sensory-motor function, in addition to that of memory. It is not surprising that memory and personality dysfunctions are affected relatively early, as "transentorhinal" disease followed by "limbic" disease comprise the first two general pathological stages, followed by an "isocortical" stage in late disease. In the effort to refine the early diagnostic features of AD, the new nosological entity, MCI (minor cognitive impairment), in which patients apparently exhibit cognitive dysfunction, is being reflected (Castellani, Rolston, & Smith, 2010).

Based on clinical, cognitive, and functional criteria, MCI is considered an intermediate state between individuals who are cognitively normal and those with a clinical diagnosis of dementia. In a literature study, the rate of atrophy in four regions of the medial temporal lobe, namely the transentorhinal cortex (TEC), the entorhinal cortex (ERC), the hippocampus and the amygdala, was followed in a number of MCI subjects by comparison with control subjects , by the longitudinal diffeomorphometry technique based on large-

deformation diffeomorphic metric mapping (LDDMM). More significant changes were noted in the TEC in terms of mean thickness and atrophy rate than in the medial regions of the ERC, and TEC was also the most discriminating measure compared to the other examined MCI areas and relative to controls. These findings suggest that TEC thickness could serve as a biomarker for AD in the prodromal phase (the period between the onset of initial symptoms and the chronicity of AD symptoms) of the disease (Tward et al., 2017); (Kulason et al., 2019).

At the microscopic level, the characteristic lesions of AD consist of senile or neuritic plaques and NFTs in medial temporal lobe structures and cortical areas of the brain, together with a degeneration of neurons and synapses. Several pathogenic mechanisms underlying these changes have been studied, including Aβ aggregation and deposition with plaque development, tau hyperphosphorylation with "tangle" formation, neurovascular dysfunction, and other mechanisms such as cell cycle abnormalities, inflammatory processes, oxidative stress, and dysfunction mitochondrial. The two types of lesions appear to form independently, with NFTs appearing first. Affected regions typically show synaptic and neuronal loss, with cholinergic and glutamatergic neurons being the most affected, as well as inflammation, gliosis, oxidative stress, and neuronal dystrophy (Blennow, de Leon, & Zetterberg, 2006).

1.2.1. Amyloid plaques and the amyloid cascade hypothesis

Aβ is a 40 or 42 amino acid peptide derived from APP after its sequential cleavage by β- and γ-secretases. The amyloidogenic process is initiated by the enzymatic breakdown of APP by β-amyloid cleaving enzyme (BACE1). This is followed by the catalytic cleavage of APP by γ-secretase to form insoluble proteins or Aβ. This accumulation of Aβ in neurons leads to the impairment of neurotransmission and implicitly to neurodegeneration (Ayaz et al., 2017). The physiological role of Aβ is related to the modulation of synaptic activity, in AD it accumulates forming intermediate soluble oligomers, which are synaptotoxic, which are the main constituent of dense-core plaques (mainly Aβ42) and cerebral amyloid angiopathy (mainly Aβ40).

Several studies suggest that Aβ peptide influences neuronal and synaptic activities, and Aβ accumulation in the brain causes a combination of aberrant network activity and synaptic depression. Deficiencies of inhibitory interneurons and aberrant stimulation of glutamate receptors, which can lead to excitotoxicity, appear to play important upstream roles in this pathogenic cascade (Huang and Mucke, 2012).

ACh is known to promote non-amyloidogenic processing of APP and to reduce tau phosphorylation by reducing the activity of glycogen synthase kinase 3-β, which phosphorylates tau proteins. Consequently, reduced ACh release by Aβ may initiate a feedback loop that increases Aβ production through APP processing, increases tau phosphorylation, and disrupts trophic factor homeostasis. It is highly likely that interactions between the cholinergic system, and events including tau phosphorylation and Aβ plaque production, influence the pathological cascade (Auld et al., 2002).

1.2.2. Hyperphosphorylation of tau protein

Tau protein is a microtubule-associated protein located in the axon, where it physiologically facilitates axonal transport by binding and stabilizing microtubules. In AD, tau protein is translocated to the somatodendritic compartment and undergoes hyperphosphorylation, misfolding and aggregation, giving rise to NFTs and neuropil fibers (Serrano-Pozo et al., 2011). Tau protein, associated with highly soluble microtubules, plays a central role in stabilizing microtubules, especially axons, and through a phosphorylation process, has been shown to be the main constituent of NFTs. Studies have also shown that tau phosphorylation protects neurons from acute apoptotic death by stabilizing b-catenin. Although tau protein and its phosphorylation play an essential role in normal physiology, its hyperphosphorylation is a pathological manifestation in neurodegenerative disorders, and specialized studies have shown for more than four decades that microtubule-based axonal transport and synaptic function could be impaired in AD.

Abnormal hyperphosphorylation of tau leads to impairment of its biological activity, resistance to degradation, induces conformational changes and promotes the formation of PHF, which is the main component of NFTs. Only 2–3 amino acid residues have been found to be phosphorylated in the healthy brain, but the accumulation of phosphorylation with nearly nine phosphates per molecule leads to AD and other tauopathies (Obulesu, Venu, & Somashekhar, 2011).

1.2.3. Neurofibrillary tangles

Neurofibrillary tangles, or NFTs, are intraneuronal aggregates of hyperphosphorylated and misfolded tau proteins that become extraneuronal (“ghost” tangles) when neurons bearing these “tangles” die. NFTs have a stereotypical spatiotemporal progression that correlates with the severity of cognitive decline, and their topographic diagnosis is used for the pathological diagnosis of AD. The morphological

study of NFTs can be done by impregnation with silver, as they are argyrophiles, by staining with fluorescent dyes such as Thioflavin S, to recognize the β-folded structure of paired helical filaments or by immunostaining with anti-tau antibodies (Serrano-Pozo et al ., 2011).

In AD, NFTs have been found in the cell body of several neurons in the cortex, hippocampus and basal forebrain and are composed of accumulations of paired helical filaments. Interesting observations have been reported regarding excitatory amino acids in relation to NFTs. Excitatory amino acids (EAAs), such as glutamate and aspartate, are the main transmitters of the cerebral cortex and hippocampus, and EAAs appear to play a role in learning and memory (Greenamyre and Young, 1989).

1.2.4. Apolipoprotein E

Apolipoprotein E or (ApoE) is a 299 amino acid protein encoded by the apoE gene. The apoE ε2, ε3, and ε4 alleles strongly and dose-dependently influence the likelihood of developing AD. Thus, the apoEε4 allele is associated with an increased risk for AD, being mediated, in large part, by the differential effects of the ApoE protein on brain Aβ accumulation, while the apoEε2 allele is associated with a lower risk (Verghese, Castellano, & Holtzman, 2011). ApoE activity manifests itself differently at the level of organs, with the highest activity in the liver, followed by the brain. Non-neuronal cells, mainly astrocytes and to some extent microglia, are the main cell types expressing ApoE in the brain. However, neurons can produce this protein under certain conditions, albeit at much lower levels than astrocytes (Kim, Basak, & Holtzman, 2009).

1.2.5. The cholinergic hypothesis

The cholinergic hypothesis of AD emerged in the mid-70s, from the desire to identify a clearly defined neurochemical abnormality, where to highlight the emerging role of ACh in learning and memory. There have been reports of neocortical deficits, the specific enzyme for ACh synthesis and choline acetyltransferase (ChAT), and subsequent findings of reduced choline uptake, ACh release, and cholinergic cell body loss in the basal nucleus of Meynert confirmed a substantial presynaptic cholinergic deficit (Francis et et al., 1999). The degeneration of cholinergic neurons in the basal brain and the associated loss of cholinergic neurotransmission in the cerebral cortex and other areas have contributed significantly to the deterioration of cognitive function in AD patients.

Specifically, presynaptic markers of the cholinergic system appear uniformly reduced, exemplified by reduced ChAT activity and ACh synthesis.

Cholinergic transmission to muscarinic acetylcholine receptors (mAChRs) has been implicated in brain functions such as learning and memory, and impaired neurotransmission at muscarinic cholinergic synapses may contribute to the devastating loss of memory and other cognitive abilities in AD. The molecular diversity of mAChRs is evidenced by the cloning of a five-gene family, m1–m5, that encodes related but distinct receptor subtypes and by the M1–M4 subtypes identified in tissues by in situ hybridization to localize mRNAs and selective antibodies in quantification and direct localization of proteins. In forebrain regions of interest for AD, m1, m2, and m4 proteins are the most abundant subtypes (Levey, 1996).

Scopolamine is a muscarinic antagonist that affects learning and memory for many tasks, supporting an important role for the cholinergic system in these cognitive functions. This substance is also an anticholinergic drug commonly used as a standard experimental drug to induce cognitive deficits in animals related to tests of visual recognition memory, visual-spatial recall, psychomotor speed, and visual perceptual function (Newman and Gold, 2016).

CHAPTER 2. THE ANIMAL MODEL OF DEMENTIA

The literature presents zebrafish as models that have been successfully used to simulate AD disease pathology as well as Tauopathy, due to their relatively simple nervous system and the optical transparency of embryos that allow real-time neurological imaging (Saleem and Kannan, 2018). As a popular aquarium species, zebrafish have been used in developmental biology for many years. Its current importance as a model organism stems from the work of Streisinger, who pioneered their use in molecular genetic studies of vertebrate embryology, and Kimmel, who published detailed descriptions of cell differentiation and nervous system organization in zebrafish.

Its quality as a model organism is that, being a vertebrate, it is more comparable to humans as opposed to invertebrate model species such as Drosophila, and is more amenable to genetic and embryonic manipulation than mammalian species such as mice, in which such procedures are more complicated and expensive. Danio rerio was first described by Francis Hamilton, a British East India Company surgeon stationed in West Bengal in the early 19th century. He published "An account of the fishes found in the River Ganges and its branches" in 1822, which included 10 Danio species. Subsequently, D. rerio was assigned to the subgenus Brachydanio, along with the other small Danio species, with short dorsals and a reduced lateral line (Kalueff et al., 2010).

2.1. Typology of experimental animal models

The generation of animal models is particularly relevant because they have been designed to test neurodegeneration with similar characteristics to those in the human brain, allowing us to design new therapeutic approaches. These models are key tools for in-depth studies of neurodegenerative diseases.

Although many studies of AD have been based on experimental models in mice, as their genome is almost 99% homologous to humans, the causes of AD remain unknown and more efforts are needed to decipher the mechanisms underlying neurodegeneration. Therefore, additional animal models with complementary advantages should be used to analyze the basis of neurodegeneration and subsequently to evaluate the effects of new drugs. In this regard, the zebrafish has become an intensively used animal in neurobehavioral studies because it exhibits neuropathological and behavioral phenotypes that are quantifiable and has recently been proposed as a valid experimental paradigm for studying AD (Santana, Rico, & Burgos, 2012).

Zebrafish possess genes orthologous to those mutated in familial AD (FAD), and research using this model has revealed unique features of these genes that have been difficult to observe in rodent models (Newman, Ebrahimie, & Lardelli, 2014).
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2.2. Conditions to be followed in experimenting with zebrafish in the laboratory

Zebrafish are a shoal/group only species, and have a natural tendency to form mixed-sex groups in the wild and in captivity, with groups forming based on both visual and olfactory cues. Zebrafish are diurnal, exhibiting the highest levels of activity throughout the day, particularly in the early morning. They sleep most frequently at night, and this clear pattern of circadian activity governs many physiological, biochemical and behavioral processes in fish and is highly dependent on the establishment of a regular photoperiod when they are maintained in artificial environments. Disruptions to the light cycle can be extremely problematic for zebrafish kept in the laboratory, particularly in terms of maintaining the reproductive cycle (Harper and Lawrence, 2011).

They are omnivores, their natural diet consisting mainly of zooplankton and insects, although phytoplankton, detrital algae and vascular plant material, invertebrate spores and eggs, arachnids, sand and silt have also been reported from gut content analysis. The high proportion of planktonic elements in their diet indicates that zebrafish feed primarily in the water column, but feeding on terrestrial insects and arachnids has also been detected, suggesting surface feeding as well (Cachat et al., 2011).

Animals used in the experiments were purchased from a local pet store and placed in 8 L aquaria (10 fish per aquaria), at 18–22 °C, with constant filtration and aeration, a natural light cycle (with approximately 13 h light / 11 h dark) and fed 14 times a week with food. At least one week of acclimatization was required from the purchase of the zebrafish to the start of the experiment. Because it is difficult to distinguish each individual fish, before the start of the experiments, the animals were placed individually in an aquarium 25 cm long, 11.5 cm wide and 15 cm high. Animal husbandry and all behavioral experiments were performed in accordance with "Directive 2010/63/EU of the European Parliament and

of the Council of 22 September 2010", on the protection of animals used for scientific purposes.

2.3. Advantages and disadvantages of using zebrafish as an AD model

The zebrafish shares structural and physiological properties more with the human organism than with invertebrate models. Most human genes have orthologs that can be found in zebrafish and typically show similar expression patterns, and the orthologous proteins are approximately 70% identical in terms of their amino acid residue sequence. Fertilization of embryos is external, and the developmental model facilitates their experimental observation and manipulation, and they can be exposed to chemicals by placing them in the embryo support medium. Unlike placental mammals, chemicals are not metabolized by the host before reaching the developing embryo. Females tend to be larger than males in both domesticated and wild populations (Conference, Aucklsi, & New, 2022).

Disadvantages include that it is not a mammal, it is poikilothermic, and developing embryos lack a placenta. This means that some drugs may be metabolized in a different manner or at least at a different rate compared to mammals and this may alter their function. Another potential disadvantage is genetic redundancy in the zebrafish genome, which results from the duplication of the fish genome following the phylogenetic divergence of fish and mammals. This redundancy can complicate the comparison of homologous developmental pathways in these taxa (Detrich, Westerfield, & Zon, 2009).
Among the new approaches, the zebrafish has emerged as a popular alternative animal model, and according to the 2010 European Commission Directive, experiments with the early life stages of some animals are not regulated as animal studies. For zebrafish, independent feeding, which begins around 5 days post fertilization (dpf), is considered the first regulated stage for animal experimentation. Beyond the known advantages of gene similarity, concordance of cellular mechanisms, and comparable tissue biology, toxicology studies using zebrafish take into account the capacity of the experimental model for regeneration, as mentioned earlier in this subchapter. This regenerative capacity can impact targets of translational toxicity, as has been demonstrated for the retina. According to behavioral studies, despite the strong association of brain regions between zebrafish and humans, the neocortex is absent in zebrafish, but the neurotransmitter systems (dopamine, GABA, glutamate, norepinephrine, serotonin, histamine, and acetylcholine) are present in the model of animal taken in our study and therefore may serve as a pharmacological and toxicological target (Cassar et al., 2020).

A disadvantage in using the zebrafish as an experimental model is the lack of a system of methods for the generation of embryonic stem cells, for the "elimination" genes through homologous recombination. In the absence of such methods, a cooperative and synergistic game of "ping-pong" between zebrafish and mammal research groups is considered. Another potential drawback is the genetic redundancy in the genome of our experimental model, which most likely resulted from the duplication of the fish genome following their phylogenetic divergence from mammals. This redundancy may complicate the comparison of homologous developmental pathways in these taxa. Alternatively, additional gene copies may simplify certain types of analysis, as complex functions in mammals may have been separated and assigned to different paralogous genes in fish (Detrich, Westerfield, & Zon, 2009).

2.4. Zebrafish, experimental model in neurological studies

This animal model has the ability to regenerate the CNS, which is recommended as a neurodegeneration model to investigate the activation state of neural stem cells and to identify molecular differences between zebrafish and mammalian stem cells for use in regenerative therapies. Zebrafish is a predominant animal model used in the analysis of gene functions together with their signaling pathways during developmental and neurodegeneration processes. Researchers have used this behavioral model to study physiological behavior such as feeding, learning, hearing, sight, touch, and emotions such as fear, pain, helplessness, courtship, social interactions, anxiety, and decision making. In the embryonic zebrafish brain, endothelial cells of continuous capillaries have barrier properties, specialized to ensure homeostasis and protection of the central nervous system (Quiñonez-Silvero, Hübner, & Herzog, 2020).

In AD, the BBB is affected by reductions in cerebral blood flow and impairment of hemodynamic responses. Recent imaging and biomarker studies suggest early BBB breakdown and vascular dysregulation in AD detectable before cognitive decline. On the one hand, the flow of neurotoxic agents, cells and pathogens into the brain and the breakdown of the BBB leads to the development of enlarged perivascular spaces and ischemic changes in the brain, and on the other hand, the dysfunction in BBB transport systems leads to the development of Aβ pathology and tau and neuronal loss (Montagne, Zhao, & Zlokovic, 2017).

The similarity of zebrafish to higher vertebrates is derived from neuronal studies of adult zebrafish spinal cord, neural differentiation, and spinal network analysis. Several

anthropomorphic analyses, have described a similar behavioral pattern between zebrafish and humans, implying a well-conserved behavioral mechanism and circuit pattern of both systems. Furthermore, studies indicate similarity between the expression patterns and axonal projections of hypocretin/orexin (Hcrt) neurons in both larval zebrafish and humans (Saleem and Kannan, 2018). Aβ deposition and tau phosphorylation decrease orexin and receptor expression in the hypothalamus, while cerebrospinal fluid orexin levels increase in AD patients. In microglial cells, orexins increase Aβ levels by suppressing Aβ uptake and degradation. In zebrafish, the Hcrt network comprises ∼16–60 neurons, which, similar to mammals, are located in the hypothalamus and innervate the striatum, brain, and spinal cord (Chen, Du, & Chen, 2019).

The zebrafish telencephalon is characterized by two massive lobes covered by a T-shaped ventricle located dorsally, while in the rat the telencephalon is formed by two bilateral hemispheres that surround centrally located ventricles. The zebrafish pallium, like its mammalian counterpart, consists mainly of four pallial divisions: the medial pallium (MP) homologous to the pallial amygdala (BLA), a dorsal pallial division (DP), which corresponds topologically to the mammalian isocortex (Ctx), and the ventral pallium (VP) and lateral pallium (LP) are homologous with the mammalian pallium (basolateral), the mammalian hippocampus, and the piriform cortex (pirCtx) respectively (Mueller, 2012).

There is some experimental evidence that the palaeal area of the brain in fish is involved in distinct learning functions. Experimental evidence from behavioral studies suggests that the medial and lateral pallium of the fish brain may have similar implications in learning as their homologous structures, the hippocampus and amygdala, respectively. Thus, the medial pallium is central to emotional learning and related processes (avoidance learning) as is the hippocampus, while the lateral pallium is involved in spatial and/or relational and temporal features of learning, as is the pallial amygdala. In addition, there is another, less studied structure of the fish telencephalic pallium, known as the dorsal pallium, which has been proposed as homologous to the mammalian isocortex and transitional cortex (Vargas, López, & Portavella, 2009).

The main obstacle to identifying pallial divisions in zebrafish is the unusual development of the teleostean telencephalon. The zebrafish telencephalon develops through a unique outward folding process called eversion. A number of eversion models have been proposed, ranging from very simple to very elaborate. As a result, there is no consensus on the accuracy of the anatomical delineation of pallial homologues, such as the teleostean pallial amygdala, hippocampus, and piriform cortex (Mueller et al., 2011).

2.5. Expression of biochemical indicators of oxidative stress and neurotrophins in zebrafish

The biology of oxidative stress induced by various particles represents an important mechanistic paradigm on which to develop a predictive model for studying the toxicity of designed substances. All aerobic organisms naturally produce reactive oxygen species (ROS). The main contributors of cellular ROS are mitochondria, where oxygen acts as the final electron acceptor in the electron transport chain (ETC). Studies support that antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX)) reduce excessive ROS damage in zebrafish (Du et al., 2017).

Similar to other vertebrates, zebrafish possess an extensive antioxidant system, including the reduced form of glutathione (GSH), which is an important antioxidant acting alone or in combination with other enzymes, such as glutathione peroxidase (GPX). Upon interaction with ROS, GSH is oxidized, resulting in the formation of glutathione disulfide (GSSG) (Massarsky, Kozal, & Di Giulio, 2017). Proteins are a major target of oxygen free radicals and other reactive species during several forms of stress. An example of stress is given by the sudden temperature difference from 28ºC to 18ºC in a study, which demonstrated how the content of carbonylated proteins increases in the first hour of acute cold shock by up to 38%, followed by a sudden decrease after 6 h exposure to cold. Subsequently, brain carbonyl content increased again and was significantly above control levels at 24 h and 72 h of cold acclimation (Tseng et al., 2011).

Ecotoxicity was studied from the perspective of oxidative stress using zebrafish embryos. The zebrafish embryo test is considered to be a good substitute for the acute fish toxicity test and is successfully used to measure the toxicity of many environmentally relevant substances. SOD together with two oxidative damage products, MDA and protein carbonyl (PC), were used to estimate the induction of oxidative stress after exposure test to different concentrations of toxic agent (eg 4-Epianhydrotetracycline, a major intermediate during the progression degradation of tetracycline, frequently detected in aquatic environments) (Wang et al., 2021).

The cholinergic system, with acetylcholine (ACh) as its neurotransmitter, is involved in cognitive processes, by activating muscarinic metabotropic and nicotinic ionotropic cholinergic receptors. The zebrafish (Danio rerio) is an urgent vertebrate model for studying several biological events, such as neurochemical changes promoted by heavy metal toxicity. This teleost only possesses the gene for AChE that has already been identified, cloned and functionally detected in the brain of this animal.

Acetylcholinesterase is an important biomarker for several environmental contaminants in zebrafish. In addition, the important role of this enzyme in diseases with an increasing incidence in the elderly population, such as AD, is also known (Richetti et al., 2011).

Numerous mammalian studies have reported that neurotrophins may play a significant role in both protection and restoration of function following neurodegenerative diseases. This protective role of neurotrophins after a traumatic brain injury event, for example, has been reported in the zebrafish model. Since the discovery of nerve growth factor (NGF) in the 1950s, the family of neurotrophic factors has grown progressively. Neurotrophins regulate neuronal differentiation and play key roles in neuronal survival, growth, and plasticity.

Adult fish brains have high regenerative properties after brain injury, indicating that differences in regenerative properties between mammalian and fish brains have been attributed to remarkable processes of neurogenesis among adults in particular. Different from mouse and human, the adult zebrafish brain shows a large number of proliferative zones mainly located in 16 neurogenic niches (Cacialli, 2021). Recent findings suggest a significant role for brain-derived neurotrophic factor (BDNF) as a mediator of brain regeneration following stab injury in zebrafish. Oxidative stress and apoptosis are two essential biological processes that are affected by BDNF activity. Thus, it can be hypothesized that increased BDNF expression could modulate oxidative stress and apoptosis in the injured zebrafish brain as well (Anand, Sahu, & Mondal, 2021).

ROS-induced oxidative damage results in the increase of 8-oxoguanine in DNA, followed by repair through the base excision repair (BER) pathway. AP endonuclease 1 (Apex1) participates directly in BER, and regulates the transcription factor Creb1. Creb1 has a close relationship with normal brain development and neuronal function, while CBP is known to regulate interneuron differentiation and survival. Apex1 regulates the levels of the protein DNA polymerase ß (Polb), the next participant in the BER pathway, through Creb1. It has also been reported that BDNF activates Creb1 and regulates Apex1 in the cerebral cortex and hippocampus of mice (Pei et al., 2019).

Neuropeptide Y (NPY) controls energy homeostasis, including orexigenic actions in mammals and non-mammalians. Recently, NPY has attracted attention as a mediator of emotional behavior and psychosomatic diseases. Zebrafish deficient in the npy gene (NPY-KO) showed several anxiety-like behaviors, such as decreased social interaction in the mirror test and decreased locomotion in the black-white test. The NPY amino acid sequence is strictly conserved across vertebrates such as birds, reptiles, amphibians and

fish. Zebrafish NPY amino acid sequences have high similarity to human NPY (89%), and several studies have suggested that zebrafish NPY functions in a similar manner to human NPY (Shiozaki et al., 2020).

The transcription factor Nrf2a induces a cellular antioxidant response and provides protection against chemical-induced oxidative stress, and also plays a critical role in development and various diseases (Mills et al., 2020). Zebrafish are an important model in studying the role of Nrf2a in these processes, and they are also an important developmental model that possesses six nrf genes, including the duplicated nrf1 and nrf2 genes. Zebrafish–human comparisons demonstrate conserved synteny (possessing shared chromosomal sequences) involving nrf2 and hox genes, indicating that nrf2a and nrf2b are co-orthologs of human NRF2 (Timme-Laragy et al., 2012).

Constantly increased amounts of oxidants activate various signaling pathways that in turn target the promoters of "redox-sensitive" genes, one of which is the early growth response transcription factor-1 (Egr-1). Egr-1 encodes the zinc finger transcription factor, which belongs to the "up-down" gene group and regulates a number of genes involved in synaptic activity and plasticity. ROS have been shown to rapidly induce Egr-1 mRNA and protein expression (Pagel and Deindl, 2012). Typically, egr1 upregulation occurs at brain levels involved in learning and memory, particularly in the mammalian hippocampus. The egr1 gene has been linked to neuronal activation in fish as well. In general, egr1 upregulation is present in the teleost brain in response to systemic kainic acid stimulation that activates glutamatergic receptors (Kress and Wullimann, 2012).

CHAPTER 3. ESSENTIAL OILS SELECTED FROM PLANTS OF THE LAMIACEAE FAMILY

The family Lamiaceae (Labiatae) includes herbaceous and shrubby plants, with quadrangular, fragrant, erect, oblique or procumbent stems. The leaves of the plants in this family are opposite, with hairs or odorous glume. The bilabiate flowers grouped in biparous or condensed cymes at the base of the leaves are usually bilaterally symmetrical, hermaphrodite, rarely polygamous, in cymes placed in the whorls or spikes. Calyx is campanulate to long tubular, with 5 concretized sepals, often bilabiate, persistent. Tubular or infundibuliform corolla, with bilabiate, rarely unilabiate or even slightly radial limb. The four stamens are fixed on the corolla tube, usually didynamous, with two longer and two shorter stamens, rarely two, with free filaments, rarely concretized and inverted anthers. The fruits are in the form of obovoid or tetrahedral nuclides grouped four in a calyx, persistent, tubular, with hard, smooth walls, rarely with verrucosities or bristles (Socaci, 2020).

3.1. Therapeutic applicability of Rosmarinus officinalis

Rosmarinus officinalis is a common aromatic herb and is frequently used in dietary formulations due to its powerful antioxidant properties. Scientific studies confirm the pharmacological actions of *R. officinalis* essential oil (REO) as antibacterial, antifungal, anticancer, antioxidant and hypoglycemic. Moreover, REO stimulates the nervous system and thus increases the ability to memorize and concentrate. This oil possesses moderate AChE inhibitory activity and can act synergistically with α-pinene and 1,8-cineole, from which the inhibitory capacity of essential oils on cholinesterases can be observed through reversible binding to the active sites of AChE / BChE enzymes, preventing -degradation of the neurotransmitter ACh. At the same time, studies claim that REO can restore the action of AChE / BChE enzymes for a long period of time and consequently, the synaptic concentration of ACh is increased, resulting in the improvement of AD symptoms (Ayaz et al., 2017).

Rosemary oil has digestive, antiseptic, antispasmodic and anti-inflammatory properties. R. officinalis is used in connection with AD and dementia for the general symptoms of old age, debility and fatigue. According to literature data, there are three types of rosemary essential oils, namely the Spanish type, rich in α-pinene, 1,8-cineole and camphor; the French type, rich in α-pinene, 1,8-cineole and nail acetate, and the African oils from Morocco, Tunisia, with a high content of 1,8-cineole. A number of 22

components were identified in rosemary oil by gas chromatography (GC-MS), which represents 95.57% of the total amount (Socaci, 2020).

Chemical analysis of different types of composition of rosemary extracts shows that the most active and powerful components are triterpenes, phenolic diterpenes and phenolic acids including rosmarinic acid, carnosic acid, rosmanol, carnosol, ursolic acid and betulinic acid. Ursolic acid, a pentacyclic triterpenoid derived from rosemary, could reduce immobility time in both the tail suspension test and the forced swim test in mice. In oxidative stress research projects, REO polyphenols such as rosmarinic acid, luteolin, carnosic acid improved mood and cognition in healthy adults. Inhaling rosemary essential oil as an anti-stress and anxiolytic therapy has fewer side effects (Ghasemzadeh Rahbardar and Hosseinzadeh, 2020).

3.2. Therapeutic applicability for Thymus vulgaris

Thymus vulgaris possesses a number of biological properties that have a positive impact on human health. Cultivated in France and other countries, six chemotypes have been reported: geraniol, linalool, c (gamma)-terpineol, carvacrol, thymol and terpinen-4-ol. The anti-inflammatory properties of T. vulgaris L. essential oil were demonstrated by suppressing the enzymatic activity of 5-lipoxygenase and reducing the secretion of pro-inflammatory cytokines TNF-, IL-1 and IL-8 in THP-1 cells, which may have a potential effect anti-Alzheimer. Moreover, this essential oil has a high percentage of thymol, with the highest antioxidant activity. The volatile constituents of essential oils (monoterpenes - thymol, carvacrol, as well as β-caryophyllene) are likely to cross the BBB easily, due to their small molecular size and lipophilicity. Their volatile nature may also allow their administration in the form of inhalant vapors, therefore the consumption of Lamiaceae plants rich in thymol and carvacrol is useful in the treatment of AD, according to literature data (TOPCU and KUSMAN, 2014).

It can be given to human patients by infusion or used externally in baths to cure rheumatic and skin diseases, but both thyme and thyme essential oil are a good source of vitamins such as: vitamin A which is needed to maintain mucous membranes and of healthy skin as well as for good visibility and vitamin C that provides resistance against microbial infections and fights harmful pro-inflammatory free radicals. In addition, it is a good source of vitamin B6 or pyridoxine, which helps maintain gamma amino butyric acid (GABA) levels in the brain and acts as a stress reliever. Other vitamins present in the composition

of these plants are vitamin K, vitamin E and folic acid (Almanea, Abd El-Aziz and Ahmed, 2019).

Thyme contains high concentrations of phenols, such as carvacrol and thymol which are the main phenolic components responsible for its antioxidant activity. In addition to these, thyme oil is widely used in herbal medicine, especially to treat and provide protection against acne, hypertension, infections and cancers. Regarding animal testing, in a study with male Wistar rats, the anxiolytic effects of T. vulgaris leaf extract were investigated using the elevated plus maze test (EPM). Fat-soluble, low molecular weight compounds readily cross the BBB, such as α-pinene, limonene, linalool, and 1,8-cineole after inhalation and accumulate in organs in mice. Results of the EPM test in mice treated with TEO demonstrated the attenuation of the effects of fatigue and anxiety induced by inflammation or stress (Satou et al., 2018).

3.3. Therapeutic applicability of Origanum vulgare ssp. hyrtum

The common name oregano describes a flavor unlike a specific botanical genus or species. Subsp. hirtum, whose name comes from the Latin word -hirtus meaning "hairy", is commonly known as Greek oregano or sweet winter marjoram, is a hardy perennial herb grown mainly in herb gardens (Origanum vulgare subsp .hirtum - Plant Finder, 2023). The essential oils of Origanum vulgare subspecies vulgare and subspecies hirtum were evaluated for their antioxidant activities (DPPH, ABTS, FRAP, CUPRAC, beta-carotene / linoleic acid, phosphomolybdenum and metal chelation), antimicrobial and inhibitory properties against acetylcholinesterase, butyrylcholinesterase , tyrosinase, α-amylase and α-glucosidase. Both essential oils showed moderate antibacterial and antifungal activities. The phenolic compounds of these oils play a vital role in the neutralization and inhibition of free radicals, but they present a wide spectrum of pharmacological properties, such as anti-inflammatory, anti-cholinesterase and cardio-protective effects. The description of the composition, antioxidant, antimicrobial activity and inhibition enzymes in the oils of O. vulgare subsp.hyrtum and subsp vulgare, could be used in the development of new functional foods and drug formulations for the prevention of chronic diseases such as Alzheimer's and type II diabetes, which are related to oxidative stress (Sarikurkcu et al., 2015).

Volatile organic compounds or plant essential oils are a group of chemically diverse organic compounds with high vapor pressures and low molecular weights that allow them to diffuse easily through the gas phase. They are classified as secondary

metabolites and often have a strong odor, are colorless, non-polar, but soluble in organic solvents. Their ease of diffusion through the gas phase and through biological systems empowers them to serve as signaling molecules that can transmit information within and between organisms (Pai, Sonkamble, & Wagh, 2020). According to studies, OEO can prevent autoxidation of polyunsaturated fatty acid esters isolated from mouse brain, due to its antioxidant compounds - carvacrol and thymol. It is believed that the antioxidant capacity properties of essential oils are related to the mechanisms in which they exert their effect on oxidative stress, among which are listed: free radical scavenging activity, modulation of enzymes with antioxidant capacity (superoxide dismutase), and inhibition of pro- oxidation (Leyva-López et al., 2017).

THE PRACTICAL PART

Chapter 4. MATERIALS AND METHODS

4.1. The experimental design

In the experimental part of the doctoral thesis, I used five batches of zebrafish for each essential oil separately and for each behavioral test separately as follows: one control batch, one batch treated with Sco (100µM), and three batches treated with essential oil in three different concentrations (25, 150, and 300 µL/L) treated simultaneously with Sco (100 µM) 30 minutes before behavioral testing (Fig. 4.1.). Administration of the essential oils in the fish tank was done by immersion with detergent TWEEN 80 concentration 1% to make them miscible with water.

According to specialist studies, Sco is an amnestic agent used to study memory formation, being frequently used in combination with nootropic drugs that improve memory. It is frequently used in memory research in laboratory animals, and in addition to the accompanying symptoms of nausea and discomfort, movement problems, the prolonged condition often causes apathy and depression (Hamilton et al., 2017). The animals were housed in laboratory B-115 of the Faculty of Biology in Iasi, where both the ambient temperature of 22°C and that of the fish tank (22-24°C) were monitored.

In the first phase, zebrafish were subjected to behavioral tests (50 in total per essential oil and per behavioral test) for the first three behavioral tests, namely the novel pool test (NTT), the Y-maze test and the novel object recognition test (NOR). All these behavioral tests were performed one after the other at a distance of one day break, due to the relatively short working time, for the NTT (1-2 days depending on the number of batches), the Y-maze test (2-3 days) and for NOR (6 days). For the T-maze, things are a little different because the working protocol aims at several days of work, with several stages and the preparation before the start of the test provides for 2-3 days of starvation.

Preparation of groups of animals for T-maze testing was preceded by essential oil pretreatment for one week, starvation for three days, and then going through the three stages. Oil treatment continued throughout the test until euthanasia. It should be mentioned that on the test days, the administration of REO, TEO and OEO was done one hour before the test, and the treatment with Sco (100 µM) started from the discrimination phase and on the days when we changed the water (once three days), until the extinction stage.

After behavioral testing of the zebrafish, they were ethically euthanized. According to the protocol (Gupta and Mullins, 2010) dedicated to zebrafish organ sectioning (brain, heart, intestine, liver), we were able to prepare brain biological samples.

The animals were anesthetized in cold water at 2° C with ice, then the head was sectioned for brain sampling (Fig. 4.3.). The body is preserved for biochemistry analysis and the brain for genetic testing and AChE activity. Biochemical samples were frozen at -20° C, and samples for genetic testing were stored at -80° C.

4.2. Behavioral assessment of zebrafish

The working steps in the NTT test consist of establishing batches of adult zebrafish and testing them in groups of interest. In the first phase, the control group is tested with untreated fish and the group treated with Sco, then the groups treated with essential oil in 3 different increasing doses, simultaneously with the Sco treatment 30 minutes before the test. After ensuring that the water is deionized and softened, with optimal pH, 1.5 L of water is placed in the test aquarium. With the help of a video camera and the animal behavior study program ANY-maze, the behavior of the fish is recorded in real time. Bring the fish (age 6-8 months) into the test room to acclimate to the new environment/aquarium (around 10 fish in 6 liters of water). The test room has an ambient environment of 25 - 27 °C, being well lit, one hour before the test. Afterwards, testing begins, according to the protocol written in the ANY-maze program (Stoelting CO, Wood Dale, IL, USA). The zebrafish are transferred to the new tank with the help of a liar. To avoid stressing the animals, we maneuvered the lure in the direction the fish were swimming. We recorded fish behavior for the desired time period (eg, 6 min) using a Logitech HD Webcam C922 Pro Stream camera (Logitech, Lausanne, Switzerland) (Cachat et al., 2010).

Spatial learning and memory have been studied for several decades, being particularly relevant from a biomedical point of view. Along these lines, the cellular, synaptic, and molecular mechanisms underlying spatial learning have been investigated, but the behavioral strategies in a spatial task still raise questions. Using the Y-maze, we tested the cognitive and memory function of zebrafish, mainly assessing the response to novelty (Brinza et al., 2020). The Y-maze method can be reliably used in zebrafish, providing a novel, fast, and independent preference/avoidance task for the study of memory in this teleost. Furthermore, the results highlight the implications of glutamatergic and cholinergic systems in the memory of this experimental model.

The arms of the Y-maze were designated as follows: (1) the start arm, in which the fish started to explore the maze, being always open (A), (2) the new arm, blocked during the first session but open during the a two sessions (C) and (3) the other arm always open (B). The central portion of the maze (neutral zone) was not considered in the analysis. This

behavioral test assessed novelty response and spatial recognition by geometric cues (circles, squares, triangles) at 1 h. During the first session (training, 5 min), the fish explored only two arms (the start arm and the other arm), while the third arm (the new arm) is closed. In the second session, fish were placed back into the same starting arm with free access to all three arms for 5 min. Training and testing sessions were recorded and analyzed using the ANY-maze® program (Stoelting CO,Wood Dale, IL, USA), in which the number of arm entries, spontaneous alternations, total distance traveled, average speed, turning angle, number of lines crossed, time spent in the start arm, time spent in the other arm, and time spent in the new arm (Cognato et al., 2012).

NOR is a behavioral test commonly used to investigate memory performance in zebrafish (Brinza et al., 2020). This novelty-based test can also be adapted to assess predator avoidance or social behavior. The novel object recognition test is based on placing fish in a cylindrical tank devoid of visual cues, either individually or in a group, and after an acclimatization period, a new stimulus is introduced. Work steps in the NOR test consist of establishing batches of adult zebrafish and testing them individually. In the first phase, untreated fish and those treated with essential oil are tested, and then the test is repeated under the effect of Sco (except for the control group that remains untreated). The water is deionized and softened with optimal pH, and 6 L of water is introduced into the test aquarium. With the help of a video camera, Logitech HD Webcam C922 Pro Stream camera (Logitech, Lausanne, Switzerlsi) and the behavior analysis program ANY-maze® (Stoelting CO, Wood Dale, IL, USA) the behavior of the fish is recorded in real time of experiment. Zebrafish were maintained at a density of 10 animals per aquarium (filled with 6 L of water), with a constant 14-10 h light-dark cycle and at a temperature of 26-28°C. Fish in the primary aquarium are divided into 5 groups: control, control+Sco, rosemary (25µg/L), rosemary (150µg/L) and rosemary (300µg/L). All batches were tested according to the following scheme: habituation in the new aquarium 3 days; the training session, where the animals were subjected to two identical objects of the same color, the exposure to the agent that favors the pathology (in our case Sco which induces dementia) and the test session, after a 24 h break, the animals are placed back in the tank test that will contain one of the familiar objects from the training phase and a new object (Faillace et al., 2017).

Color preference in zebrafish was assessed using objects of different colors, and zebrafish showed a preference for the color red. In the conditioned place preference (CPP) test it spent more time in the red and green background compartments relative to the yellow background and showed a strong aversion to the blue background compartments. The T-

maze test is also seen as an investigation of spatial learning and memory, where animals are taught to discriminate between the two arms based on visual cues - colors, stimulating their olfactory, tactile or auditory senses during consecutive trials (What we can learn from zebrafish in a T-maze | Noldus, 2021). In our study, we took Colwill's protocol as a model, where we used two groups of red and green colors to track acquisition, extinction, and extinction by reversing visual discrimination in adult zebrafish. Initially the animals had to learn which of the two arms to choose according to the reward, represented by food. In each trial the fish were placed in turn in the start space of the T-maze. The Plexiglas door was released and then closed after the fish left the start space. Once the fish entered one of the arms of the maze, it was blocked with a Plexiglas gate to prevent the fish from exiting. This was rewarded after 30 seconds of entering the arm and then re-entering the start space (Colwill et al., 2005).

Over time, animal experiments in the T-maze test, using Sco as an anxiolytic agent, have been performed, especially in rodents, to elicit a certain type of memory. For example, one study investigated the effects of Sco on spontaneous alternation in the T-maze test, in which rodents alternate the arm of the maze they are exploring depending on the arm they explored in the previous trial, due to a preference for the option newer. Such a response uses working memory, a situation related to the arm explored in the previous process, which involves a muscarinic cholinergic mechanism (Caramillo, 2017).

Taking the rodent results as a model for Sco use in T-test tasks and results for spatial learning and response to change, and other similar tests such as the Y-test, we confidently used the model of zebrafish by using Sco to demonstrate the use of the species in researching cognitive impairments related to neurodegenerative diseases such as AD.

4.3. Analysis of enzyme activity in biological samples from zebrafish treated with essential oil of R. officinalis, T. vulgaris and O. vulgare ssp. hirtum

After recording the behavioral data, the zebrafish were euthanized and their brains were isolated for analysis of biochemical parameters. Collected brain samples were gently homogenized on ice with a Potter homogenizer (Cole-Parmer, Vernon Hills, IL, USA) at 200 rotations/minute for 2 minutes in 0.1 M potassium phosphate buffer solution (pH 7, 4) with 1.15% KCl. The homogenates were centrifuged at 14000 revolutions/minute for 15 minutes at 4ºC. The supernatant was used to estimate the specific activities of AChE, superoxide dismutase (SOD) and catalase (CAT), along with total reduced glutathione (GSH), carbonylated proteins and malondialdehyde (MDA) content. The protein content

was estimated by the Bradford method (Sigma-Aldrich, Darmstadt, Germany) (Boiangiu et al., 2020). An appropriate volume of 0.1 M phosphate buffer solution (pH 7.4) with 1.15% KCl was added to each weighed sample (0.1 g tissue: 1 ml buffer solution). After homogenization, the cell homogenates had were transferred to 1.5ml Eppendorf tubes and kept on ice until all homogenates were obtained. After centrifugation, the clear soluble phase of the samples was again transferred to Eppendorf tubes and kept on ice/freezer at -20 ºC.

4.3.1. Determination of protein concentration

For the quantitative determination of the soluble proteins extracted from the tissues belonging to the Danio rerio species under study, the Bradford method was used, which is based on the observation that in an acidic environment, the dye Coomasie Brilliant Blue G-250 reacts with arginine radicals and later with the remains of histidines, lysine, tyrosine, tryptophan and phenylalanine from the protein structure, forming a complex whose absorption maximum is located at λ= 595 nm.

4.3.2. Determination of superoxide dismutase (SOD) activity

Determination of SOD activity was done according to the method of Winterbourn, Hawkins, Brian and Carrell adapted by Vlad Artenie. The principle of the method has as its first aim the determination of SOD activity based on the ability of the enzyme to inhibit the reduction of NBT by the superoxide radicals generated in the reaction medium by the photoreduction of riboflavin.

4.3.3. Determination of catalase activity (CAT)

Catalase (EC 1.11.1.6) is an antioxidant enzyme, widespread in aerobic organisms, which, along with peroxidase and glutathione peroxidase, is involved in the detoxification of ROS, which are formed both in normal metabolism and in numerous pathological conditions, under the action of various oxidases and of SOD. The spectrophotometric determination of catalase activity by the Sihna method refers to the fact that the CAT enzyme acts on H2O2, after which it is inactive by adding a mixture of potassium bichromate - acetic acid. The amount of oxygenated water, remaining undecomposed after stopping the CAT action, reduces potassium bichromate to chromic acetate in the acid medium, which can be determined spectrophotometrically at λ = 570 nm. By dividing the initial and final amount of hydrogen peroxide in the reaction medium, the amount of hydrogen peroxide decomposed by catalase is found.

4.3.4. Determination of glutathione peroxidase (GPX) activity

The biological function of GPX is to reduce hydrogen peroxide to water. The determination of GPX activity was carried out by the method of Fukuzawa and Tokumura, according to which GPX catalyzed the decomposition of H2O2 by using GSH as a reducing agent. At the end of the reaction we obtained oxidized glutathione (G-S-S-H) and water. The remaining excess reduced GSH reacted with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) forming a yellow colored complex. In the spectrophotometer, at a λ=412 nm, the color intensity of the formed complex was measured. The difference between the initial and final amount of GSH is directly proportional to GPX activity (Artenie et al., 2021).

4.3.5. Determining the level of reduced glutathione (GSH)

Increased oxidative stress related to AD has been attributed to decreased levels of the brain antioxidant, glutathione (GSH). GSH (E.C. 1.11.1.9) is a major endogenous antioxidant catalyzed by enzymes, which plays a fundamental role in ROS detoxification and regulates the intracellular redox environment (Saharan and Mșial, 2014). The method for determining the total content of reduced GSH is based on the reaction of GSH with DTNB to form glutathione disulfide (GSSG) and 5-thionitrobenzoic acid (TNB), detected spectrophotometrically at 412 nm (Salbitani, Bottone, & Carfagna, 2017).

4.3.6. Determination of malondialdehyde (MDA) level

MDA is one of the reactive carbonyl compounds, decomposed as a result of the instability of lipid peroxides originating from polyunsaturated fatty acids. MDA is widely used as an indicator of lipid peroxidation and is important to use as a method in the investigation of oxidative stress (Artenie et al., 2021). MDA was measured spectrophotometrically using the thiobarbituric acid assay described by Ohkawa et al. in 1979. The reaction of lipid peroxides in animal tissues with thiobarbituric acid is dependent on the pH of the reaction mixture as in the case of linoleic acid hydroperoxide, and the optimum pH was found to be 3.5. Based on these details, a standard procedure was developed for the determination of the level of peroxidized lipids in animal tissues by their reaction with thiobarbituric acid (Noctor and Foyer, 2016).

4.3.7. Determination of the level of carbonylated proteins

Carbonylated protein content is widely used as both a marker of oxidative stress and a measure of oxidative damage. In the AD brain, studies show that increased protein oxidation (protein-carbonyl and 3-nitrotyrosine), lipid peroxidation, DNA oxidation, and

ROS formation occur. Increased levels of carbonylated proteins have been consistently reported in the hippocampus and neocortex in the context of AD (Sharma et al., 2020).

One of the procedures used to evaluate carbonylated proteins in biological samples involves the derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (DNPH), which forms a stable product dinitrophenyl hydrazone (DNP). This fragment is readily detected at an absorbance of 370 nm, and the assay can be coupled to protein fractionation by high performance liquid chromatography for greater sensitivity and specificity (Bizzozero, 2009). The method that was used in our study to quantify carbonylated proteins was described by Oliver et al. in 1987 and modified by Luo and Wehr (2009).

4.3.8. Determination of acetylcholinesterase (AChE) activity

The cholinergic system, with ACh as neurotransmitter, is involved in cognitive processes, by activating metabotropic muscarinic cholinergic receptors and ionotropic cholinergic nicotinic receptors. The reaction responsible for maintaining ACh levels is catalyzed by two cholinesterases: acetylcholinesterase (AChE) (E.C. 3.1.1.7) and butyrylcholinesterase (BuChE) (E.C. 3.1.1.8). Zebrafish possess only the gene for AChE, which is responsible for the entire degradation of ACh, BuChE being absent (Richetti et al., 2011). The Ellman method proposes detailed kinetic studies of AChE activity, where the progress of hydrolysis is followed by measurement of a reaction product. The principle of the method consists in measuring the rate of production of thiocholine, by the continuous reaction of thiol with 5 : 5 dithiobis-2-nitrobenzoate ion, to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The rate of color production is measured at 412 μm in a photometer (Ellman et al., 1961).

4.4. Evaluation of the expression of genes of interest of some molecular markers by quantitative real-time polymerase chain reaction (RT-qPCR)

In the present study, we aimed to evaluate the impact of selected essential oils from the Lamiaceae family, REO, TEO, respectively OEO, on the expression of npy, bdnf, creb, nrf2a and egr1 genes in zebrafish, through the RT-qPCR technique.

4.4.1. Extraction of total RNA from zebrafish brain samples

After completion of the four behavioral tests, groups of animals were euthanized. Later, brain samples were taken, and for genetic analysis they were preserved in order to stabilize the RNA (RNA Save-Biological Industries, CT, USA) and stored at - 80º C.

During the extraction process, from these brain tissue samples, total RNA was purified to assess npy, , bdnf, creb, nrf2a and egr1 gene expression. RNA was extracted using the Maxwell® 16 Tissue LEV Total RNA Purification Kit (Promega, Madison, USA) following the manufacturer's instructions.

4.4.2. RT-qPCR and quantification of expression levels

The RT-qPCR technique has been widely used for gene expression analysis due to its specificity, sensitivity and reproducibility. Zebrafish is a suitable vertebrate animal model for studies of molecular genetics and gene functions (Hu, Xie, & Yao, 2016). RT-qPCR, combines the effects of reverse transcription and quantitative/real-time PCR to amplify and detect specific targets. In this work, the GoTaq® 1-Step RT-qPCR kit was used to perform reverse transcription and amplification in one-step amplification reactions following the manufacturer's instructions.

4.5. Statistical analysis of experimental data

In our case, statistical analyzes were performed using the GraphPad Prism program version 8.3 (La Jolla, CA, USA), the data being expressed as means ± standard error of the mean (S.E.M.). Statistically significant differences between groups were identified using one- or two-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. A statistically significant difference was considered for a statistical significance (p) threshold of less than 0.05 ($p < 0.05$). Pearson's correlation coefficient (r) was used to correlate behavioral and biochemical parameters.

CHAPTER 5. RESULTS AND DISCUSSIONS

5.1. Effects of REO, TEO and OEO solutions on the anxious response in the novel aquarium immersion test (NTT)

In NTT testing of zebrafish, they show a strong response to novelty-induced anxiety. The test itself is based on the animal's natural instinct to seek protection in an unfamiliar environment by diving into the lower half of the new aquarium, alternating with periods of immobility and reduced aquarium exploration (Cachat et al., 2010).

By performing the NTT test, a dose-dependent improvement in upper zone exploration time can be observed in the REO-treated groups ($p = 0.0229$) compared to the scopolamine group (Fig. 5.1.B). Scopolamine treatment resulted in a significant increase in time spent exploring the lower area of the novel aquarium, denoting anxious behavior, compared to control ($p < 0.0001$). Scopolamine also affected the locomotor function of zebrafish by decreasing the total distance traveled (Fig. 5.1.C, $p = 0.0061$) and the average speed – magnitude (Fig. 5.1.D, $p < 0.0001$).

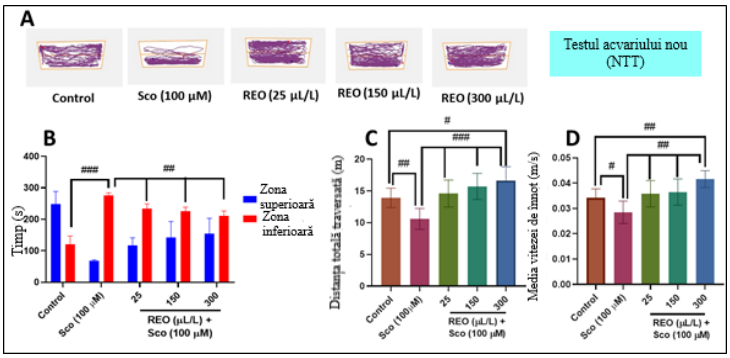

Fig. 5.1. Improvement of locomotor parameters and combating anxiety by the three doses of *Rosmarinus officinalis* essential oil (REO: 25, 150 and 300 µL/L) in the NTT test

From the results obtained on TEO in the NTT test, regarding the time spent in the upper area of the new aquarium (Fig. 5.2.B), an improvement in anxious behavior can be observed ($p < 0.0001$), unlike the group of scopolamine that recorded significantly lower values in the exploration of the upper zone, which reflects the anxious behavior of the animals in the NTT test. Scopolamine also affected the locomotion of the fish, with them being more immobile on the bottom of the new aquarium than moving around exploring the new environment. As a result, the average movement speed was significantly decreased ($p = 0.0061$) (Fig. 5.2.D), but also the total distance traveled ($p = 0.0156$) (Fig. 5.2.C).

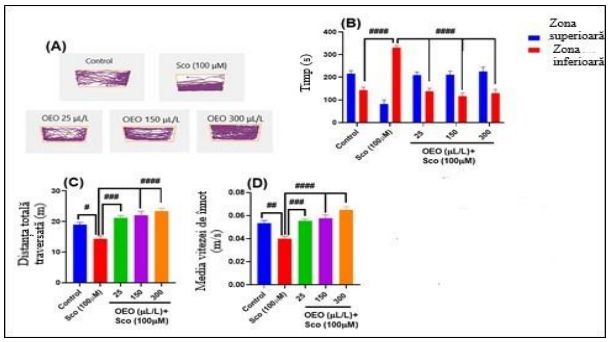

Fig. 5.2. Improvement of locomotor activity and reduction of anxiety response as a result of administration of *Thymus vulgaris* essential oil (TEO: 25, 150 and 300 µL/L) in the NTT test.

Based on recordings of locomotor parameters using the ANY-maze animal behavior analysis program, depicted in Fig. 5.3.A, it is observed that the group of zebrafish treated with scopolamine shows a high level of anxiety characterized by significant exploration of the lower area of the novel aquarium, in contrast to the control group. At the same time, we can observe an intensification of the exploration of the upper area of the new aquarium, in the case of batches treated with OEO and pre-treated with Sco (100µM), compared to the batch treated only with Sco (100µM). These data further correlate with the data obtained in Fig. 5.3.B, analyzed by one-way ANOVA test, demonstrating differences in exploration of the two zones of the novel aquarium, with a significant decrease in the time of exploration of the lower zone in the OEO-treated groups compared to the Sco-treated group (100µM) ($p < 0.0001$). The group treated with Sco (100µM) intensively explored the lower area of the NTT, which indicated the anxiogenic profile, evidenced by a significant increase in the time of exploring the lower area compared to the control group ($p < 0.0001$).

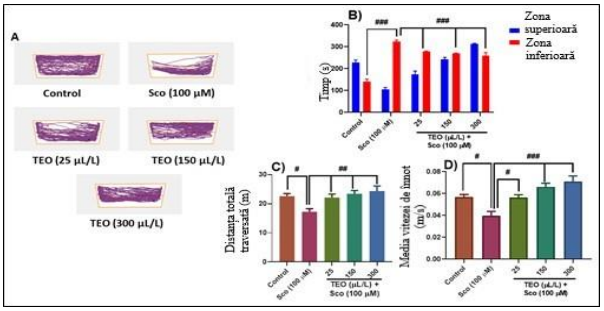

Fig. 5.3. Improvement of locomotor parameters and reduction of anxiety in the NTT test under the action of *Origanum vulgare* ssp. *hirtum* essential oil (OEO: 25, 150 and 300 µL/L)

5.2. Effects of REO, TEO and OEO solutions on spatial memory performance in the Y-test

The Y-maze is a test investigating spatial learning and memory and is used reliably in zebrafish, providing a novel, fast, and independent preference/avoidance task for the study of memory in this teleost. Furthermore, the results highlight the implications of glutamatergic and cholinergic systems in memory in this experimental model.

As in the previous test, we tested the three batches of zebrafish treated with rosemary, thyme and oregano oil respectively in three different concentrations, alongside the normal control and the one treated with scopolamine, where we followed both the response of this experimental model to the element of novelty of the Y test by the time spent in the new arm, as well as the essential motor activity of swimming by the distance covered and average speed.

By performing the Y-maze test in animal models treated with essential oils and Sco before testing (Fig. 5.4.B.), it can be seen how Sco affected the exploration time of the novel arm, as opposed to the normal control-arm exploration again, however, it was improved in groups treated at all three doses with REO ($p < 0.0001$). The Sco-treated group had numerous entries in the start arm, indicating a memory deficit. Sco also affects locomotion, as evidenced by the decrease in the total distance traveled ($p < 0.0001$) (Fig. 5.4.C) and the turning angle when passing between the three arms ($p < 0.0001$) (Fig. 5.4. D). REO treatment significantly counteracted Sco-induced locomotion and memory deficits by improving novel arm exploration ($p < 0.0001$), while total distance traveled ($p < 0.0001$) was significantly improved in REO 150 µL groups /L and REO 300 µL/L by comparison with zebrafish treated with Sco (100 µM).

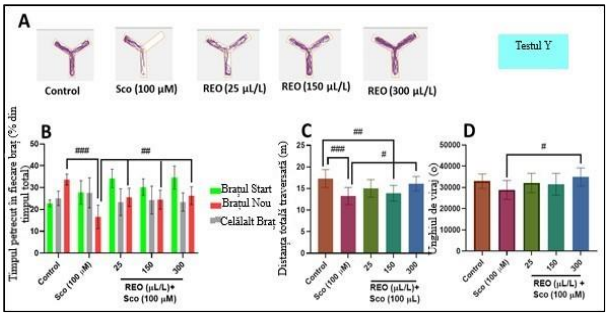

Fig. 5.4. Improvement of memory and locomotion in the Y test under the action of *Rosmarinus officinalis* essential oil in the three doses (REO: 25, 150 and 300 µL/L)

Thyme essential oil significantly improved memory deficits by increasing novel arm exploration time in a dose-dependent manner, with the best performance occurring at the highest doses TEO 150 and 300 µL/L ($p < 0.0001$). Furthermore, thyme essential oil attenuated Sco-induced hypolocomotion by increasing total maze walking distance ($p < 0.001$).

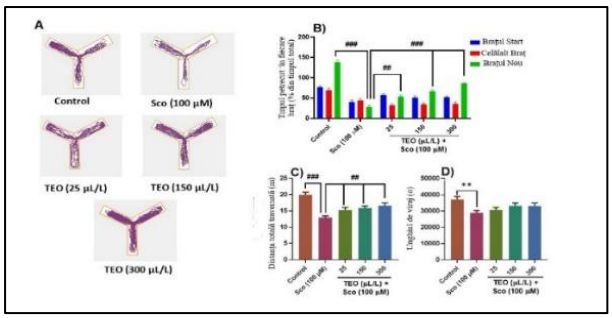

Fig. 5.5. Improvement of locomotor activity and memory performance as a result of administration of *Thymus vulgaris* essential oil (TEO: 25, 150 and 300 µL/L) in the Y-maze test

Regarding the Y-maze test, from the recordings represented in Fig. 5.6.A, the group treated with Sco (100µM) showed a deficit in the exploration of the new arm, in contrast to the groups treated with OEO, in which an improvement in spatial memory and exploratory memory was evident. The benefits of oregano essential oil in all three doses used in the present study are represented by the time spent in each arm, namely the start arm, the other arm and the new arm (Fig. 5.6.B), where the emphasis obviously fell on the exploration of the new arm, and high exploratory interest was found in the OEO 150 and 300 µL/L group ($p < 0.0001$) and OEO 25 µL/L ($p < 0.001$) in contrast to the group treated only with Sco.

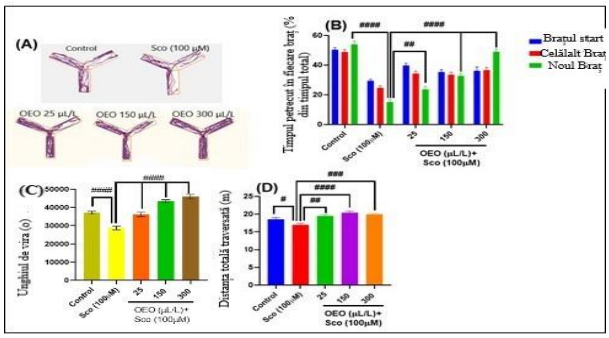

Fig. 5.6. Improvement of exploratory behavior and spatial memory following administration of Origanum vulgare ssp. hirtum essential oil (OEO: 25, 150 and 300 µL/L) in the Y-maze test

5.3. Effects of REO, TEO and OEO solutions on memory performance in the novel object recognition (NOR) test

Object recognition memory is a simple form of memory present in various vertebrates, such as rats, mice, requiring the use of cortical structures such as the perirhinal, entorhinal, and inferior temporal cortex. Assessment of locomotion in the NOR test (Fig. 5.7.A) illustrates the differences in exploring the familiar object (FO) and the novel object (NO), with the group treated with Sco (100µM) showing a strong preference to explore the FO, suggesting a memory deficit. Regarding the percentage of preference (Fig. 5.7.C), one-way and two-way ANOVA tests revealed the effect of improving memory and concentration as a result of treatment with rosemary essential oil in all doses, both in approaching the new object more than the familiar one ($F(4, 45) = 8.971$, $p < 0.0001$), as well as in the exploration times of NO, ($F(4, 90) = 44.33$, $p < 0.0001$). Animals treated with Sco (100 µM) showed lower NO preference percentages ($p < 0.1$ and $p < 0.0001$) and shorter NO exploration times ($p < 0.0001$) compared to the control group. control, while administration of REO especially at the dose of 150 µL/L improved NO preference and exploration time, suggesting improved memory.

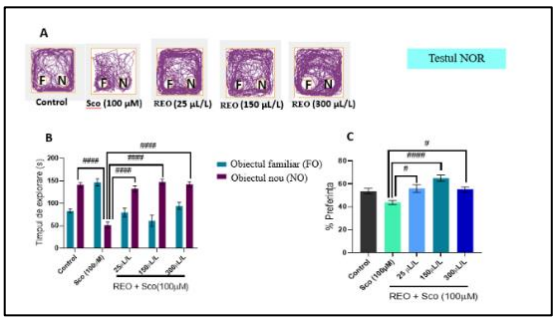

Fig. 5.7. Improvement of novel object recognition memory following administration of *Rosmarinus officinalis* essential oil (REO: 25, 150, 300 µL/L) in the NOR test

In Fig. 5.8.A is graphically represented the locomotion in the NOR test evaluated by the differences between the exploration of the familiar object (FO) and the new object (NO). The group treated with Sco (100µM) showed a high preference to explore the FO, indicating a memory deficit. Regarding the percentages of preference for NO, one-way ANOVA statistical analysis revealed significant effects of essential oil treatment ($F(4,45) = 3.99$, $p < 0.001$) (Fig. 5.6.C), but also significant effects on NO exploration time ($F(4,90) = 31.38$, $p < 0.0001$) (Fig. 5.8.B). Animals treated with Sco (100 µM) showed lower NO

preference percentages ($p < 0.01$) and shorter NO exploration times ($p < 0.0001$) compared to the control group, while administration TEO, especially at 150 and 300 µL/L ($p < 0.01$) improved NO preference and exploration time, suggesting a memory-enhancing profile.

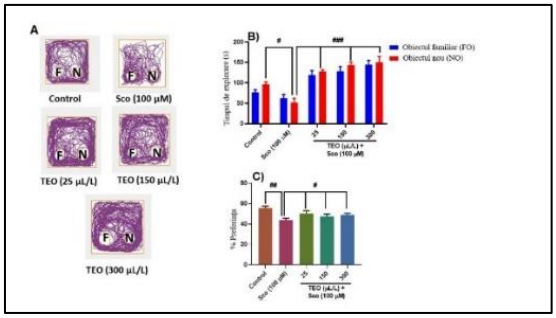

Fig. 5.8. Improvement of memory in the novel object recognition test (NOR) due to the action of *Thymus vulgaris* essential oil (TEO: 25, 150 and 300 µL/L)

In Fig. 5.9.A shows schematic representations of the locomotor activity of fish in the NOR test, characterized by exploration of the familiar object (FO) and exploration of the new object (NO). The preference for FO is significant for fish in the Sco-treated group, in contrast to the control or OEO-treated groups that show interest in NO exploration. One-way ANOVA analysis revealed in terms of exploration time (Fig. 5.9 B), a high FO exploration score by the Sco group (100 µM) compared to NO ($p < 0.0001$), which suggested a deficit in recognition memory. Groups pretreated with Sco (100 µM) and then treated with OEO at the doses of 25 and 300 µL/L mainly explored NO more than FO ($p < 0.0001$), which suggested a recognition profile much better. Regarding the percentage of preference, the lowest was evident in the group treated with Sco ($p < 0.0001$), according to Fig. 5.9 C, while the groups treated with OEO in all three doses, showed a high percentage of preference for NO.

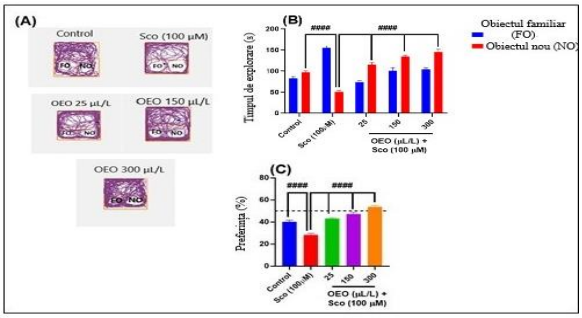

Fig. 5.9. Improvement of recognition memory in the NOR test by administration of *Origanum vulgare* ssp. *hirtum* essential oil (OEO: 25, 150 and 300 µL/L)

5.4. Effects of selected essential oils on oxidative stress in scopolamine-induced dementia model

Based on animal and human studies, it can be stated that oxidative stress plays an important role in the onset and development of most diseases. Reactive oxygen species (ROS) are important for maintaining homeostasis because they function as a second messenger in the cascade. of intracellular signaling. ROS are very unstable and reactive, presenting a very short half-life, however, the species they generate, after the oxidation of biomolecules, are much more stable than them and can be used as biomarkers of oxidative damage, such as lipids, nucleic acids and proteins in their oxidized states. Altered levels of antioxidant enzymes such as catalases, superoxide dismutase and glutathione peroxidase, as well as other antioxidant substances, can be considered as biomarkers (Teixeira et al., 2019).

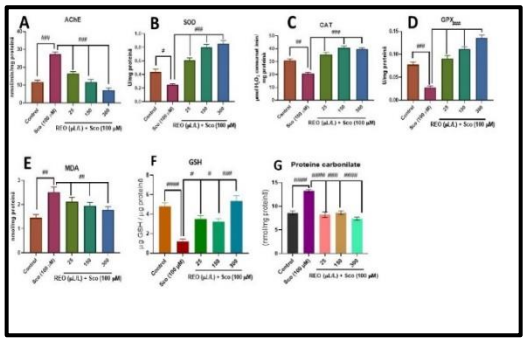

Fig. 5.10. *Rosmarinus officinalis* essential oil (REO: 25, 150 and 300 µL/L) exerted an anti-AChE effect, and improved the antioxidant status in the zebrafish brain

Our experimental results demonstrated that in the brain of fish from the group treated with Sco, the activity of AChE increased pronouncedly (Fig. 5.10.A) in contrast to the control group ($p < 0.0001$), which denotes a degradation of acetylcholine. Treatment with rosemary essential oil in a dose-dependent manner was able to significantly decrease AChE activity ($p < 0.0001$) in contrast to the Sco group. This demonstrates to us the anti-AChE profile of REO, along with the improvement of memory ability in zebrafish, as previously demonstrated in the NTT, Y-maze, and NOR tests.

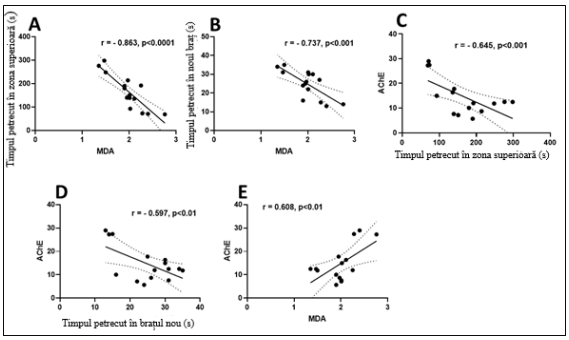

Fig. 5.11. Pearson correlation between behavioral parameters and biochemical parameters (n = 10 animals/lot). Data obtained for AChE are expressed in nmol/min/mg protein, for MDA are expressed in nmol/mg protein, and time spent in both the upper zone and the new arm are expressed in (s)

Pearson's correlation coefficient (r) was used for linear association between cognition, antioxidant enzymes and lipid peroxidation. According to Fig. 5.11.A and Fig. 5.11.B a significant negative correlation can be observed between the time spent in the upper zone of NTTvs. MDA ($r = - 0.863$, $p < 0.001$), as well as between the time spent in the new arm vs. MDA ($r = - 0.737$, $p < 0.001$), $n = 10$. Significant negative correlations were revealed by linear regression and in the case of AChE vs. time spent in the upper NTT area ($r = - 0.645$, $p < 0.001$) and vs. time spent in the new arm ($r = - 0.597$, $p < 0.01$), $n = 10$, according to Fig. 5.11.C and Fig. 5.11.D.

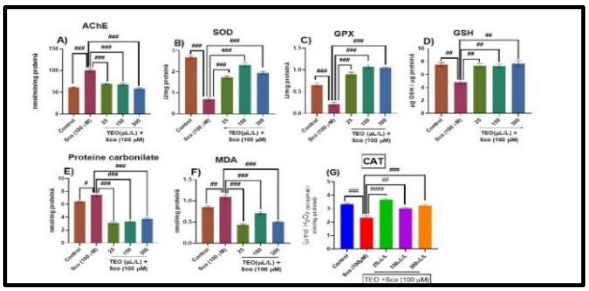

Fig. 5.12. Thyme essential oil (TEO: 25, 150 and 300 µL/L) exerted an anti-AChE effect, and improved the antioxidant status in zebrafish brain

The underlying mechanism of TEO - mediated memory enhancement in Sco-treated zebrafish was also examined through the levels of biochemical markers related to cholinergic function and oxidative stress. Specialized studies support the modification of AChE activity with the progression of AD disease (Gauthier, 2002), a fact proven by the dysfunction of cholinergic neurons in the forebrain, mentioned as the most primordial

pathological events occurring in the pathogenesis of AD. These are followed by decreased choline acetyltransferase (ChAT) activity and ACh levels in the brain of AD subjects (Nyakas et al., 2011).

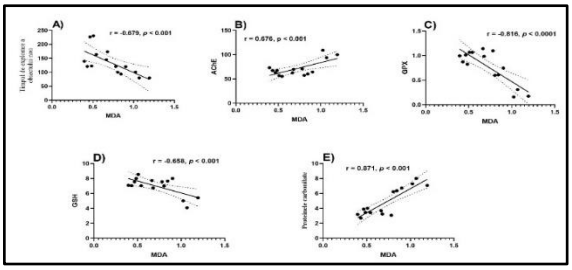

Fig. 5.13. Pearson correlation between behavioral and biochemical parameters (n = 10). The data presented are: new object exploration time (s), AChE (nmol/min/mg protein), GPX (U/mg protein), GSH (µg GSH/µg protein), carbonylated proteins (nmol/mg protein), and MDA (nmol/mg protein)

In Fig. 5.13. a significantly negative correlation can be observed between the exploration time of the new object vs. MDA ($r = -0.679$, $p < 0.001$), which denotes an improvement in the behavioral response in the NOR test, correlated with a low level of lipid peroxidation. Also, a strong negative correlation was highlighted by the linear regression, in the case of GPX vs. MDA ($r = -0.816$, $p < 0.0001$) and between GSH vs MDA ($r = -0.658$, $p < 0.001$) (n=10) which denotes a significant correlation between the activity of antioxidant enzymes and a low level of lipid peroxidation . At the opposite pole, a significantly positive correlation was observed between AChE vs. MDA ($r = 0.676$, $p < 0.001$) and between carbonylated proteins vs. MDA ($r = 0.871$, $p < 0.001$), n=10, which suggested that the decrease in AChE activity and carbonylated protein level correlates with the decrease in MDA level.

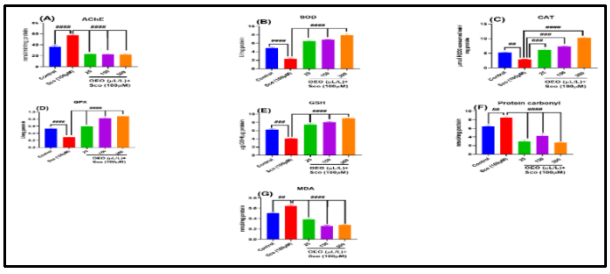

Fig. 5.14. Antioxidant effect of *Origanum vulgare* ssp. *hirtum* essential oil (OEO: 25, 150 and 300 µL/L)

Overall, significant differences can be observed from the results obtained in the one-way ANOVA analysis, between the group treated with Sco and the groups treated with OEO, for SOD ($p < 0.0001$) - Fig. 5.14.B, CAT ($p < 0.0001$) - Fig. 5.14.C, GPX ($p < 0.0001$) – Fig. 5.14. D, . Significant differences were also obtained for the GSH level according to Fig. 5.14.E ($p < 0.0001$). Moreover, the one-way ANOVA analysis revealed important differences also for carbonylated proteins ($p < 0.0001$) – Fig. 5.14.F and the level of MDA ($p < 0.0001$) – Fig. 5.14.G. The levels of carbonylated proteins and lipid peroxidation were increased in fish treated with Sco (100µM), in contrast to the control group, and groups pretreated with Sco and then treated with OEO, which showed decreased levels of carbonylated proteins and MDA ($p < 0.0001$), which confirms the antioxidant effect of OEO.

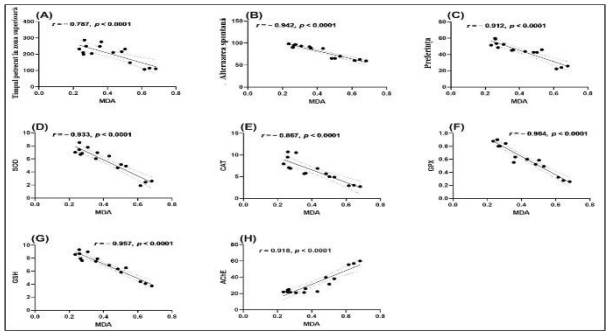

Fig. 5.15. Pearson correlation between behavioral and biochemical parameters (n =10). The data presented are: time spent in the upper zone (s) vs. MDA (nmol/mg protein), spontaneous alternation (%) vs. MDA (nmol/mg protein), novel object preference (%) vs. MDA (nmol/mg protein), SOD (U/mg protein) vs. MDA (nmol/mg protein), CAT (µmol H2O2 consumed/min/mg protein) vs. MDA (nmol/mg protein), GPX (U/mg protein) vs. MDA (nmol/mg protein), GSH (µg GSH/µg protein) vs. MDA (nmol/mg protein) and AChE (nmol/min/mg protein) vs. MDA (nmol/mg protein)

It should be mentioned that the data obtained for the value of the coefficient r, helped us to demonstrate that the treatment with OEO had the effect of increasing the memory capacity of zebrafish pretreated with Sco, in close connection with the increase in the activity of antioxidant enzymes, respectively the decrease the level of lipid peroxidation.

5.5. The effect of Rosmarinus officinalis, Thymus vulgaris and Origanum vulgare ssp. hirtum solutions on associative memory in the T-maze test

Learning abilities were tested in the T-maze test, as well as studying both short- and long-term memory and memory plasticity in zebrafish (Kundap et al., 2017). The work protocol we followed in studying the effect of essential oils of rosemary, thyme and oregano respectively on the associative memory of zebra fish, is based on a Pavlovian-type conditioned learning model, where an arbitrary stimulus (the color green, or the color red) become associated with a meaningful stimulus for the animal (food) so that the two stimuli evoke the same innate behavioral responses (foraging) (Pilehvar, Town and Blust, 2020).

Depending on the length of the retention interval, the T-maze test can estimate the strength of spatial working memory correlated with associative learning (Lalonde, 2002). According to Figure 5.16, the shorter the average time required for the zebrafish to complete each work session, the greater the effectiveness of the essential oil treatment. From this point of view, the control group had a good response time, unlike the Sco group. Similarly, in the case of REO treated groups in all three doses they showed a good execution time, a sign that the effects of the Sco treatment were counteracted. During the two pre-training sessions, zebrafish showed a decreased response time to perform the task, especially on the last day, and Sco treatment implicitly affected this first stage. In the discrimination sessions, the tendency to increase the execution time of the task was especially potentiated in the first 5 stages for the Sco group. In stage 9, the REO group (300 µL/L) took a significantly longer time to make the correct choice in one of the arms of the maze, then the trend remained constant. It can be seen that both treatment and time affect this effect in the discrimination phase. Also, the average time required for the zebrafish to complete the task was significantly shorter in the final session 16 compared to session 1.

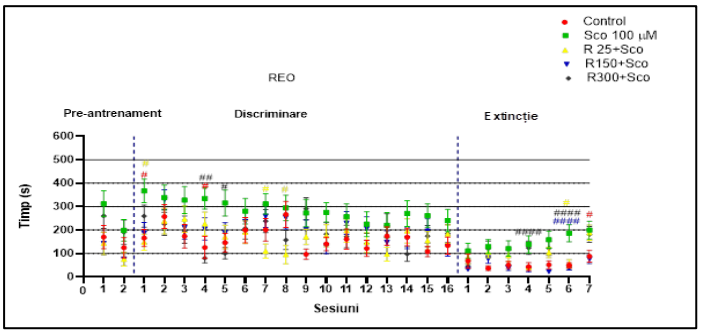

Fig. 5.16. Mean time taken by zebrafish to complete each session of each stage (pre-training, discrimination and extinction). Data shown are expressed ±SD, n=10, # indicates

significant differences ($p < 0.1$) between control, rosemary essential oil treated groups in the three concentrations (25, 150 and 300 µL/L) versus treatment with Sco within the same session (two-way RM-ANOVA)

In figure 5.17., we have represented the average time required for the zebrafish to complete the sessions dedicated to each sample separately. A good time was recorded in the case of the control batch as in figure 5.16., but also in the case of the batches treated with TEO especially in the highest dose of 300 µL/L. In the pre-training phase, a mean effective time (short) is observed for all groups in habituation of the fish to the T test arms without color with food reward only, except for the Sco group which had higher test completion times. In contrast, in the discrimination sessions, this time trend is increasing in contrast to pre-training, due to the color marking of the maze arms. Further, the control group records a short time to enter the arms of the maze in the correct ABBA order, followed by reward, in contrast to the Sco group who record high task completion times, sometimes recording the maximum time of 600 seconds.

In the first 6 stages of the discrimination, for the groups treated with thyme essential oil, the response time of the zebrafish increased significantly, with an effective time average, indicating an inhibition of the Sco effect. From stage 7 to the end of discrimination, the time-mean trend remained constant for TEO treatments, with no large differences between treatment doses. However, it can be concluded that the TEO 25 µL/L dose registered a higher time average trend, unlike TEO 300 µL/L which had the lowest time average or TEO 150 µL/L which kept almost constant as time without much contrasts. At the same time, we can also state that for the control and TEO-treated groups, the average time required for the zebrafish to complete all the sessions in each work stage was significantly shorter in the final stage 16, compared to the first stage, which proves to us the effectiveness of -training and the T-test itself to teach the fish tasks.

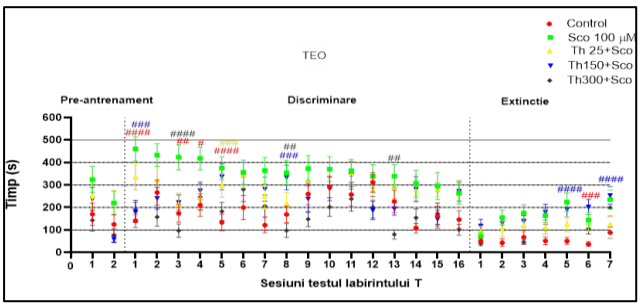

Fig. 5.17. Mean time taken by zebrafish to complete each session of each stage (pre-training, discrimination and extinction). Data presented are expressed ± SD, n = 10, #

indicates significant differences ($p < 0.1$) between control, thyme essential oil treated groups in the three TEO concentrations (25, 150 and 300 µL/L), vs. Sco treatment within the same session (two-way RM-ANOVA)

Further, in Fig. 5.18., the average time required for zebrafish to complete the sessions dedicated to each process in the T-test, under the action of oregano essential oil, was represented. A good time was recorded by the control group as in Fig. 5.16. and Fig. 5.17., the average short execution time of the work tasks, also being recorded in the case of batches treated with OEO especially in the dose of 25 µL/L, in most of the work sessions in discrimination and in the dose of 150 µL/ L from stage 6 discrimination and especially in extinction. In the pre-training stage the mean total time was short at all essential oil doses, with a decreasing trend.

Starting with the color discrimination stage, the trend of the time average started to increase, a phenomenon found in all three essential oils selected in our study. For TEO and OEO treated groups the response time of zebrafish in the discrimination sessions increased more significantly than REO, observed in the early stages of the first six stages. These increased time means are observed for Sco treated groups vs. control ($p < 0.0001$ and $p < 0.001$) persisting through stage 16, and for OEO 150 µL/L treated groups at stage 5 vs. OEO 25 µL/L ($p < 0.001$), in stage 6 for OEO 300 µL/L vs. OEO 150 µL/L ($p < 0.01$) and for OEO 25 µL/L in stage 7 -a compared to OEO 300 µL/L ($p < 0.001$), after the time trend being in a decrease and maintaining approximately constant without significant differences. We can conclude that both time and treatment significantly affected this effect in the discrimination phase. In the last stage of extinction, the trend of the time average has a significant decrease in the first 5 days, then increasing slightly towards the end of the 2 days of testing. Overall the Sco group experienced the highest mean time compared to the control and essential oil treated groups in all 3 stages of the T-test.

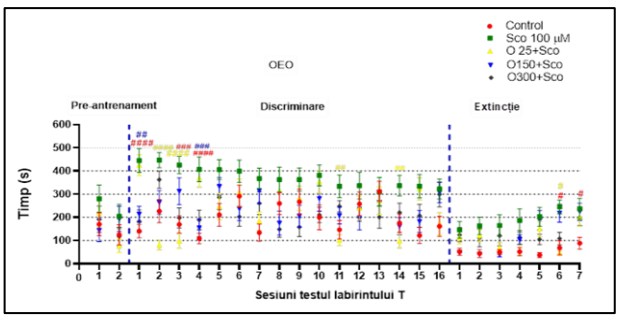

Fig. 5.18. Mean time taken by zebrafish to complete each session of each stage (pre-training, discrimination and extinction). Data shown are expressed ± SD, n = 10, # indicates

significant differences ($p < 0.1$) between the control, groups treated with oregano essential oil in the three concentrations (25, 150 and 300 µL/L), compared to the treatment with Sco within the same session (two-way RM-ANOVA)

As a final conclusion we will detail in table 1 and 2, the time averages obtained by all the groups studied in the work sessions of the discrimination and extinction stage to see the color preferences according to the essential oil used as treatment.

Table 1. Differences in time between groups of fish in completing discrimination training in the T-maze test based on color preference

LOTS	*TIME MEANS – DISCRIMINATION STAGE*		*SIGNIFICANT DIFFERENCES*
	Green Color	**Red Color**	
Control group	72,74 sec	101,3 sec	### p < 0,001
Scopolamine group	113,8 sec	169,9 sec	#### p < 0,0001
Rosemary 25 µL/L	84,47 sec	114,1 sec	## p < 0,01
Rosemary 150 µL/L	87,08 sec	101 sec	ns p = 0,1
Rosemary 300 µL/L	94,40 sec	113,3 sec	# p < 0,1
Thymus 25 µL/L	129 sec	142,5 sec	ns p = 0,1
Thymus 150 µL/L	82,06 sec	105,9 sec	ns p = 0,8
Thymus 300 µL/L	117,1 sec	115,7 sec	## p < 0,01
Origanum 25µL/L	132,2 sec	120,9 sec	ns p = 0,1
Origanum 150 µL/L	104 sec	119,9 sec	ns p = 0,1
Origanum 300 µL/L	117.9 sec	119,9 sec	ns p = 0,8

Table 2. Differences in time between groups of fish in completing tasks in the last stage of extinction, within the T-maze test based on color preference

LOTS	*TIME MEANS – EXTINCTION STAGE*		*SIGNIFICANT DIFFERENCES*
	Green color	**Red color**	
Control group	37 sec	51 sec	ns p =0,08
Scopolamine group	117,1 sec	208 sec	## p < 0,01
Rosemary 25 µL/L	56,32 sec	71 sec	ns p = 0,3
Rosemary 150 µL/L	22,86 sec	23,83 sec	ns p = 0,1
Rosemary 300 µL/L	111,1 sec	112,8 sec	ns p = 0,06
Thymus 25 µL/L	83,47 sec	132,2 sec	ns p = 0,1
Thymus 150 µL/L	166,9 sec	137,8 sec	ns p = 0,8

Thymus 300 µL/L	115,5 sec	56,84 sec	## p < 0,01
Origanum 25µL/L	184,1 sec	161,5 sec	ns p = 0,4
Origanum 150 µL/L	140,8 sec	107,9 sec	ns p = 0,4
Origanum 300 µL/L	140 sec	119,4 sec	ns p = 0,6

5.6. Effects of rosemary, thyme and oregano essential oils on genetic markers in brain samples from zebrafish immersed in scopolamine to induce AD dementia

5.6.1. Effect of REO, TEO and OEO on bdnf gene expression

BDNF along with other neurotrophins have important roles in neurogenesis, neuronal growth, survival, differentiation, maturation, neuronal migration and influence dendritic growth, density, neuronal growth, connectivity and neuroplasticity throughout life (Ahmed et al., 2015). In zebrafish, bdnf mRNAs were present from the embryonic stage, as the presence of bdnf transcripts in the forebrain, midbrain, and hindbrain was demonstrated at early developmental stages with whole-mount in situ hybridization experiments (Cacialli et al. , 2016). Sco administration stimulates the cholinergic neuronal pathway and memory circuits of the CNS, but can also reduce the expression of response binding protein for BDNF and CREB in the brain.

In Fig. 5.19, we followed the effects of REO (25, 150 and 300 µL/L), TEO (25, 150 and 300 µL/L) and OEO (25, 150 and 300 µL/L) on bdnf gene expression in the treated zebrafish brain with Sco. According to the T-maze protocol, we investigated the results obtained from groups trained on the one hand to choose the green arm and on the other hand, groups trained to choose the red arm. As we can see, zebrafish with green arm preference have much higher gene expression value than zebrafish with red arm preference. In both cases, Sco treatment significantly reduced the mRNA copy number of the bdnf gene, compared to the control groups ($p < 0.1$ and $p < 0.0001$). From each essential oil we observe important enhancements of the negative effects of Sco on bdnf gene expression by significantly increasing mRNA copy number ($p < 0.01$ and $p < 0.0001$) for REO at concentrations of 150 and 300 µL/L; $p < 0.0001$ for TEO at 25 and 300 µL/L concentrations; $p < 0.0001$ for OEO at concentrations 150 and 300 µL/L).

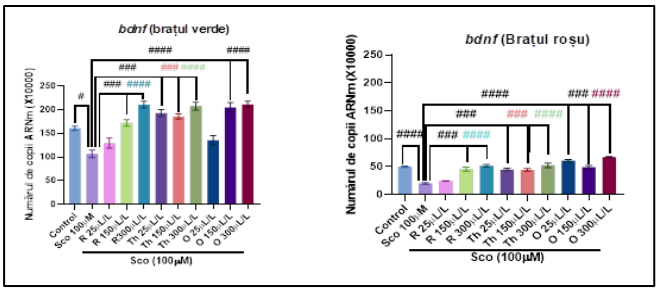

Fig. 5.19. Gene expression in zebrafish brain determined by RT-qPCR, representing bdnf mRNA copy number plots, with F = 21.89, p<0.0001 for green arm and F = 32.56, p <0.0001 for red arm

5.6.2. Effect of REO, TEO and OEO on creb_1 gene expression

CREB is essential for important functions of cognition such as memory and synaptic plasticity, therefore in neurons it has been correlated with various intracellular processes, including proliferation, differentiation, survival, long-term synaptic potentiation, neurogenesis and neuronal plasticity (Wang et al ., 2018).

In our study, creb mRNA expression was overexpressed for Sco-treated groups compared to control groups ($p < 0.01$ and $p < 0.0001$). For fish treated with essential oils REO (25 µL/L) - red arm, TEO (25 and 150 µL/L) - green arm and OEO (25 and 150 µL/L) - green arm, creb mRNA expression was similar to that of the Sco groups (no significant difference). This confirms that Sco and essential oil doses were dependent on AD dementia symptoms, amnesic effects, which contributed to the loss of memory function in fish, after we learn that the animals choose the correct color associated with the food reward. In another case, REO ($p<0.0001$ at the concentration of 150 and 300 µL/L), TEO ($p < 0.001$ at the concentration of 25 µL/L and $p < 0.0001$ for the concentration of 150 and 300 µL/ L) and OEO ($p < 0.001$ at 150 µL/L concentration and $p < 0.0001$ for 300 µL/L) significantly reduced red arm expression as seen in Fig. 5.20.

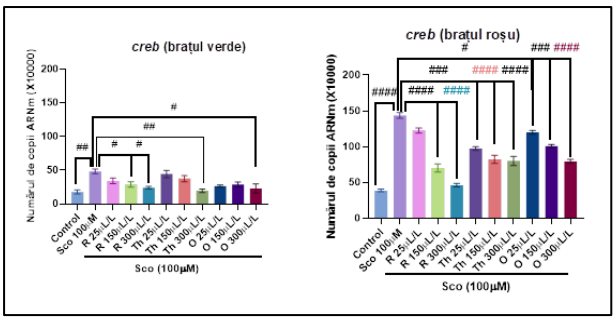

Fig. 5.20. Gene expression in zebrafish brain determined by RT-qPCR representing mRNA copy number plots for creb_1 with F = 6.084 ($p < 0.01$) for green arm and F = 67.23 ($p < 0.0001$) for the red arm

Considering the functional role of polyphenols in the regulation of learning and memory, activation of CREB signaling could explain the neuroprotective effects of our essential oils on Sco-induced memory deficits.

5.6.3. Effect of REO, TEO and OEO on npy gene expression

Neuropeptides exert neuroprotective roles mainly by preventing Aβ accumulation, increasing neuronal glucose transport, increasing neurotrophin production, inhibiting endoplasmic reticulum stress and autophagy, modulating potassium channel activity, and hippocampal long-term potentiation (Chen, Du and Chen, 2019).

As seen in Fig. 5.21., the mRNA copy number of the npy gene was significantly reduced for the red arm-trained groups as opposed to the green arm-trained groups, and also for the groups treated with Sco (100 µM) for both colors. For green arm trained groups, we observe a significant increase in gene expression from zebrafish brains treated with essential oils at a concentration of 300 µL/L, vs. Sco ($p < 0.0001$), but we have a non-significant increase in mRNA copy number for REO (25 µL/L)-green arm, TEO (25 µL/L) and OEO (25 µL/L)-red arm.

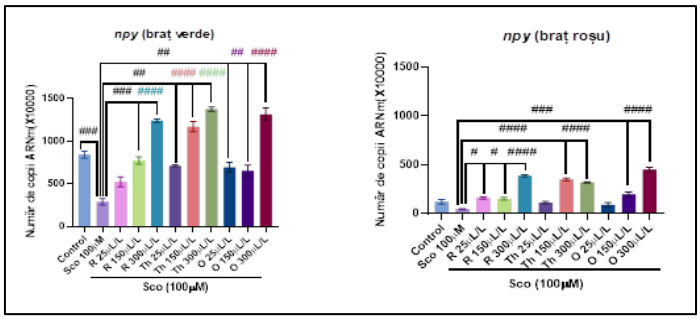

Fig. 5.21. Gene expression in zebrafish brain determined by RT-qPCR, representing npy mRNA copy number plots, with $F = 52.52$ ($p < 0.0001$), in groups of green arm-trained fish and $F = 71.96$ ($p < 0.0001$) for groups of trained fish for red arm

In conclusion, NPY is useful in laboratory animal model experiments against dementia symptoms typical of AD and may be recommended as a target therapy in neurological disorders due to its close relationship with acetylcholine.

5.6.4. Effect of REO, TEO and OEO on nrf2a gene expression

In specialized studies, the zebrafish was used as an experimental animal model in the study of oxidative stress as a mediator of toxicity, demonstrated by the deficiency of nrf2a, as a key gene in the antioxidant response, as reported by Yamashita et al. (2019) .

As we can see in Fig. 5.22., there are no big differences regarding the number of mRNA copies of the nrf2a gene, in the control groups both in choosing the green and the red color, these groups having a profoundly increased activity. Instead, the number of mRNA copies was significantly reduced for groups treated with Sco (100 µM) due to the toxicity emanating in the body of fish lacking gene activity, unlike the control and groups treated with essential oil. Significant differences in nrf2a gene activity are present for the red color trained groups especially at REO 150 µL/L $p<0.001$), OEO (concentration 150 µL/L $p < 0.1$) and for all high doses of REO essential oil (300 µL/L $p < 0.0001$), TEO (300 µL/L $p < 0.1$) and OEO (300 µL/L $p < 0.0001$); while for the groups trained for green color, significant differences are present in TEO ($p < 0.01$) and OEO ($p < 0.01$) at high concentrations of 300 µL/L in contrast to Sco groups.

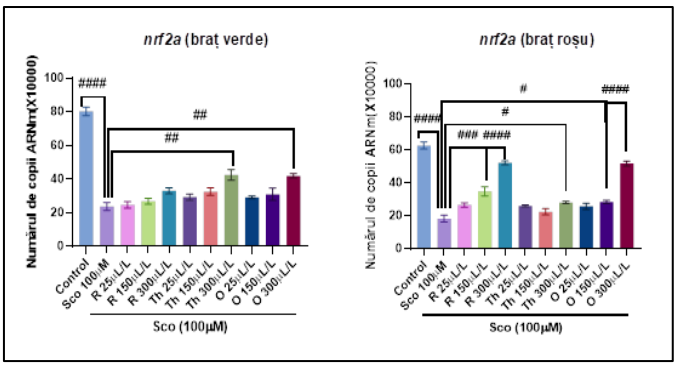

Fig. 5.22. Gene expression in zebrafish brain determined by RT-qPCR, plotting mRNA copy number plots for nrf2a, with $F = 49.54$, $P < 0.0001$ for green arm and $F = 73.47$, $P < 0.0001$ for the red arm

5.6.5. Effect of REO, TEO and OEO on egr1 gene expression

Egr-1 (also known as zif268, NGFI-A, krox-24, ZENK) is a transcription factor that functions as part of the immediate-early gene response, the first wave of gene expression induced in a neuron by stimulation. Egr-1 expression is induced by a variety of natural experiences, sensory stimuli, and during the production of behaviors (Burmeister and Fernald, 2005).

In Fig. 5.23. we evaluated the expression level of the egr1 gene in the brain of zebrafish pretreated with Sco and essential oils from the Lamiaceae family. We observed that the mRNA copy number of this gene was significantly increased for groups trained to choose the color green, especially at high concentrations of essential oil treatment 300 µL/L (REO, TEO and OEO $p < 0.0001$). Instead, the mRNA copy number of this gene was

significantly reduced in Sco (100 µM) treated groups as opposed to control groups for zebrafish groups trained for both colors.

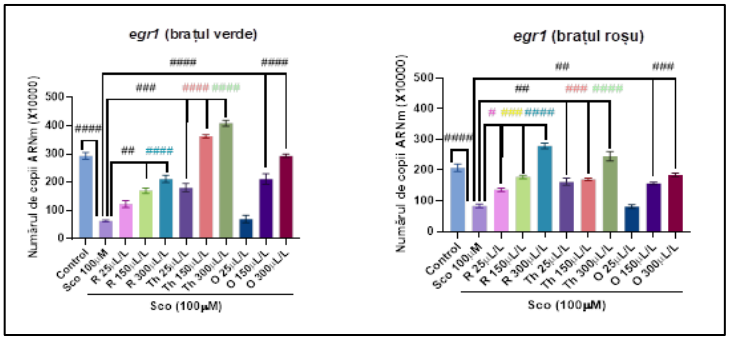

Fig. 5.23. Gene expression in zebrafish brain determined by RT-qPCR, representing mRNA copy number plots for egr1, with F=94.12, ($p<0.0001$) for green arm and F= 47.36 , ($p < 0.0001$) for the red arm

CONCLUSIONS

The present study aimed to evaluate the effect and impact of the essential oils of R. officinalis, T. vulgaris and O. vulgare ssp. hirtum selected from the Lamiaceae family, both on the behavior and on the biochemical and molecular parameters in a fish animal model zebrafish of AD induced by immersion in Sco solution (100 µM). Based on the results obtained, the following general conclusions can be formulated:

1. On the basis of behavioral studies, the ability of zebrafish to memorize, to perform tasks, due to their natural reflexes, of a behavior learned or learned as a result of a reward/punishment, is accurately observed, clues that help us understand the activity of the cholinergic system of the animals but also the effect of the pharmacological agents used. According to the results obtained in the tests of the NTT, Y, NOR and T mazes, the evolution of the behavior of the fish under study can be observed in comparison with the control groups.

2. All three concentrations 25 µL/L, 150 µL/L, 300 µL/L of essential oils of R. officinalis, T. vulgaris and O. vulgare ssp. hyrtum, showed their potential to counteract oxidative stress induced by the administration of Sco, by decreasing the level of carbonylated proteins and the level of MDA in the body of zebrafish and by increased levels of the total content of reduced glutathione, together with the intensification of the specific activities of glutathione peroxidase, superoxide dismutase and catalase, highlighting the antioxidant capacity of essential oils.

3. The administration of the essential oils of R. officinalis, T. vulgaris and O. vulgare ssp. hyrtum caused significant decreases in the enzymatic activity of AChE in the brain tissue of zebrafish, in contrast to the effect of Sco, which produced oxidative stress and increased AChE activity. At the same time, the administration of the three essential oils selected from the Lamiaceae family demonstrated pro-cognitive and antioxidant properties due to the increase in the expression of bdnf, npy, creb_1, nrf2a and egr1 genes in response to the cytotoxic action of Sco inducing oxidative stress.

4. All these results indicate to us that these essential oils represent a suitable therapeutic alternative for ameliorating the symptoms associated with AD especially by improving the mechanisms underlying memory formation – cholinergic system, specific gene expression and reducing ROS.

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Scientific activity

Articles published in extenso from the subject of the doctoral thesis:

- Căpățînă L, Boiangiu RS, Dumitru G, Napoli EM, Ruberto G, Hritcu L, Todirascu-Ciornea E, 2020, Rosmarinus officinalis essential oil improves scopolamine-induced neurobehavioral changes via restoration of cholinergic function and brain antioxidant status in zebrafish (Danio rerio) , Antioxidans (Basel), 9(1): 62, doi: 10.3390/antiox9010062. (Q1; AIS: 0.910; IF: 6.313)
- Căpățînă L, Todirascu-Ciornea E, Napoli EM, Ruberto G, Hritcu L, Dumitru L, 2020, Thymus vulgaris essential oil protects zebrafish against cognitive dysfunction by regulating cholinergic and antioxidants systems, Antioxidans (Basel), 9(11): 1083, doi: 10.3390/antiox9111083. (Q1; AIS: 0.910; IF: 6.313)
- Căpățînă L, Napoli EM, Ruberto G, Hritcu L, 2021, Origanum vulgare ssp. hirtum (Lamiaceae) essential oil prevents behavioral and oxidative stress changes in the scopolamine zebrafish model, Molecules, 6(23): 7085, doi: 10.3390/molecules26237085 . (Q2; AIS: 0.671; IF: 4.927)

Participation in scientific events:

- Oral presentation: Căpățînă L, Boiangiu RS, Todirascu-Ciornea E, Dumitru G, Napoli EM, Ruberto G, Hritcu L - "Effects of Rosmarinus officinalis essential oil in memory formation and relieving brain oxidative stress in zebrafish model" - October 21-22 2019, "Conference Life Sciences in the Dialogue of Generations: Connections between Universities, Academia and Business Community", Chisinau.
- Poster presentation: Căpățînă L, Boiangiu RS, Todirascu-Ciornea E, Dumitru G, Napoli EM, Ruberto G, Hritcu L - "Effects of Rosmarinus officinalis essential oil in memory formation and relieving brain oxidative stress in zebrafish scopolamine model"- 21-24 September 2019," The 48th Annual General Meeting of the European Brain and Behavior Society", Prague, EBBS.
- Poster presentation: Căpățînă L, Boiangiu RS, Mihășan M, Maniu C, Napoli EM, Hritcu L -"Rosmarinus officinalis and Thymus vulgaris essential oils attenuate cognitive deficits and brain oxidative stress in scopolamine zebrafish model: underlying mechanisms"- 11-15 July 2020 , "FENS 2020 Virtual Forum", Glasgow.

Workshops and seminars:

- Participation in the workshop held by Valerie Moreau (Noldus), on the use of the EthoVisionXT video tracking software, 19/02/2019, Faculty of Biology, "Alexandru Ioan Cuza" University in Iasi;
- Participation in the 5th Bioinformatics Seminar "Bioinformatics tools for exploring protein biology" organized by the Romanian Society of Bioinformatics (RSBI) during 04/04/2019-04/05/2019 within the Faculty of Biology of the University "Alexandru Ioan Cuza" from Iasi;
- Participation in the RoBioinfo conference organized by the Romanian Society of Bioinformatics (RSBI) between May 11-13, 2023 in Bucharest at the "Nicolae Simionescu" Institute of Cellular Biology and Pathology, Bucharest.