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The impact of some natural flavonoids with favorable action in neurodegenerative diseases

SUMMARY OF THE DOCTORAL THESIS

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Contents:

KEY WORDS

ACh – acetylcholine;

AChE – acetylcholinesterase;

AD – Alzheimer's disease;

APOE – apolipoprotein E;

APP – amyloid precursor protein*;*

Aβ – Beta amyloid;

Baic – baicalein 5,6-dimethyl ether;

BBB - blood-brain barrier;

BDNF – brain-derived neurotrophic factor;

(BDNF – human/rodent BDNF protein; Bdnf – Bdnf protein from zebrafish; *BDNF – human BDNF gene*; *Bdnf - Bdnf gene* from rodents*; Bdnf - Bdnf gene* from zebrafish*);*

CAT – catalase;

CREB – the receptor protein for binding cAMP (CREB – human/rodent CREB protein; Creb – Creb protein from zebrafish; *CREB – human CREB gene*; *Creb - Creb gene* from rodents*; creb - creb gene* from zebrafish*);*

EGR-1 - early growth response protein 1 (EGR-1 – human/rodent EGR-1 protein; Egr-1 – the Egr-1 protein from zebrafish; *EGR-1 – human EGR-1 gene*; *Egr-1 - Egr-1 gene* from rodents*; Egr-1 - Egr-1 gene* from zebrafish*);*

Fab - agatisflavone ;

GAL – galantamine;

GPX - glutathione peroxidase;

GSH – glutathione;

GSK3β - glycogen synthase kinase 3 beta;

GST - glutathione S – transferase;

CSF – cerebrospinal fluid;

MAPK/MEK - mitogen-activated protein kinases;

MCI – minor cognitive deficit;

MDA- malondialdehyde;

NOR – the new object recognition test, also known as NOP ;

NPs - neuritic amyloid β plaques;

NPY – AD (NPY – human/rodent NPY protein; Npy – Npy protein from zebrafish; *NPY – human NPY gene*; *Npy - Npy gene* from rodents*; npy - npy gene* from zebrafish*);*

NRF2 - nuclear factor 2 linked to erythroid factor (NRF2 – human/rodent NRF2 protein; Nrf2 – the Nrf2 protein from zebrafish; *NRF2 – the human NRF2 gene*; *Nrf2 - Nrf2 gene* from rodents*; Nrf2 - Nrf2 gene* from zebrafish*);*

NTT – the test of immersion in a new aquarium;

OF – familiar object;

ON – new object;

OS – oxidative stress;

ROS – reactive oxygen species;

Sco-scopolamine;

CNS - central nervous system;

PNS - peripheral nervous system;

SOD – superoxide dismutase;

TNF-α – tumor necrosis factor α.Tumor necrosis factor α;

TREM2 - the trigger receptor expressed on myeloid cells type 2;

Trk - receptor tyrosine kinase family;

INTRODUCTION

Alzheimer's disease (AD) is a complex, multifactorial neurodegenerative disorder characterized by a slow and irreversible progression, being the most widespread form of dementia (1). The average life span from onset of clinical symptoms to death is approximately 8.5 years (2). AD is manifested by a gradual decline in cognitive functions, accompanied by behavioral disturbances (3), and is associated with cerebral atrophy, decrease in brain volume and weight, expansion of the cortical grooves and the ventricular system (4).

Among the pathological signs of the disease, neurofibrillary tangles (NFTs) can be distinguished, which are formed as a result of hyperphosphorylation of the tau protein (τ), the amyloid plaques that are formed from the β-amyloid peptide (Aβ), the presence in the cortex of neurofibrillary degeneration, granulo-vacuolar degeneration, mitochondrial dysfunction, cholinergic dysfunction and the establishment of oxidative stress (OS) (5). These changes lead to the loss of neurons in the cerebral cortex and hippocampus, and the remaining neurons show synaptic degeneration, dendrite and neuropil damage, thus contributing to the cognitive decline and behavioral and neuropsychiatric disturbances seen in AD patients (6).

Risk factors for developing AD include advanced age, family history, genetic mutations, oxidative damage, and neurovascular disease (7).Despite intensive research, there is currently no effective treatment for AD, drugs targeting Aβ and tau proteins have not provided significant clinical results, and available treatments, which include cholinesterase inhibitors and psychotropic drugs, provide only limited symptom relief (8). An important factor in AD is the nuclear protein CREB1, which plays essential roles in various brain regions (9). CREB1 regulates the transcription of essential genes (10) such as *bdnf* (11), *egr-1*, *nrf2* (12) and *npy* (13). Both BDNF, EGR-1 and NPY are neuropeptides that. BDNF (brain-derived neurotrophic factor) is crucial for neuronal survival and differentiation during development and regulates synaptic transmission and activity-dependent plasticity (14). EGR-1 controls the expression of genes involved in cell growth, survival and the maintenance of long-term potentiation (LTP), a process essential for long-term memory (15). NPY modulates the excitability of hippocampal neurons and acts as a modulator of neuroplasticity and neurotransmission(16). NRF2 is involved in neuronal protection against oxidative and inflammatory stress and regulates mitochondrial pathways (17).

Current treatments for neurodegenerative diseases, including AD, are ineffective (18) or have numerous adverse effects (19). Recently, due to the high efficiency of flavonoids(20)there is a high interest in the selection of these natural compounds as potential neuroprotective agents (21). Experimental studies have shown that flavonoids such as roifolin (Rho), baicalein 5,6-dimethyl ether (Baic) and agatisflavone (Fab) exhibit neuroprotective properties. Fab protects neurons against glutamate-induced excitotoxicity (26),Rho protects neurons from Aβ peptide-mediated neurotoxicity (34), and Baic can ameliorate Aβ-related pathology and cognitive dysfunction (27, 28).

The zebrafish (*Danio rerio*) is increasingly used in neuropharmacological research due to its neurophysiological and genetic similarities to humans, as well as ease of handling (29). This fish exhibits a blood-brain barrier (BBB) similar to that of humans and all major neurotransmitter systems, making it a suitable model for studies of neurodegeneration (30).

The doctoral thesis presents the latest news from the national and international scientific literature regarding dementia associated with AD, as well as the mechanisms of action of flavonoids in improving the symptomatology of this disease. Also, this thesis proposes the use of an animal model of dementia exemplified by the zebrafish which is induced by immersion administration of a muscarinic cholinergic receptor blocker represented by scopolamine (Sco). The research methodology proposed for studying the impact of the three flavonoids on the behavioral, biochemical and molecular aspects of AD in the brain of the animal model is also presented.

The study aimed to evaluate the state of anxiety, learning and memory processes, oxidative status, absolute gene expression of *bdnf, npy, egr-1, nrf2α* and *creb1*, and the relative level of Creb1 protein, relative to GAPDH (through tests specific behavioral, biochemical and molecular).

CHAPTER I. DEMENTIA ASSOCIATED WITH ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is the most common neurodegenerative disorder (27), irreversible (28) and progressive (29), being the main cause of dementia in the elderly (30). AD affects about 10% of people over 65 and over 32% of people over 85 (31). In 2021, it was the fifth leading cause of death in the elderly (32). The clinical course of the disease begins with subtle memory impairment (mild cognitive impairment) that progresses to language impairment and visual-spatial deficits, and in the final stage, patients become rigid and unconscious, requiring help with basic tasks (8).

Neuropathological signs include brain atrophy and loss of cholinergic neurons, particularly in the hippocampus and cortex, associated with the accumulation of amyloid plaques and neurofibrillary tangles (NFTs) (37). Critical factors in the development of AD include neurovascular dysfunction, genetic mutations, oxidative stress (OS), and inflammatory processes. Although there are five medications to manage the symptoms, they cannot stop the progression of the disease (37). The course of the disease can last from one to 25 years, but the typical duration of the disease is 8-10 years (32)Therapeutic approaches to manage AD symptoms are limited due to the protective nature of the BBB that prevents drugs from targeting neurons (34).Death usually occurs from secondary complications, and the global costs of caring for AD patients exceed one billion dollars (35).

I.1 Prevalence and incidence of Alzheimer's disease

The prevalence of AD increases exponentially with age, doubling every five years (41). In China, the prevalence of AD ranges from 0.2% in people aged 55–59 to 48.2% in those aged 95–99 (42). In the West, prevalence is below 0.6% in those under 65, but exceeds 33% in those over 85 (2). AD is more common in women than men, according to a 2017 meta-analysis showing that women are twice as likely to develop the disease as men (38).

The incidence of AD varies significantly by region, with 2.0 to 16.8 new cases reported per 1,000 persons over 60 years of age in the US (39), Europe (38) Japan (40) and China (41). This variation is due to differences in population characteristics, age ranges, periods studied, and diagnostic criteria (40). A meta-analysis from Europe showed that the incidence of AD increases with lifespan (42). The estimated annual rate of dementia ranges from 10.5% in North America to 8% in China (48). In 2015, the global number of AD cases exceeded

32.8 million, with an anticipated increase to 13 million by 2050 as the elderly population doubles (48).

I.2 The global impact of Alzheimer's disease

AD has one of the fastest growing mortality rates, with a median survival time of 3–6 years after diagnosis (5, 44), and patient care imposes significant global costs, estimated to exceed $2.8 trillion by 2030 (49). Almost half of patients require intensive care, either in institutions or at home, with large variations depending on the economic development of countries (50).

I.3 Etiology

AD has a multifactorial etiology, with a complex pathogenesis involving genetic, environmental, metabolic and vascular factors (47). AD can have a late onset (LOAD) or early onset (EOAD), with the familial form (FAD) being rare and associated with specific genetic mutations, while the sporadic form (SAD) is much more common and influenced by variable factors (48). Age is the main risk factor, with over 95% of cases being diagnosed in people over 60 years old (53). Clinical manifestations include progressive memory loss, cognitive decline, and behavioral disturbances. Although there are several hypotheses to explain the etiology of AD, the exact pathogenesis remains unclear (50).

I.3.1 Hypothesis of oxidative stress and mitochondrial dysfunction

Mitochondria play an essential role in cellular energy production through oxidative phosphorylation and in the regulation of cellular signaling, including calcium and reactive oxygen species (ROS) (51). They contain a semi-autonomous genome (mtDNA) encoding components of the electron transport chain (ETC) (52). In addition to ATP production, mitochondria are involved in various cellular processes such as OS and hypoxic response, cell cycle control, and apoptosis (53). Imbalance between ROS production and antioxidant defense leads to OS, which is associated with aging and AD pathogenesis (54). This imbalance causes oxidative damage to DNA and proteins, impairing cognitive function and contributing to the formation of beta-amyloid (Aβ) plaques and NFTs, hallmark markers of AD, accelerating cell death by apoptosis (55).

I.3.2 Hypothesis of metallic ions

The homeostasis of Cu^{2+}, Zn^{2+} and Fe^{2+} ions is crucial for normal brain functions, and their imbalance may contribute to the onset and progression of AD (60). This imbalance

affects essential cellular pathways, leading to Aβ misfolding and NFT formation (42). In the brain these ions are concentrated in amyloid plaques, and changes in their levels are observed in both cerebrospinal fluid (CSF) and neocortical matter (61).

I.3.3 The cholinergic hypothesis

The cholinergic hypothesis suggests that reduced cholinergic functions in certain parts of the brain, particularly the neocortex and hippocampus, are associated with disease severity (58, 59). This cholinergic deficit is caused by the atrophy and degeneration of cholinergic neurons, which play a crucial role in memory, learning and spatial orientation (31, 62). Cholinergic neurons modulating activity through acetylcholine (ACh), an essential neurotransmitter, whose decrease in AD is linked to neuronal death, τ protein hyperphosphorylation, and amyloid plaque deposition (61). Increased activity of acetylcholinesterase (AChE), which degrades ACh, contributes to cognitive dysfunctions, suggesting a synergistic relationship between decreased ACh levels and progression of AD pathology (62).

I.3.4 The amyloid hypothesis

The Aβ peptide, consisting of 38 to 43 amino acids, originates from the fragmentation of the precursor protein APP (66). APP contains a large extracellular N-terminal domain and a shorter C-terminal domain, with roles in synapsis, neuronal transport, and iron export. On the neuronal membrane, APP is cleaved by β-secretase (BACE) and γ-secretase, generating $A\beta_{40}$ and, to a small extent, $A\beta_{42}$. γ-secretase, a multimeric complex, produces mostly $A\beta_{40}$ (70), and ADAM10-mediated α-secretase cleaves APP to prevent amyloid plaque formation (74). Mutations in APP and PSEN1 increase the production of $A\beta_{42}$, which accumulates in plaques. $A\beta_{42}$ (66) it aggregates into oligomers, protofibrils and amyloid fibrils, which play a significant role in neuronal and vascular degeneration, disrupting enzyme activity and brain receptors (73, 75). This linkage causes a number of downstream effects, including OS, cholinergic dysfunction, τ protein hyperphosphorylation and microglial activation. Activation of microglia leads to the production and release of pro-inflammatory cytokines such as IL-1β, TNF-α and IFN-γ, which in turn stimulate adjacent astrocytes to produce more $A\beta_{42}$ oligomers (71).

I.3.5 The tau protein hypothesis

The τ (tau) protein is essential for the stabilization of microtubules in axons by interacting with tubulin (70). Normally, the τ protein is regulated by post-translational

modifications such as glycosylation and phosphorylation, but in AD, this phosphorylation is significantly increased, with up to nine phosphate groups per molecule (71). Hyperphosphorylation disrupts the balance between kinases and phosphatases, favoring the fibrillization and aggregation of τ protein into NFTs which are insoluble fibers within neurons (48). NFTs are composed of paired helical filaments and can form occasional single filaments (SF), which contain an abnormal and hyperphosphorylated form of the τ protein (72).

Enlargement of SF and NFT is associated with loss of neurons and synapses, brain atrophy and dilation of lateral ventricles due to loss of brain tissue (87). These abnormalities are related to the severity of dementia and the duration of the disease, directly affecting brain function (74). NFTs and SFs are present in the hippocampus, where they contribute to memory impairment, and in the association cortex, where they affect higher cognitive functions (75). Progressive deterioration of different cortical areas is associated with various cognitive dysfunctions. However, it is not clear whether NFTs directly contribute to neuronal death or are a response mechanism of damaged affected neurons (76).

I.3.6 The neuroinflammatory response hypothesis

Neuroinflammation plays a crucial role in the development of AD, amplifying Aβ and τ pathology. Imaging studies have demonstrated increased microglia activity and inflammation in the brains of AD patients (91). Over 50% of genes involved in FAD are associated with immune and microglial functions, including APOE and TREM2, whose dysfunctions exacerbate Aβ pathology by reducing phagocytic clearance (93). Elevated levels of pro-inflammatory cytokines are observed in both serum and brain of AD patients (79). The inflammatory response in AD includes the activation of various cells and systems, generating NO, ROS and proinflammatory cytokines, leading to infiltration of peripheral immune cells, neuroinflammation, thereby contributing to disease progression and neuronal death (98).

I.4 Risk factors for Alzheimer's disease

Numerous studies indicate an association between several risk factors and AD (104). Epidemiological and genetic studies reveal several statistically significant correlations between AD prevalence and advanced age (47). Simultaneously, pathogenetic mechanisms, such as inflammation and free radical generation, suggest a causal link between atherosclerotic disease, diabetes, hypertension (HA), obesity, arterial fibrillation (AF),

education level, physical activity, alcohol consumption and smoking. on AD prevalence (105). In addition, AD is also correlated with genetic risk factors, of which the most significant role belongs to the APOE4 allele (54).

I.5 Pathology

From a pathological point of view, AD is characterized by cerebral atrophy with a decrease in brain volume and weight, atrophy of the convolutions of the cortex with expansion of the cortical grooves and the ventricular system (4). In AD, the most affected brain regions are those associated with higher mental functions, especially the neocortex and hippocampus (31). Pathological, specific and cardinal features of the disease include reduced cholinergic functions, mitochondrial dysfunction (2), the presence in the brain of amyloid plaques formed by Aβ and NFT (5). These pathological molecular clusters are located especially in the frontal, temporal, parietal cortex, in the hippocampus and in the cholinergic nuclei of the basal forearm (4, 175). Amyloid plaques do not appear to have a defined pattern of deposition, probably due to the many mechanisms capable of removing these proteins at the extracellular level, whereas NFT deposition appears to follow a well-defined pattern (84). Numerous studies have indicated that one of the earliest changes in AD involves the loss of synapses (4) which correlates with cognitive decline, eventually leading to marked cell loss in multiple areas of the brain. It is also noted that there are no forms of AD in the absence of other coexisting disease processes, such as other forms of dementia, cardiovascular disease or diabetes (85).

I.5.1 Molecular characteristics

I.5.1.1 Brain-derived neurotrophic factor (BDNF)

Brain-derived neurotrophic factor (BDNF) is the most abundant neurotrophin in the CNS and plays a major role in LTP, regulating synaptic plasticity, axonal and dendritic growth and guidance, and participates in neurotransmitter release, neuronal survival and differentiation (86). BDNF plays a major role in energy metabolism, pain and apoptosis (87). Regarding AD, BDNF has been shown to enhance the survival and differentiation of basal brain cholinergic neurons (88). BDNF stimulates ACh release, suggesting that deficiencies in BDNF synthesis may participate in the impairment of cellular homeostasis triggering AD (89).

I.5.1.2 Neuropeptide Y (NPY)

Neuropeptide Y (NPY) is the most abundant neuropeptide in the CNS, having 36 amino acid residues and being highly conserved among mammals (90). NPY plays a crucial role in learning, memory, and various physiological functions, including mood regulation, cardiovascular homeostasis, and gastrointestinal motility. Studies have shown that AD patients have low levels of NPY compared to healthy controls (91). NPY mediates intracellular Ca^{2+} concentrations, reduces glutamate excitotoxicity, protects hippocampal cells and promotes neurogenesis by activating specific receptors, attenuates neuroinflammation and contributes to neuronal protection in AD (92).

I.5.1.3 Early growth response protein 1 (EGR1)

Early growth response protein 1 (EGR-1), regulates genes involved in growth, survival and synaptic plasticity, including maintenance of LTP (15). EGR-1 is constitutively expressed in the cortex and rapidly induced in the prefrontal lobe and hippocampus following stimuli such as exposure to novelty or fear conditioning (93). EGR-1 regulates PSEN2 gene expression and influences cholinergic functions, significantly impacting learning and memory (94). Reducing EGR-1 expression impairs LTP and memory in tasks such as spatial navigation and object recognition, and EGR-1 mutant mice do not retain information about spatial location or object features. EGR-1 improves learning and memory by remodeling synapses and growing new synaptic connections (95).

I.5.1.4 Nuclear factor 2 related to erythroid factor 2 α (NRF2)

Nuclear factor 2 related to erythroid factor 2 (NRF2), a member of the Cap'n'collar (CNC) family of transcription factors, regulates the expression of more than 250 genes that contain the antioxidant response element (ARE) in their promoters (96). These genes are involved in detoxification, glutathione metabolism, NADPH production, fatty acid oxidation, iron metabolism, and proteasomal and autophagic processes. NRF2 levels are reduced in AD patients(97). Mouse studies show that lack of NRF2 exacerbates the accumulation of Aβ and τ proteins (98), and cognitive deficits in AD models. Activation of NRF2, through genetic and pharmaceutical interventions, provides a neuroprotective role in AD (99).

I.5.1.5 cAMP response element binding protein (CREB)

CREB1 (cAMP Response Element-Binding Protein) is a 43 kDa nuclear protein expressed in all brain cells, which regulates the expression of genes involved in neuronal survival and synaptic plasticity, being essential for learning and memory (100). Alteration of enzymatic pathways leading to CREB phosphorylation and interaction with transcription machinery impairs CREB function and contributes to synaptic dysfunction and memory loss in AD (101). Studies have shown that CREB expression is reduced in AD, with decreased total and phosphorylated CREB levels in the prefrontal lobe and hippocampus (102). Cognitive deficits in AD are associated with decreased CREB activity, affecting BDNF expression and being mediated by Aβ oligomers that block CREB function (103) and enhancing CREB function could ameliorate memory deficits in AD (104).

I.6 Symptoms of Alzheimer's disease

Symptoms of AD include cognitive decline, behavioral and psychological changes, seen in all stages of the disease (105). Common manifestations include aphasia, executive dysfunction, apathy, and personality changes. Although non-memory cognitive deficits may appear early, memory decline is considered the main symptom (106). Other symptoms include deterioration of language and social skills, decreased enthusiasm and increased aggression. Visuospatial skills gradually deteriorate and executive dysfunction begins early and worsens over the course of the disease (105).

I.7 Diagnosis and treatment

Diagnostic tests based on the assessment of cognitive skills, such as the MMSE test (35). The diagnosis includes different combinations of impairment of cognitive, emotional and social skills, subsequently the diagnosis of AD is confirmed or not with the help of *in vivo* measurement techniques of neuropathology, using various biomarkers of AD (107) such as: recognition memory (108) and long-term memory (109).

AD treatment is based on cholinesterase inhibitor drugs such as donepezil, rivastigmine and GAL, and memantine (110). These drugs are FDA-approved and help relieve symptoms, but do not promote neuronal proliferation or inhibit apoptosis (10). Donepezil, rivastigmine, and GAL work by inhibiting cholinesterase, thereby increasing ACh levels in the brain. Memantine works by blocking overstimulated NMDA channels.

CHAPTER II. FLAVONOIDS AND ALZHEIMER'S DISEASE

II.1 Flavonoids. General aspects

Flavonoids are bioactive plant polyphenols with important roles in nature and health, originating from the metabolism of plants and fungi (111). They influence the color and aroma of flowers and fruits, regulate plant growth and provide protection against biotic and abiotic stress (112). These polyphenols have a long history of medical use due to their antioxidant effects, preventing the formation of free radicals and influencing cellular function by interacting with receptors and intracellular signaling pathways (113). Flavonoids act through various antioxidant mechanisms, including scavenging free radicals, chelating metal ions and inhibiting pro-oxidant enzymes, and have been successfully used in nutraceutical and pharmaceutical applications (114).

II.1.1 Chemical structure, bioavailability and metabolism of flavonoids

Flavonoids have a skeleton of 15 carbon atoms and a basic structure consisting of two benzene rings and one pyranic ring (69). These polyphenols are biosynthesized from phenylalanine via the shikimic acid pathway. Their structure can include hydroxyl, methoxy and glycosidic groups, and their classification is based on the degree of oxidation and saturation of the pyran ring (115). Flavonoids can have varied structures, and differences in structure affect their bioavailability and biochemical and pharmacological activities (116).

Absorption, metabolism and excretion of flavonoids involve complex processes that modify the structure of these compounds in various tissues and cellular compartments. The bioavailability of flavonoids is essential for evaluating their effects as chemopreventive agents (117). Flavonoids undergo extensive transformations, including in the liver and intestine, where they are hydrolyzed into more lipophilic aglycones, facilitating absorption (118). These are subsequently conjugated and detoxified to improve water solubility, and their metabolites are excreted in urine and bile. Their bioactivity varies depending on their chemical structure and target tissue, influencing their therapeutic effects and toxicity (117).

II.1.2 Localization of flavonoids in brain tissue

Some flavonoids and their metabolites can cross the BBB and directly access brain tissues, and their ability to do so depends on their lipophilicity (120). Examples include the detection of quercetin-3-O-glucuronide in brain tissue and the accumulation of flavonoids in various brain regions in animals (121). Although brain levels of flavonoids are often very

low, some classes are retained more in neural tissue than in plasma, amplifying their direct effects on the brain (122,123).

II.2 Flavonoids and Alzheimer's disease

Flavonoids are being investigated as neuroprotective agents due to their ability to influence neurological processes by regulating neuronal and glial signaling pathways (124). They improve vascular function, stimulate synaptic plasticity, and may prevent oxidative damage and mitochondrial dysfunction (18). These natural polyphenols can alter neurodegenerative and cognitive processes by inhibiting cholinesterases and modulating signaling pathways such as ERK and PI3K/Akt (116).

Numerous flavonoids have demonstrated the ability to ameliorate Aβ pathology by promoting non-amyloidogenic processing and inhibiting aggregation, resulting in a decrease in $A\beta_{42}$ levels (125). They also exhibit neuroprotective effects by modulating GSK-3β activity and reducing Aβ-mediated phosphorylation (126). Simultaneously, flavonoids protect neurons by modulating cell signaling pathways, interacting with MAPK signaling pathways, particularly MEK1 and MEK2, which control cellular processes by translating extracellular signals into intracellular responses (127). In addition, activation of anti-apoptotic ERK1/2 and PI3K/AKT pathways, and down-regulation of pro-apoptotic JNK pathways by flavonoids provide protection against oxidative stress (128).

II.3 Roifolin

Roifolin ($C_{27}H_{30}O_{14}$), also known as apigenin-7-O-β-neohesperidoside, is a yellow dihydroxyflavone glycoside with a molar mass of 578.5 g/mol, belonging to the apigenin family (129). The name "roifolin" comes from the Japanese tree Rhus succedanea, from where it was isolated for the first time, and was later identified in the leaves of other plants of various botanical families, such as: Theaceae, Urticaceae, Euphorbiaceae, and in the juices and extracts of citrus fruits, bananas, tomatoes and grapes (130).

Rho exhibits a number of remarkable pharmacological properties, including antiviral, antibacterial, anti-inflammatory, anti-proliferative activities (131) and antioxidants (132) having beneficial effects on viral infections, diabetes, inflammation and OS (133). Rho has also demonstrated anticancer effects (134), antihypertensive and hepatoprotective, as well as a neuroprotective potential against Aβ peptide-mediated neurotoxicity (131).

II.4 Baicalein 5,6-dimethyl ether

Baicalein 5,6-dimethyl ether (Baic, $C_{17}H_{14}O_5$) is a yellow O-methylated flavonoid with a molar mass of 298.0841 g/mol, originally isolated from the roots of the plant Scutellaria baicalensis (family Lamiaceae), used in traditional medicine in the Northeast of Asia for various ailments (135). Baic has also been found in other species of Scutellaria and in plants of the genus Alnus (family Betulaceae), known for their antioxidant, anti-inflammatory, antimicrobial, antiviral and hepatoprotective properties (136).

Baic excels in antioxidant properties, demonstrating the ability to protect mitochondria and reduce oxidative stress. In addition, it showed remarkable hepato-protective effects, preventing hepatic steatosis and reducing lipid peroxidation. The antidiabetic properties of Baic include significantly lowering blood sugar and improving gut microbiota (137). Baic also has remarkable anti-inflammatory effects, significantly reducing inflammatory markers such as NF-κB, TNF-α, IL-6 and IL-1β. In terms of neurovascular protection, Baic has demonstrated neuroprotective effects, protecting neurons and improving cognitive function (138). However, there is no current evidence to suggest that Baic could ameliorate amnesic symptoms in Sco-treated zebrafish.

II.5 Agatisflavone

Agatisflavone (Fab, C27H30O14), also known as 6,8"-Biapigenin, is a yellow biflavonoid with a molar mass of 538.457 g/mol and a density of 1.656 g/cm³, found in the leaves, stems, fruits and roots of plants such as Caesalpinia pyramidalis, Anacardium occidentale and Rhus parviflora, and can be extracted with polar or bipolar solvents such as methanol and ethanol (139).

Fab exhibits notable biological activities, including antiviral, antimicrobial, and neuroprotective effects (140). Fab protects neurons *in vitro* from excitotoxicity by upregulating glutamate and influencing microglia toward an anti-inflammatory M2 phenotype, thereby reducing cell death and proinflammatory cytokine levels (22).

CHAPTER III THE ANIMAL MODEL

III.1 The use of animal models for the study of Alzheimer's disease

Despite advances in non-animal study methods, the use of animal models remains essential in neuroscientific research due to the complexity of the brain (141). Animal models, such as mice and zebrafish, provide essential insights into the mechanisms of AD and the effectiveness of treatments, being more accessible and easier to genetically manipulate than other mammals (54).

III.2 Danio rerio - animal model in biomedical research

In biomedical research, animal models remain essential for understanding the pathogenesis of human diseases and developing new therapies, despite advances in alternative methods (142–144). Zebrafish (*Danio rerio*) has become increasingly popular due to its advantages such as rapid development, genetic diversity and tactility in genetic manipulation (141). This model organism is useful for studying neurodegenerative processes and for evaluating new therapeutic substances due to metabolic pathways conserved with mammals (145).

III.2.1 Danio rerio, as an animal model of AD

Zebrafish is rapidly emerging as a promising model organism to study various CNS disorders, including AD (146). The brain neurochemistry of these fish is very similar to that of mammals, showing all the major neurotransmitter systems, transmitters, receptors, synthesis and metabolism enzymes that are similar to those of humans (147). Also, the main cell types found in the mammalian brain, such as microglia, oligodendrocytes, motor neurons and astrocytes, are also present in the CNS of these fish (148). Another important similarity between mammals and this fish is the development of the BBB, with selective permeability to different macromolecules (149). Also, the typical behavior of zebrafish can be considered as an essential indicator of establishing models of dementia or verifying the effectiveness of drugs. In addition, these fish respond in a similar manner to all classes of neurotropic drugs, including antipsychotics, stimulants, hallucinogens, anxiolytics, antidepressants, antiepileptics, sedatives, hypnotics, anesthetics, analgesics, and cognitive enhancers (150).

III.2.2 Similarities between zebrafish and mammalian behavior

Although the zebrafish brain is smaller and simpler than that of mammals, the genetic, neural and physiological mechanisms responsible for behavioral responses are similar to those of mammals (151). Zebrafish show homologous brain regions that perform similar functions to those in the mammalian brain, such as regulation of emotional states and learning and memory processes (108).

III.2.3 Similarities between the neuroanatomy of zebrafish and that of maifers

Due to its phylogenetic proximity to humans, the zebrafish is recognized as a reliable model for studies of behavior, neural circuits and neurodegenerative diseases (148). Although fish differ from mammals in the organizational structure of the CNS, several brain nuclei in the zebrafish brain, including the basal ganglia, striatum, hippocampus, and amygdala, show high homology with those of mammals (152).

III.3 Disadvantages of using zebrafish as an animal model in neuroscience

Zebrafish offers numerous advantages as a model system for the study of AD, including ease of administration of drugs in the aqueous environment, which allows assessment of efficacy, bioavailability, and toxicity at various stages (146). However, there are also significant limitations, such as differences in the route of drug administration compared to humans, the possibility of absorption of drugs through the skin or other organs, and the complexity of interpreting the circuit-behavior interaction due to the incomplete development of certain brain regions and CNS structure in zebrafish (150).

CHAPTER IV MATERIAL AND METHODS

IV.1 In silico studies

Research into natural chemical compounds with pharmaceutical potential is a complex 12-20 year process involving drug discovery and development. Compounds are often rejected due to inadequate pharmacokinetic behavior, which is why these studies are performed as early as possible to select only compounds with high potential for development (153).

In the context of the computational analysis, we adopted a simplified method of representing the chemical structures using the canonical SMILE system (simplified molecular inline insertion system) for Sco, GAL and the three natural flavonoids (Rho, Baic and Fab) (Table IV.1) .

Table IV.1 SMILE data forscopolamine (Sco), galantamine (GAL), roifolin (Rho), baicalein 5,6-dimethyl ether (Baic) and agatisflavone (Fab)

INGREDIENTS	SMILE (Simplified Inline Molecular Insertion System)
Scotland	CN1C2CC(CC1C3C2O3)OC(=O)C(CO)C4=CC=CC=C4
Welshman	CN1CCC23C=CC(CC2OC4=C(C=CC(=C34)C1)OC)O
Rho	CC1C(C(C(C(O1)OC2C(C(C(OC2OC3=CC(=C4C(=C3)OC(=CC4=O)C5=CC=C(C=C5)O)O)CO)O)O)O)O)O
Bike	C1=CC=C(C=C1)C2=CC(=O)C3=C(O2)C=C(C(=C3O)O)O
Fab	C1=CC(=CC=C1C2=CC(=O)C3=C(O2)C=C(C(=C3O)C4=C(C=C(C5=C4OC(=CC5=O)C6=CC=C(C=C6)O)O)O)O)O

IV.1.1 Evaluation of the physico-chemical properties of the compounds

Physicochemical and molecular properties are critical factors that influence pharmacokinetic and pharmacodynamic processes, thus affecting drug safety and efficacy. Evaluation of these properties, along with ADME analysis, is essential in drug discovery, helping to prioritize high-potential candidates.

Free online platforms such as pKCSM (https://biosig.lab.uq.edu.au/pkcsm/) and SwissADME (http://www.swissadme.ch/index.php#), are used to assess the essential characteristics of compounds, providing information on solubility, hydrophobicity, and chemical interactions.

IV.1.2 Evaluation of the biomedical alert structures of the tested compounds

To evaluate the potential biomedical alerts of Sco, GAl, Rho, Baic and Fab compounds, the SwissADME platform was used. The evaluation included detection of interfering compounds by the PAINS method, identification of problematic structural fragments according to the Brenk list, and lead similarity analysis to determine drug potential. The synthetic accessibility (SA) of the compounds was also estimated, providing a score that reflects the likelihood of synthesis difficulties (154).

IV.1.3 Prediction of compounds, analogy with drugs

The concept of "drug-likeness" assesses the likelihood that a molecule will become an oral drug, focusing on bioavailability. Tools like SwissADME and MolSoft MolSoft (https://www.molsoft.com/) are used to analyze structural and physicochemical criteria, applying rules such as those of Lipinski, Weber, Ghose, and Egan, to filter out molecules that do not meet the necessary conditions for an adequate pharmacokinetic profile. These evaluations help identify and select compounds with the greatest potential to become effective therapeutic agents (155).

IV.1.4 Pharmacokinetic profile estimated in silico of the compounds

In the computational analysis, we used the simplified molecular in-line insertion system (SMILES) for Sco, Gal, Rho, Baic and Fab which were retrieved from the Pubchem platform (https://pubchem.ncbi.nlm.nih.gov/). I used 4 free computers (PASS Online (https://www.way2drug.com/PASSOnline/predict.php), in the computational analysis of Sco, GAL, Rho, Baic and Fab compounds, we used the SMILES representations taken from the PubChem platform. We used four free calculators, including PASS Online, pKCSM, ADMETlab 2.0, and ProTox-II, to obtain predictions related to various pharmacokinetic and toxicological properties of these compounds. To facilitate the comparison of the predictions obtained from these platforms with the experimental data, it was essential to standardize the measurement units and convert some parameters into binary categories. Properties analyzed included water solubility, Caco2 permeability, intestinal absorption, skin permeability, toxicity and interactions with CYP enzymes.

IV.1.5 Prediction of biological activity of compounds by means of PharmMapper and PASS platforms

In the computational analysis of the compounds, we used (https://www.lilab-ecust.cn/pharmmapper/), PASS (Prediction of Activity Spectra for Substances) (https://www.way2drug.com/PASSOnline/predict.php), to assess biological activity and side effects. PharmMapper (156) analyzes molecular interactions by comparing the structure of compounds with pharmacophore models from extensive databases, using Z-scores to assess the relevance of drug targets. passim (157) estimates the probability that a substance has certain biological activities, giving Pa and Pi scores that indicate the chance of being active or inactive. This information helps in determining the therapeutic potential and various actions of the compounds.

IV.1.6 Protein target prediction of studied compounds by means of Molinspiration and SwissTargetPrediction platforms

To evaluate the potential protein targets of the studied compounds, we used Molinspiration platforms (https://www.molinspiration.com/) and SwissTargetPrediction (http://www.swisstargetprediction.ch/). Molinspiration (158) provided ratings of the molecules' biological activities, indicating that scores greater than 0.00 suggest significant biological activity. SwissTargetPrediction (159) performed 2D and 3D similarity analyzes to predict protein targets, using a Combined Score that identified possible common targets and guided compound selection for further experiments.

IV.1.7 Prediction of the studied compounds from the research stage to their possible approval on the market through the miDruglikeness platform

The prediction of the possibility of approval on the pharmaceutical market of Sco, GAL, Rho, Baic and Fab compounds was carried out using the miDruglikeness platform (http://www.pkumdl.cn:8000/midruglikeness). This platform provides a comprehensive assessment of drug potential, analyzing *in vivo* potency, investigational new drug (IND) potential, and estimates for market approval (160). miDruglikeness provides critical information to guide drug development resources and decisions, thus contributing to the selection of compounds with the greatest potential for commercial success.

IV.2 Plant material

The study looked at three natural flavonoids, Rho, Baic and Fab, known for their beneficial health effects and absence of adverse reactions. Rho reduces inflammation and protects neurons from neurotoxicity (161), Baic has anti-neuroinflammatory effects and regulates the expression of TNF-α and IL-6 (162), and Fab stimulates neuron generation and protects against glutamate excitotoxicity (163). These flavonoids were isolated and characterized at Ain Shams University, CairoEgypt, under the scientific coordination of Prof. univ. Dr. Omayma Eldahshan.

IV.3 Animal model and method of treatment administration

In this study, we investigated the impact of chronic administration of three natural flavonoids on a zebrafish model of dementia.120 adult short-finned zebrafish (Danio rerio), 3 to 4 months of age, were used and lengths of 3-4 cm at the beginning of the experiment, in the proportion of 50% females and 50% males, purchased from an authorized breeder (Pet Product SRL, Bucharest, Romania). After acquisition, fish were acclimated in two 70 L aquaria equipped with Tetratec® air pumps (Tetra, Melle, Germany) in a test room at the Faculty of Biology. Dechlorinated water, treated with Tetra AquaSafe (Tetra, Germany), was replaced every two days and water quality parameters were monitored daily. The fish were maintained under a natural photoperiod with a 14-h light-10-h dark cycle and fed twice daily with NovoMalawi algae flakes (JBL, Neuhofen, Germany).

After 10 days of acclimation, the fish were randomly divided into 11 experimental groups, each with 10 animals, placed in 10 L aquaria containing dechlorinated water treated with Tetra AquaSafe, replaced every two days. Of the 120 fish, 10 were used as control group (Ctr), 10 were treated with Sco (100 μM; Sigma-Aldrich, Germany), 10 were treated with Sco (100 μM) + Galantamine (GAL, 1 mg/L; Sigma-Aldrich, Germany) and the remaining 90 were divided into another 9 groups as follows: (a) Sco (100 μM) + Rho (1, 3 and 5 μg/L); (b) Sco (100 μM) + Baic (1, 3 and 5 μg/L); and (c) Sco (100 μM) + Fab (1, 3 and 5 μg/L). Natural flavonoids (Rho, Baic and Fab) were administered chronically for 10 days before behavioral tests, diluted in 1% dimethylsulfoxide (DMSO) solution. The dementia-like state was induced by acute administration of Sco (100 μM) for 30 min before the NTT test and euthanasia procedure, and for the Y maze and NOR tests, Sco (100 μM) was administered 30 min after training session. The control group was exposed only to dechlorinated water with 1% DMSO solution.

The study was approved by the Animal Ethics Committee of the Faculty of Biology, Alexandru Ioan Cuza University, Iași, Romania (Project approval number: 370/4.02.2022), and all measures were taken to minimize animal pain and discomfort, according to Directive 2010/63/EU on the protection of laboratory animals. No deaths or signs of intoxication were recorded during the experiment.

IV.4 Evaluation of behavioral parameters

To evaluate the impact of chronic administration of the three natural flavonoids on the behavior of the animals included in this study, we monitored the activity of zebrafish using a Logitech HD Webcam C922 Pro Stream digital camera. This camera has a Full HD resolution of 1080 pixels and a frame rate of 30 per second and is manufactured by Logitech, Lausanne, Switzerland. The resulting videos were subsequently analyzed using ANY-maze® software, version 6.3, provided by Stoelting Co., Wood Dale, IL, USA.

IV.4.1 New aquarium immersion test (NTT)

Studies have shown that anxiety tests in rodents are essential for identifying anxiety-related behavioral profiles and evaluating the impact of drugs (164), and zebrafish, using partially analogous assays, give comparable results. These assays demonstrated significant similarities in measuring behaviors such as reduced exploration and thigmotaxis, underscoring the utility of zebrafish in studies of anxiety and in the assessment of genetic and pharmacological factors (165).

To investigate the effect of Rho, Baic and Fab compounds on the state of anxiety in zebrafish acutely treated with Sco (100 µM), we applied the protocol established by Cachat (166). I used a trapezoidal aquarium made of clear glass with precise dimensions: height 15.1 cm, base length 23.9 cm, top length 28.9 cm, and width 6.1 cm. The aquarium was divided horizontally into two equal sections: an upper and a lower one. During the experiment, we filled the test tank with 1.5 liters of water taken from the fish's housing aquarium and placed it on a flat white surface. Each fish was evaluated individually for a duration of 6 minutes.

During the test, we measured anxiety behavior by means of time spent in the upper/lower area of the aquarium (s), latency period (s) and distance traveled in the upper part of the aquarium (m). In addition, in the nine-point test (NTT), we assessed the locomotion-related phenotypes of zebrafish by recording parameters such as: total distance traveled (m), mean swimming speed (m/s) and immobility/freezing duration. These

measurements allowed a detailed examination of both the anxiety behavior and the locomotor response of the fish in the novel environment.

IV.4.2 Y-maze test

To evaluate the pharmacological properties of Rho, Baic and Fab on the cognitive processes of amnesic zebrafish, we used the Y-maze test, following the protocol previously described by Cognato *et al*, (167).

The Y-maze test is based on the zebrafish's natural tendency to explore in response to novelty (168). The main task of this test involves the involvement of several brain regions such as the hippocampus, septum, basal brain and prefrontal cortex, being very sensitive to impairment of hippocampal functions and amnesic drugs (169).

The aquarium used for this test was constructed of clear glass and had the shape of the letter Y, having three equal arms, each measuring 25 cm long, 8 cm wide, and 15 cm high, forming an angle of 120 degrees. To facilitate the video analysis, the bottom of the aquarium was covered with a white plastic material, and the other surfaces were covered with black plastic, on which visual landmarks in the form of squares, circles and triangles were applied. In the training phase, the fish were allowed to explore only two of the three arms of the maze—the start arm and the familiar arm—while the third arm, representing the novel arm, was blocked. In the test session, fish were reintroduced to the start arm with free access to all three arms for 5 min. Locomotor activity was assessed by recording the total number of arm entries, the total distance traveled (m) and the turning angle (˚). To evaluate the learning and memorizing processes of the zebrafish, the percentage of spontaneous alternation (%) was recorded. To evaluate the spatio-temporal memory, the number of crossed lines and the time spent in the 3 arms (s) were recorded.

IV.4.3 Novel Object Recognition Test (NOR)

The new object recognition test (NOR) is based on the natural preference of vertebrates to explore new objects (ON) to the detriment of familiar ones (OF) (170). NOR is a test used to evaluate cognitive changes in animals following nervous system disorders, neurodegenerative or those induced by certain chemicals. Also, with the help of this test it is possible to evaluate any type of memory, including short and long-term memory by simply manipulating the retention interval, defined as the time period between the training session and the test session (54).

To evaluate the impact of the three flavonoids on recognition memory in zebrafish, we implemented the NOR test, according to the protocol established by Stefanello (171). I used a glass tank with a cubic shape, having dimensions of 30 cm in width and height. The external walls of the tank were covered with a black fabric to reduce fish stress and minimize experimental errors. The tank was placed on a flat surface and filled with water taken from the fish's housing tank, with the water level maintained at 5 cm from the upper edge of the test tank.

The test was conducted over four days. During the first three days, each fish was familiarized with the test tank for 5 min twice a day with a 5 h interval between sessions. The training session, conducted 12 hours after the last familiarization session, involved exploring two identical objects (yellow cubes of 2.5 cm side) for 10 minutes. The cubes were placed in two opposite corners of the tank, oriented parallel, with a distance of 10 cm between them. After training, there was a one-hour retention period in which one of the yellow cubes was replaced by a blue cube. Thus, the yellow cube was considered familiar (OF), and the blue cube, new (ON). In the test session, each fish had the opportunity to explore the two cubes for 10 min. Recognition memory was assessed as percent preference,calculated according to the formula: Exploration time in ON/(Exploration time in OF + Exploration time in ON)*100; Therefore, fish with better reference memory will remember the OF and spend more time exploring the ON.

IV.5 Evaluation of biochemical parameters in zebrafish brain samples

Immediately after performing the behavioral tests, the fish were euthanized by rapid cooling. The procedure involved lowering the water temperature to 2-4°C by adding ice (5 parts ice to 1 part water), avoiding direct contact of the fish with the ice before they became unconscious (172). After euthanasia, fish were dissected according to the method described by Gupta and Mullins (173). The fish were removed from the cold water and transferred to a dissection rack. After decapitation, heads were subjected to precise dissection in 1X phosphate buffered saline (PBS), pH 7.4. Whole brains were extracted and placed in 0.5 ml microtubes. In biochemical tests, three zebrafish brains were considered as one sample. Brain tissue (represented by the 3 brains) was weighed (~15 mg) and homogenized (1:10, w/v) for 1 min at 1000 rpm, on ice, in an extraction buffer (phosphate buffer of potassium 0.1 M, pH 7.4 with 1.15% KCl), using the Mikro-Dismembrator U mill (Sartorius, New York, USA) equipped with 3 mm magnetic balls (Sartorius Stedim Biotech GmbH,

Goettingen, Germany). The homogenate was centrifuged for 15 min at 14000 rpm and 4°C for clarification, and the supernatant was later used for the determination of total protein content, evaluation of the specific activity of SOD, CAT, GPX, and AChE enzymes, and estimation of total GSH content , MDA and carbonylated proteins.

IV.5.1 Determination of the concentration of soluble proteins. The Bradford method

Protein content was quantified using the Bradford method (174). The Bradford method is a colorimetric technique used for protein quantification, based on the color change of a staining reagent, Coomassie Brilliant Blue, which binds to proteins, generating a measurable absorbance at 595 nm, proportional to the protein concentration in the sample

IV.5.2 Determination of superoxide-dismutase activity

In this study, superoxide dismutase (SOD, EC 1.15.1.1) activity was determined based on the protocol described byWinterbourne *et al*,. (175). Briefly, the ability of the enzyme to inhibit the reduction of Nitro Blue Tetrazolium (NBT) by superoxide free radicals that were generated in the reaction medium by riboflavin photoreduction was monitored. Enzyme activity was expressed as units/mg protein.

IV.5.3 Determination of catalase activity

For the determination of catalase activity (CAT, EC 1.11.1.6) we used a simple colorimetric method, which was first described by Sinha (176). The given method is based on the fact that potassium dichromate is reduced in acidic medium (acetic acid) to chromic acetate in the presence of heat and H_2O_2 and can be determined spectrophotometrically at 570 nm.

IV.5.4 Determination of glutathione peroxidase activity

In this study aglutathione peroxidase (GPx, EC 1.11.1.9) activity was assessed using the protocol described by Fukuzawa and Tokumura (177). This method is based on the fact that GPx catalyzes the decomposition of H_2O_2 with GSH as the reductant, thus resulting in oxidized glutathione (GSSG) and water. This reaction is important in removing low levels of H_2O_2 that could damage the cell. The remaining excess GSH reacts with DTNB to form a yellow complex. The color intensity was measured spectrophotometrically, since the difference between the initial and the final amount is directly proportional to the enzyme activity. The enzymatic activity of GPx was expressed as units/mg protein.

IV.5.5 Determining the level of reduced glutathione

In this study, the method described by Salbitani *et al* was used to determine the content of reduced glutathione (γ-L-glutamyl-L-cysteinyl-glycine-GSH, EC1.8.4.4) (178). GSH is an essential component of the cellular antioxidant defense system and plays a direct role in maintaining redox homeostasis and regulating low ROS concentrations.The amount of GSH was related to the protein concentration and was expressed as µg GSH/mg protein.

IV.5.6 Determination of malondialdehyde level

The determination of Malondialdehyde (MDA, EC 542.78.9) levels in zebrafish brains was performed according to the protocol described by Ohkawa *et al*., (179). This protocol is based on the interaction of lipid peroxides from animal tissues with thiobarbituric acid, generating a pink color, which was measured at 532 nm.

IV.5.7 Determination of the level of carbonylated proteins

To assess the level of carbonylated proteins in zebrafish brains, we applied the method described by Oliver *et al.,* (180). This involves the reaction between 2,4-dinitrophenylhydrazine and protein residues in fish brains, generating 2,4-dinitrophenylhydrazones. These complexes were quantified at 370 nm, using a mixture of GuHCl and KH_2PO_4 as reference. Results were expressed in nmoles of DNPH per mg of protein.

IV.5.8 Determination of acetylcholinesterase activity

To determine the activity of acetylcholinesterase (AChE EC 3.1.1.7) from the homogenates, the photometric method described by Ellman *et al*.,(181) was used. ACh iodide substrate (Sigma, USA) and dithiobisnitrobenzoic acid (DTNB) reagent (Sigma, USA) were added together with phosphate buffer (pH 7.4). ACh iodide was hydrolyzed to thiocholine and ACh acetate. The enzyme acts on ACh, forming thiocholine and acetate in the medium. The resulting thiocholine rapidly reacts with DTNB in the reaction medium and leads to the formation of the yellow colored 5-thio-2-nitrobenzoate anion produced from the reaction between thiocholine and DTNB. The rate of color development was used as a measure of AchE activity. A kinetic profile of enzyme activity was studied spectrophotometrically at 412 mm. Enzyme activity was expressed in nmoles ATC/min/mg protein.

IV.6 Evaluation of gene expression

IV.6.1 Extraction of total RNA from zebrafish brain samples

Extraction of total RNA from zebrafish brains was performed using EZ-RNA II Total RNA Isolation Kit (chloroform-free) Cat. No.: 20-410-100 (182). Individually weighed brains (approx. 5 mg) were homogenized in 0.25 ml of denaturing solution and incubated for 5 min. After addition of saturated phenol and 1-bromo-3-chloropropane, the mixture was centrifuged, and the supernatant was treated with isopropanol and centrifuged again. Pellets were washed with 75% ethanol and air-dried, and RNA was dissolved in nuclease-free water. RNA samples were normalized to 100 ng/µl, and concentration and purity were measured by NanoDrop. RNA was converted to cDNA using the Omniscript Reverse Transcription Kit (QIAGEN).

IV.6.2 Evaluation of bdnf, npy, egr-1 nrf2, and creb1 gene expression

Total RNA samples were slowly thawed at 4 °C and subsequently quantified. To exclude gene expression quantification errors caused by differences in the concentration of RNA introduced into the amplification reactions, all samples were normalized to the concentration of 100 ng/µl. To quantify the absolute expression of the genes of interest (*bdnf, npy, egr-1, nrf2α* and *creb*), both reverse-transcription and RT-qPCR amplification were performed using a GoTaq® 1-Step RT kit -qPCR System (Promega, Madison, WI, USA), on a HRM Rotor-Gene 6000 (Corbett, CA, USA) 5-plex real-time rotary PCR machine. Reaction mixtures were made in duplicate, each reaction mixture having a volume of 10 µl. As an internal control in RT-qPCR I use β-actin. Thus, we improved the accuracy and reliability of our gene expression data.

IV.7 Immunodetection of CREB1 protein, transferred onto nitrocellulose membranes

Detection of the proteins of interest, CREB-1 and GAPDH, was performed by immunoblotting techniques, according to the analysis previously used by Koehler (183). Brains were individually homogenized with a ball mortar (Mikro-Dismembrator U; Sartorius, New York, USA) equipped with 3 mm magnetic balls (Sartorius Stedim Biotech GmbH, Goettingen, Germany) in an extraction buffer (0, 1 M potassium phosphate buffer, pH 7.4, with 1.15% KCl and SIGMAFAST™ protease inhibitors, Protease Inhibitor Tablets, S8820), containing soluble protease inhibitors (AEBSF, E-64, bestatin, leupeptin, aprotin and EDTA (sodium salt)) with broad specificity for inhibition of serine, cysteine and

metalloproteases. The homogenate was centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant (~130 µl) was collected in sterile 1.5 ml tubes. The optimal concentration of protein extract for loading into the wells was determined using the Bradford method (174). Equal amounts of protein were diluted in loading buffer (200 mM Tris-HCl, pH 6.8, 10% SDS, 0.4% bromophenol blue, 40% glycerol, and 1M beta-mercaptoethanol) and heated at 90°C for of 10 minutes to facilitate denaturation. After denaturation, samples were centrifuged at 2500 rpm for 10 seconds and then separated by electrophoresis on a 10% polyacrylamide gel using SDS-PAGE electrophoresis.

Proteins, ordered according to their molecular masses on the polyacrylamide gel, were transferred electrostatically to a nitrocellulose membrane (0.45 µm) (Watman, Germany) using a semi-dry migration system. Membranes were blocked for 30 min in a blocking buffer solution (TTBS) containing 500 mM TRIS-HCl, 1.5 M NaCl, and 0.1% Tween 20%. Then, they were incubated for two hours at room temperature in TBS buffer solution with two primary antibodies (anti-GAPDH and anti-CREB-1, produced in rabbit) as described in Table 2. After washing with TTBS solution, membranes were incubated for one hour in TBS buffer with secondary antibody (IgG, anti-rabbit, produced in goat). After a final wash, nitrocellulose membranes were incubated for 10 min at room temperature in carbonate buffer (0.1 M $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8). Finally, nitrocellulose membranes were developed in a development buffer (7.5 g BCIP in 20 ml carbonate buffer + 60 µl NBT), shaking gently until bands appeared (~2–5 minutes). Bands were imaged with Fluor-S MultiImager (Bio-Rad, USA), and image analysis for quantification of proteins of interest was performed using Image Lab software (Bio-Rad, USA).

IV.8 Statistical analysis

Results were expressed as mean ± standard error of the mean (SEM). Data on behavior, cholinergic and oxidative status, *bdnf, npy, creb1, egr1*, *nrf2* gene expression and Creb1 protein level were analyzed by one-way analysis of variance (ANOVA) followed by post hoc multiple comparison test of Tukey's, factoring Rho, Baic and Fab treatment.

All statistical analyzes were performed using GraphPad 9.2 software (GraphPad Software 9.2, Inc., San Diego, CA, USA), and statistical significance was set at a $p < 0.05$. correlationbetween anxiety-like state, neurocognitive performance, OS of cholinergic status, expression of genes of interest *bdnf, npy, creb1,egr1,nrf2α* and CREB1 protein levelwas established by means of Pearson correlations (r).

CHAPTER V. RESULTS AND DISCUSSIONS

V.1 Physico-chemical properties of roifoline, baicalein 5,6-dimethyl ether and agatisflavone

This work complements previous research by comparing the physicochemical properties of Sco, GAL, Rho, Baic and Fab compounds using data from PubChem(https://pubchem.ncbi.nlm.nih.gov/) and analyzing them with the pKCSM online platform (https://biosig.lab.uq.edu.au/pkcsm/) and the SwissADME online platform (http://www.swissadme.ch/index.php#).

The study focused on the natural compounds Rho, Baic and Fab, due to their historical presence in the human diet. Figure 5 shows the bioavailability radar for Sco, GAL, Rho, Baic and Fab, which presents six physicochemical properties: lipophilicity (LIPO), size (SIZE), polarity (POLAR), solubility (INSOLU), flexibility (FLEX) and saturation (INSASTU). The results show that Sco and GAL are orally soluble compounds, while natural flavonoids (Rho, Baic, Fab) are not orally bioavailable due to their high polarity, large size and other physicochemical characteristics.

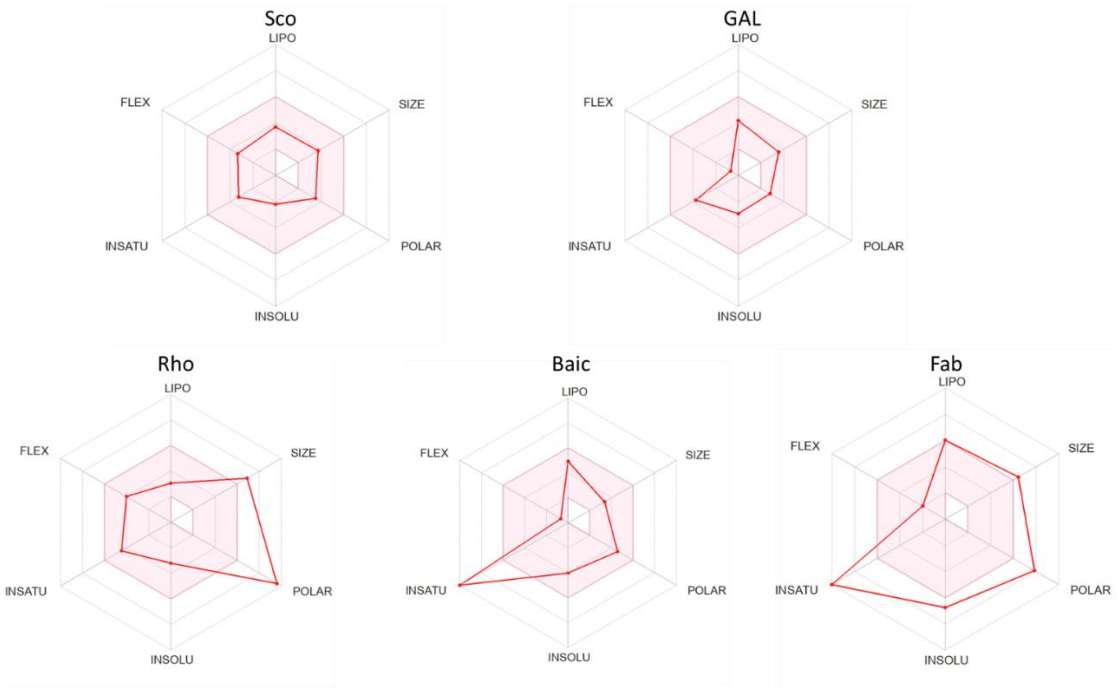


Figure 5.1. The pink area represents the optimal range for each of the properties (lipophilicity: LogP between −0.7 and +5.0, size: Molecular weight between 150 and 500 g/mol, polarity: TPSA between 20 and 130 Å, solubility: LogS no more greater than 6, saturation: fraction of carbon atoms in sp3 hybridization not less than 0.25 and flexibility: not more than 9 rotatable bonds In this example, the compound is predicted not to be orally bioavailable because it is too flexible and too polar.

Table V.1 details the essential physicochemical properties for the evaluation of compounds with pharmaceutical potential, according to the criteria established by Xiong *et al*., (191). These criteria include molecular weight (100-600 g/mol), number of hydrogen bond acceptors and donors, solvent accessible polar surface area (TPSA), solubility, lipophilicity and number of rotatable bonds. These properties are crucial for the optimization and selection of compounds with therapeutic potential.

Table V.1 Physicochemical properties of scopolamine (Sco), Galantamine (GAL), roifoline (Rho), baicalein 5,6-dimethyl ether (Baic) and agatisflavone (Fab) obtained using the Pkcsm and SwissADME platforms.

descriptive	Scotland	Welshman	Rho	Bike	Fab
Molecular formula	C17H21NO4	C17H21NO3	C27H30O14	C15H10O5	C30H18O10
Molecular weight	303,358	287,359	578,523	314,293	538,464
NH	5	4	14	6	10
nHD	1	1	8	2	6
Number of heavy atoms	22	21	41	20	40
Number of heavy aromatic atoms	6	6	16	16	32
LogP	0.9181	1.8503	-1.0983	2.8884	5.134
LogS	-2,596	1,243	-3,834	-3,441	-4,310
Csp3 fraction	0.59	0.53	0.44	0.00	0.00
Rotary links	4	1	6	3	3
Molar refraction	83.48	84.05	137.33	73.99	146.97
Surface	129,371	1.8503	231,312	130,682	222,664
TPSA	62.30 Å²	41.93 Å²	228.97 Å²	90.90 Å²	181.80 Å²

LogP (octanol/water partition coefficient)

TPSA (Surface Area of Donors and Acceptors)

V.2 Highlighting the biomedical alert structures of the 3 flavonoids

Among the analyzed compounds, only Baic presents a PAINS alert related to catechol-O-methyltransferase (COMT) inhibition, suggesting a reduced potential compared to the other compounds, as can be seen in Table V.2.

Table V.2 Properties associated with the biomedical alert structures of scopolamine, galantamine, roifoline, baicalein 5,6-dimethyl ether and agatisflavone obtained using the SwissADME platform.

compound	Scotland	Welshman	Rho	Bike	Fab
PAINS	0 alert	0 alert	0 alerts	1 alert: catechol_A	0 alerts

Brenk	1 alert: Three members_heterocycle	1 alert: isolated_ alkene	0 alerts	1 alert: Catechol	0 alerts
Like lead	Yes	Yes	Not; 1 violation: MW>350	Yes	Not; 2 violations: MW>350, LogP>3.5
SADDLE	4.03	4.57	6.33	3.02	4.17

MW- molecular weight

Sco has a Brenk Alert linked to a three-membered heterocyclic core, which may negatively influence pharmacokinetics and indicate toxic potential. GAL has a Brenk alert for "alkene_ isolate", suggesting possible adverse effects on its pharmacology, and Baic has a "Catechol" alert, indicating similar risks. Rho and Fab have no Brenk alerts, but both flavonoids have high molecular weight and low lipophilicity, indicating low solubility and difficulty in lead optimization, although all compounds are synthetically accessible according to validated SA scores (192).

V.3 Predictions of the biomedical alert structures of the 3 flavonoids

The prediction results of the biomedical alert structures of Sco, GAL, Rho, Baic and Fab are presented in Table V.3. The analysis showed that Sco, GAL and Baic fulfill the five pharmacokinetic filters, being considered as potential candidates for drug development, while Rho and Fab do not completely follow these rules and have moderate bioavailability. The Abbot bioavailability score suggests a moderate likelihood of oral bioavailability for Sco, GAL and Baic, but a low likelihood for Rho and Fab (Figure 5.2). These *in silico* analyzes provide a preliminary assessment and require confirmation by laboratory experiments to validate the potential of these compounds as drugs.

Table V.3 Predictions associated with the biomedical alert structures of scopolamine, galantamine, roifoline, baicalein 5,6-dimethyl ether and agatisflavone obtained using the SwissADME platform.

Compounds	**Scotland**	**Welshman**	**Rho**	**Bike**	**Fab**
Lipinski	Yes; 0 violations	Yes; 0 violations	Not; 3 violations: MW > 500, NorO > 10, NHorOH > 5	Yes; 0 violations	Not; 2 violations: MW>500, NHorOH>5
Ghose	Yes	Yes	Not; 4 violations : MW > 480, WLOGP < -0.4, MR > 130, #atoms > 70	Yes	Not; 2 violations: MW>480, MR>130
Weber	Yes	Yes	Not; 1 violation: TPSA >140	Yes	Not; 1 violation: TPSA>140
Egan	Yes	Yes	Not; 1 violation: TPSA>131.6	Yes	Not; 1 violation : TPSA>131.6

Muegge	Yes	Yes	Not; 3 violations: TPSA >150, H-acc >10, H-don >5 2	Yes	Not; 3 violations: XLOGP3 > 5, TPSA >150, H-don>5
Abbot	0.55	0.55	0.17	0.55	0.17

Using Molsoft(193)enabled the prediction of essential properties of molecules, such as bioavailability and protein affinity, contributing to the selection of the most promising compounds for further development.

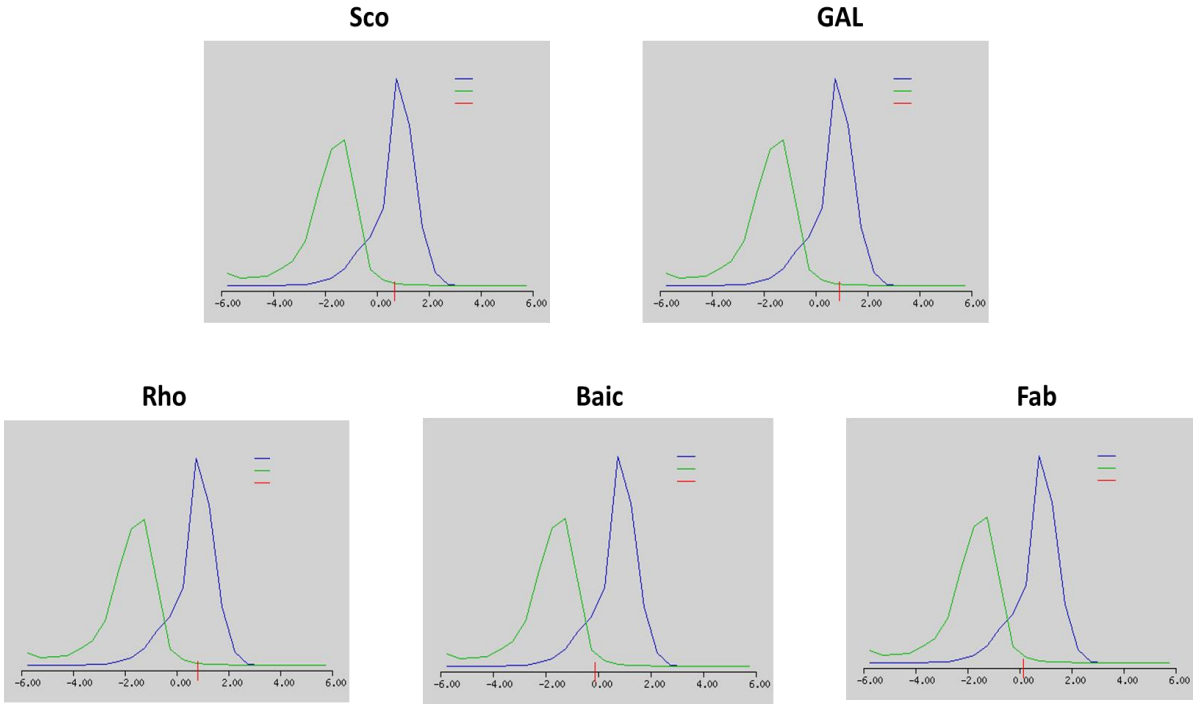


Figure 5.2 Analogy representation of scopolamine (Sco), Galantamine (GAL), roifoline (Rho), baicalein 5,6-dimethyl ether (Baic) and agatisflavone (Fab) with drugs. The blue line indicates the score for drugs, the green line indicates the score for non-drug compounds and the red line indicates the location of the analyzed compound. The drug-like model score was: 0.68 for Sco, 0.90 for GAL, 0.81 for Rho, -0.10 for Baic and 0.14 for Fab.

V.4 Pharmacokinetic profile estimated in silico of the 3 flavonoids

Molecules with medicinal potential influence cellular targets by binding to receptors and adjusting cellular mechanisms, but must travel a complex pathway in the body to manifest their therapeutic effect. Pharmacokinetics studies the steps of absorption, distribution, metabolism and excretion (ADME) of drugs, starting with absorption from the intestinal tract and continuing with hepatic metabolism and distribution in the body. After generating the therapeutic effect, drugs must be efficiently eliminated to prevent bioaccumulation and toxic side effects, which may also occur through interference with other

drugs due to enzyme induction. Thus, it is essential to monitor and manage these interactions to ensure the safety and effectiveness of treatment (194).

To evaluate the pharmacokinetic potential of Sco, GAL, Rho, Baic and Fab, we analyzed properties such as absorption, distribution, metabolism, excretion and toxicity. Solubility, an essential and easily measured parameter, can be classified into unbuffered, buffered and intrinsic solubility, and the water solubility data of these compounds are shown in Table V.4.

Table V.4 Pharmacokinetics. Pkcsm/ ADMETlab 2.0 and ProTox-II: Water solubility, Caco2 permeability prediction, intestinal absorption, P-glycoprotein substrate, glycoprotein PI/II inhibitor, skin permeability, VDss, blood-brain barrier (BBB) permeability, CNS permeability, substrate CYP3A4, CYP1A2 inhibitor, total clearance, renal OCT2 substrate, AMES toxicity max. tolerated dose (human), acute oral toxicity in rat (LD50), chronic oral toxicity in rat (LOAEL), hepatotoxicity, skin sensitization.

Owned	**Compound The name of the property**	**Scotland**	**Welshman**	**Rho**	**Bike**	**Fab**	**United**
Absorption	Solubility in water	-1,601	-2,641	-2,862	-3,302	-2,892	Numerical (log mol/L)
	Caco2 permeability	0.059	1,594	-0.942	1,219	0.057	Numerical (log Papp in 10-6 cm/s)
	Intestinal absorption (human) (low <30%, high >30%)	72,626	94,994	24,308	94,004	96,086	Numerical (% absorbed)
	P-glycoprotein substrate	Yes	Not	Yes	Yes	Yes	Definitely (Yes/No)
	PI/II glycoprotein inhibitor	Not	Not	Not	Not	Yes	Definitely (Yes/No)
	Skin permeability	-4,097	-3.75	-2,735	-2,737	-2,735	Numeric (log Kp)
Distribution	VDss (human)	0.583	0.89	1.14	0.369	-1.052	Numerical (log L/kg)
	Unbound Fraction (Human)	0.414	0.36	0.152	0.071	0.271	Numeric (Fu)
	BBB permeability (log BB > 0.3 crosses the BBB, log BB < 0.1 does not cross the BBB)	-0.043	-0.081	-1,702	-0.448	-1,825	Numeric (log BB)
	CNS permeability (log PS > -2, penetrates CNS, log PS < -3 does not penetrate)	-3.031	-2,511	-4,798	-2.29	-3.133	Numeric (log PS)

Metabolism	Substrates of CYP2D6	Not	Not	Not	Not	Not	Definitely (Yes/No)
	CYP1A2 inhibitor	Yes	Yes	Not	Not	Yes	Definitely (Yes/No)
	CYP2C19 inhibitor	Not	Not	Not	Yes	Not	Definitely (Yes/No)
	CYP2C9 inhibitor	Not	Not	Not	Not	Not	Definitely (Yes/No)
	CYP2D6 inhibitor	Not	Not	Not	Yes	Not	Definitely (Yes/No)
	CYP3A4 inhibitor	Not	Not	Not	Not	Not	Definitely (Yes/No)
Excretion	Total clearance	1,096	0.991	-0.005	0.675	0.407	Numerical (log ml/min/kg)
	OCT2 renal subsubstrate	Not	Yes	Not	Not	Not	Definitely (Yes/No)
Toxicity	AMES toxicity	Not	Not	Not	Not	Not	Definitely (Yes/No)
	Max dose tolerated (human) (minimum < 0.447, maximum > 0.477)	-0.319	-0.423	0.492	0.094	0.438	Numeric (log mg/kg and day)
	Acute oral toxicity to rat (LD50)	2,234	2,728	2,498	2.21	2,507	Numerical (mol/kg)
	Rat Chronic Oral Toxicity (LOAEL)	0.736	0.966	4,443	1,746	3,353	Numerical (log mg/kg_bw/day)
	Hepatotoxicity	Not	Yes	Not	Not	Not	Definitely (Yes/No)
	Skin sensitization	Not	Not	Not	Not	Not	Definitely (Yes/No)

According to the pkCSM platform, the water solubility of the compounds at 25°C varies, with Sco having the highest solubility (-1,601 log mol/L) and Baic the lowest (-3,302 log mol/L). The permeability coefficient of Caco-2, indicating intestinal absorption, varies significantly, with Baic and GAL having high values, suggesting efficient absorption, while Sco and Fab have low coefficients, indicating poor absorption. GAL and Baic can cross the BBB, while Sco, Rho and Fab have low permeability.

In terms of pharmacokinetics, Sco presents a variable bioavailability depending on the route of administration, the highest being by intranasal application. GAL has a linear pharmacokinetic profile with high oral bioavailability. The Vdss value is high for most compounds, with GAL having the highest volume of distribution, while Fab has the lowest

value. The unbound fraction in plasma (Fu) varies, with Sco and GAL having higher values compared to Baic and Rho.

Sco, GAL and Fab act as inhibitors of CYP1A2, without being substrates for CYP enzymes, and Baic inhibits CYP2C19 and CYP2D6, but is not a substrate for any CYP enzyme. GAL is significantly metabolized by CYP2D6 and CYP3A4. Sco clearance is high, GAL having significant renal clearance. GAL is an OCT2 substrate, whereas the other compounds are not.

AMES tests indicate absence of mutagenic potential for all compounds. Human tolerance varies, with Rho having the highest tolerance and Sco and GAL the lowest. Acute oral toxicity varies, with Baic and Sco having the lowest LD50 and the highest GAL. Chronic oral toxicity is low for Sco and GAL, but higher for Rho, Fab and Baic. The prognosis suggests that Sco and flavonoids are not toxic to the skin and liver, but GAL may affect the liver.

V.5 Prediction of the in silico biological activity of the 3 flavonoids

Predictions obtained using the PharmMapper platform suggested an impressive number of targets for the analyzed compounds. Thus, Sco could interact with approximately 5184 ligands, GAL with 9651, Rho with 5281599, Baic with 5282150 and Fab with 5281605 ligands.

For our study we selected Sco, GAL, Rho, Baic and Fab AChE and cholinesterase as predictable targets. Figure 5.3 shows the Z-score of matching the analyzed compounds with the protein targets. In descending order, Sco (1.27597) had the highest AChE match Z score, which was followed by Rho (0.610554), Fab (0.497188), Baic (0.368358) and GAL (0.27972). Regarding the match score for cholinesterase, the highest value was for GAL (0.628381), Rho (0.178299) and Baic (0.0301671) and the lowest for Fab (-0.501857) and Sco (-0.104149).

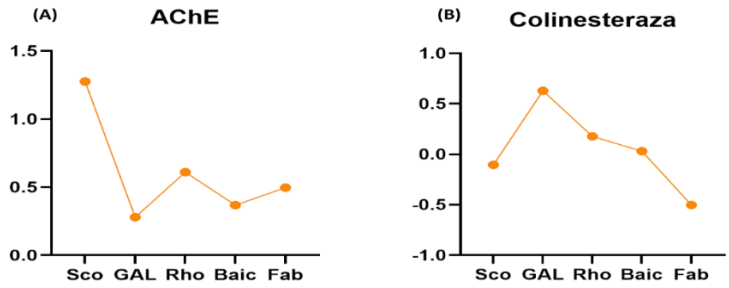


Figure 5.3 Z score of interaction of scopolamine (Sco), Galantamine (GAL), roifoline (Rho), baicalein 5,6-dimethyl ether (Baic) and agatisflavone (Fab) with (A) Acetylcholinesterase (AChE) and (B) Cholinesterase, evaluated using the PharmMapper platform.

By using Pa and Pi scores, PASS prediction provides an efficient tool for evaluating the active or inactive potential of a compound in the context of specific biological actions (157). The PASS prediction results are represented in Figure 5.4.

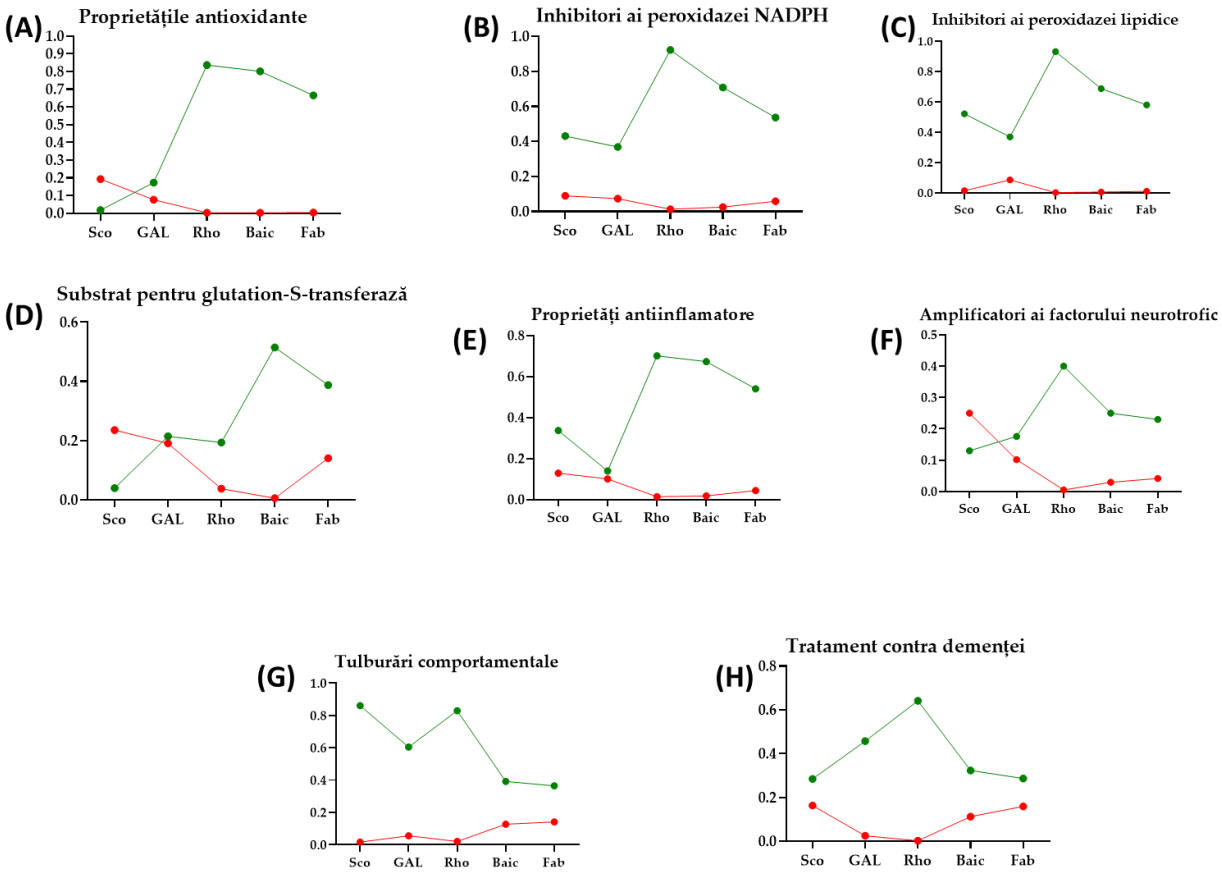


Figure 5.4PASS prediction results of scopolamine (Sco), Galantamine (GAL), roifoline (Rho), baicalein 5,6-dimethyl ether (Baic) and agatisflavone (Fab) on (A) antioxidant properties, (B) potential to inhibit NADPH peroxidase (nicotinamide adenine dinucleotide phosphate), (C) to inhibit lipid peroxidase, (D) the possibility of being a substrate for glutathione-S-transferase, (E) on anti-inflammatory properties, (F) the potential to amplify neurotrophic factor, (G) the potential to induce behavioral disorders and (H) the pharmacological potential on dementia.

PASS prediction suggested that Sco (Pa = 0.018; Pi = 0.193) and GAL (Pa 0.173; Pi = 0.076) do not exhibit antioxidant properties, while Rho (Pa = 0.837; Pi = 0.003), Baic (Pa = 0.801; Pi = 0.003) and Fab (Pa = 0.665; Pi = 0.004) can be considered effective antioxidant agents (Figure 5.4 A). At the same time, the compounds Sco (Pa = 0.431; Pi = 0.009) and GAL (Pa = 0.369; Pi = 0.074) cannot be considered NADPH inhibitors, while Rho (Pa = 0.923; Pi = 0.013), Baic (Pa = 0.709; Pi = 0.025) and Fab (Pa = 0.537; Pi = 0.059) can effectively inhibit NADPH peroxidase (Figure 5.4 B). With the exception of GAL (Pa = 0.369; Pi = 0.085) all other tested compounds were shown to inhibit lipid peroxidase (Pa =

0.931; Pi = 0.002 for Rho, Pa = 0.687; Pi = 0.005, for Baic, Pa = 0.580 ; Pi = 0.010 for Fab and Pa = 0.269 for Sco) (Figure 5.4 C). According to PASS predictions, only Baic (Pa = 0.515; Pi = 0.006) can constitute a substrate for glutathione-S-transferase, (Pa = 0.04; Pi = 0.236), while the other analyzed compounds cannot constitute a substrate for glutathione-S-transfer (Figure 5.4D). Also, Sco (Pa = 0.338; Pi = 0.130) and GAL (Pa = 0.140; Pi = 0.130) do not show anti-inflammatory effects, while Rho (Pa = 0.702; Pi = 0.015), Baic (Pa = 0.674; Pi = 0.019) and Fab (Pa = 0.541; Pi = 0.045) can be considered as candidates for studies aimed at selecting compounds with anti-inflammatory potential (Figure 5.4 E).

Regarding the interaction with the neurotrophic factor, only Rho shows the potential to amplify it (Pa = 0.400; Pi = 0.005), but the other compounds seem to be ineffective (Figure 5.4 F). induces behavioral changes (Pa = 0.861; Pi = 0.016) (Figure 5.4 G). With the exception of Baic (Pa = 0.392; Pi = 0.127) and Fab (Pa = 0.365; Pi = 0.141) all other compounds are prone to induce behavioral changes (Pa = 0.861; Pi = 0.016 for Sco, Pa = 0.830; Pi = 0.002, for Rho, and Pa = 0.605; Pi = 0.055 for GAL) (Figure 5.4 G). Also, according to the PASS prediction, only Rho could show the potential to serve as an anti-dementia drug (Pa = 0.642; Pi = 0.003) (Figure 5.4H).

V.6 Prediction of in silico protein targets of the three flavonoids

The activity of all tested compounds was subjected to rigorous analysis, taking into account four distinct criteria for the activity of a potential breakthrough drug. These criteria include G protein-coupled receptor (GPCR) ligand activity, ion channel modulation, inhibition of kinases, proteases, and other enzymes, as well as nuclear receptor ligand activity. For this purpose, we used Molinspiration Cheminformatics software to calculate the drug activities associated with the 21 drug complexes, and these results are presented as graphs in Figure 5.5. For the interpretation of these medicinal activities, we adopted a specific approach based on bioactivity scores. Thus, a molecule with a bioactivity score greater than 0.00 is likely to exhibit significant biological activities. For molecules with values between -0.50 and 0.00, moderate activity is predicted, while scores below -0.50 generally indicate a higher likelihood of inactivity (158).

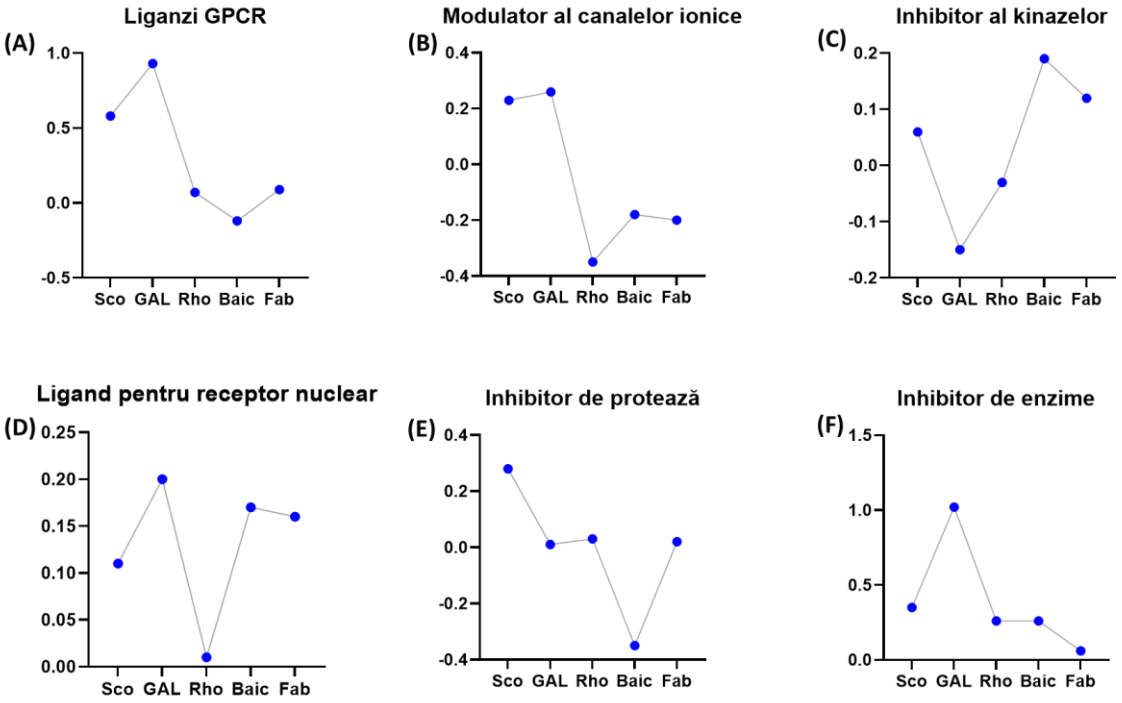


Figure 5.5 Bioactivity score of scopolamine (Sco), Galantamine (GAL), roifolin (Rho), baicalein 5,6-dimethyl ether (Baic) and agatisflavone (Fab) calculated using Molinspiration Cheminformatics software on (A) Ligand for the receptor G protein-coupled (GPCR), (**B**) Ion channel modulation, (**C**) kinase inhibition, (**D**) Nuclear receptor ligand, (**E**) Protease inhibitors, and (**F**) Enzyme inhibitors.

The bioactivity score of compounds on GPCR ligand activity, calculated using Molinspiration Cheminformatics software indicates that Sco (0.58) and GAL (0.93), Rho (0.07) and Fab (0.09) have a score greater than 0.00 and by therefore, they are likely to exhibit significant biological activities on this ligand. In contrast, Baic (-0.12) is predicted to exhibit moderate activity on ligand activity (Figure 5.5 A). Regarding the modulation of ion channels, the highest score was obtained by Sco (0.23) and GAL (0.26) (Figure 5.5 B). While the three natural flavonoids showed a moderate effect on the possibility of ion channel modulation (-0.35 for Rho, -0.18 for Baic and -0.20 for Fab) (Figure 5.5 B). GAL shows the lowest potential to inhibit kinase activity (-0.15) compared to Sco (0.06), Rho (-0.03), Baic (0.19) and Fab (0.12) (Figure 5.5 C). According to Figure 5.5 D, all compounds obtained a bioactivity score greater than 0.00 (0.11 for Sco, 0.20 for GAL, 0.01 for Rho, 0.17 for Baic and 0.16 for Fab) and thus are likely to exhibit significant biological activities on the ligand for the nuclear receptor (Figure 5.5 D). Regarding the potential to inhibit proteases, Baic proved to be the least effective (-0.35), while Sco (0.28), GAL (0.01), Rho (0.03) and Fab (0.02) could inhibit in a significant way the activity of proteases (Figure 5.5 E). Also, all

analyzed compounds could significantly inhibit the activity of different enzymes (0.35 for Sco, 1.02 for GAL, 0.26 for Rho, 0.26 for Baic and 0.06 for Fab).

The obtained results clearly highlight that the physiological actions of drug complexes may involve multiple mechanisms, possibly due to interactions with GPCR ligands, nuclear receptor ligands and inhibitors of proteases and other enzymes. The bioactivity scores provided are significantly suggestive of moderate interaction with all drug targets, providing crucial information for understanding the therapeutic potential and mechanisms of action associated with the analyzed compounds.

To improve and extend the predictions regarding the increased efficiency and probability of protein targets associated with both Sco and GAL and the three natural flavonoids studied, we resorted to the use of the SwissTargetPrediction online tool. The results indicate that Sco is equally likely to inhibit proteases and phosphatases (20.0 %) or act on G-type receptor-coupled proteins (GPCRs) (20.0 %) (Figure 5.6 A). GAL, on the other hand, has a fair estimated probability to act on GPCR targets (36.0 %) and to a lesser extent on phosphodiesterases and other enzymes (12.0 %) (Figure 5.6 B). On the other hand, Rho and Baic present a higher probability of acting on ligases (24.0 %) or may also intervene in the modulation of the activity of other enzymes (20.0 %) (Figure 5.6 C and D). Rho also shows a significant probability of acting as a GPCR target (16.0 %) (Figure 5.6C), while Baic is more likely to act as a kinase modulator (16.0 %) (Figure 5.6 D). In contrast, Fab has a higher predicted probability of acting as a Kinase modulator (20.0 %) and as a GPCR target (16.0 %) (Figure 5.6 E). These results highlight the diversity of possible actions of Sco, GAL, Rho, Baic, and Fab, suggesting that they might interact with multiple protein targets, providing ample perspectives for further exploration of their mechanisms of action.

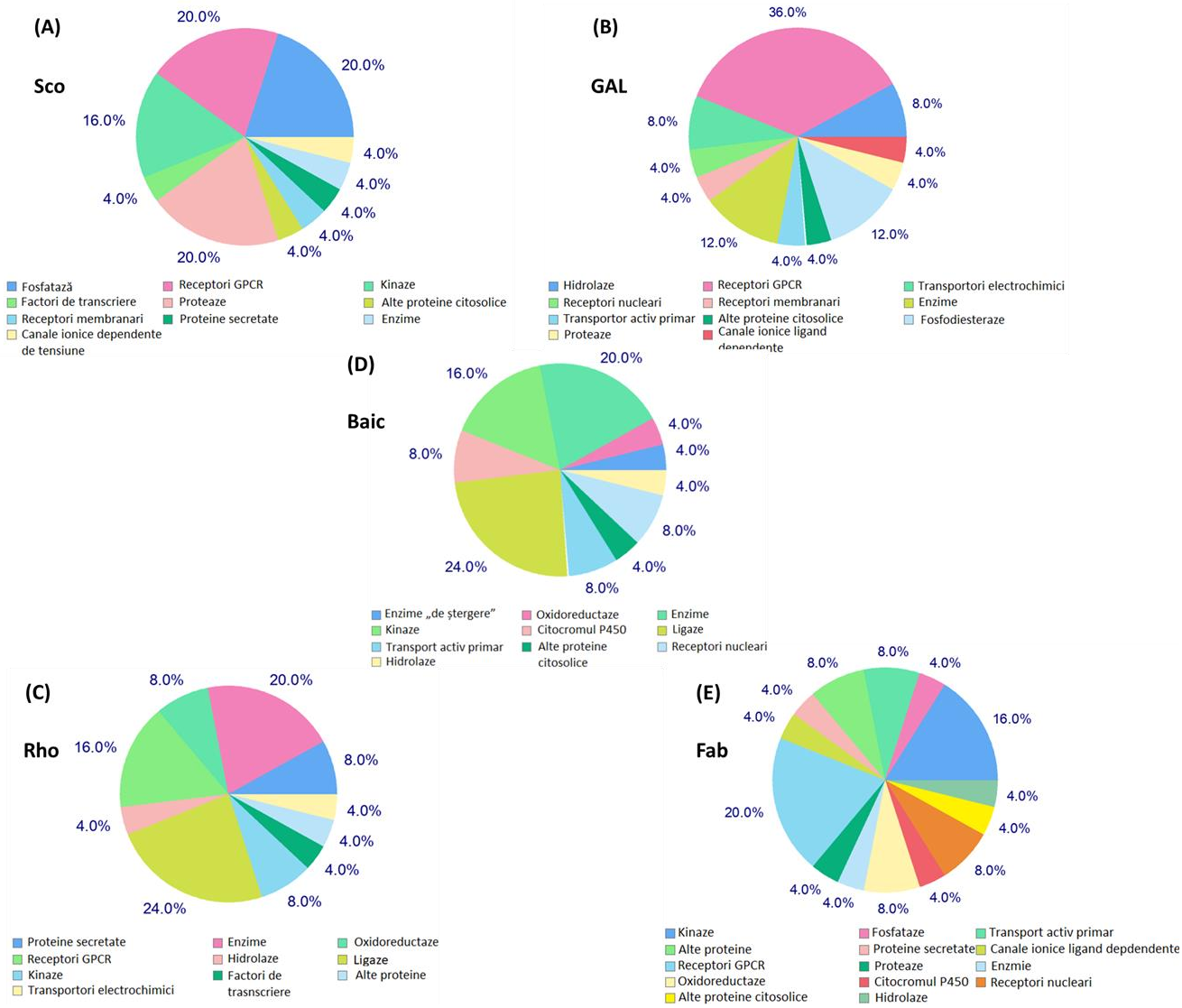


Figure 5.6 Predicted biological targets of (A) scopolamine (Sco), (B) Galantamine (GAL), (C) roifoline (Rho), (D) baicalein 5,6-dimethyl ether (Baic) and (E) agatisflavone (Fab) using the SwissTargetPrediction online tool.

V.7 Prediction of pharmaceutical market approval of roifoline, baicalein 5,6-dimethyl ether and agatisflavone

Figure 5.7 shows the results of predicting the similarity of Sco, GAL, Rho, Baic and Fab to drugs for different subdivision stages of their research. According to the miDlikeness online tool, all analyzed compounds show the probability of showing effects in *in vivo* studies (0.6626 for Sco, 0.7241 for GAL, 0.8487 for Rho, 0.9992 for Baic and 0.9872 for Fab) (Figure 5.7 A).

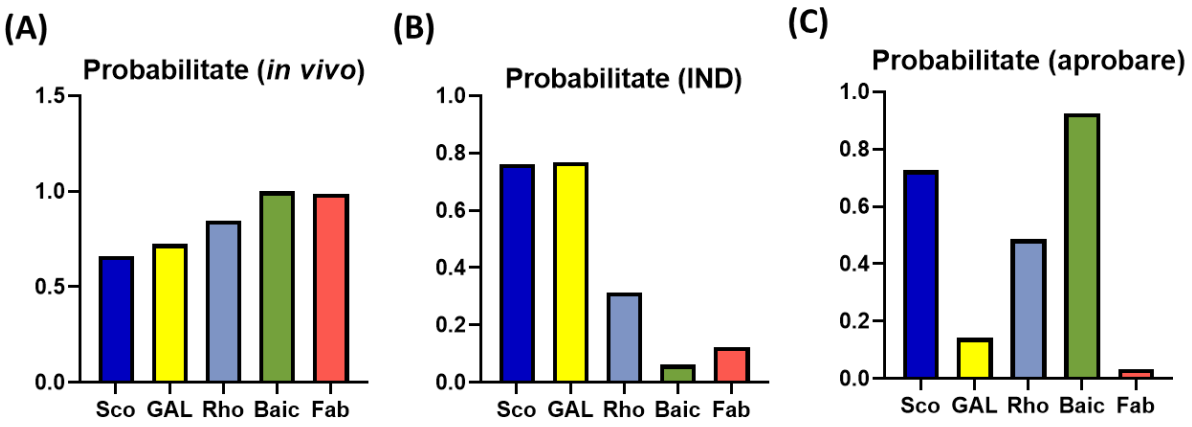


Figure 5.7 Drug similarity prediction of scopolamine (Sco), Galantamine (GAL), roifolin (Rho), baicalein 5,6-dimethyl ether (Baic) and agatisflavone (Fab) for different subdivision steps of their research. (A) predicting *in vivo* effects, (B) predicting IND (Investigational New Drug) interest, and (C) predicting the potential of the compound to be approved on the pharmaceutical market using the miDlikeness online tool.

Regarding the interest for IND of the analyzed compounds, the results obtained through miDlikeness suggest that the most pronounced interest will be Sco and GAL (0.7625 and 0.7683) (Figure 5.7 B). On the other hand Rho may present a more moderate interest (0.3145) (Figure 5.7 B), while Baic and Fab indicated a low probability for IND (0.0609 and 0.1235) (Figure 5.7 B). Surprisingly, Baic has the highest probability of being approved in the pharmaceutical market (0.9255), followed by GAL (0.7265), Rho (0.4872), Sco (0.1417) and finally Fab (0.0341) (Figure 5.7 C).

V.8 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on anxiety-like state and memory

For the behavioral assessment, in this study we used several mazes, thus for the assessment of anxiety we used the NTT test, for the assessment of locomotor activity and spatial memory we used the Y maze, and for the assessment of reference memory we used the NOR test.

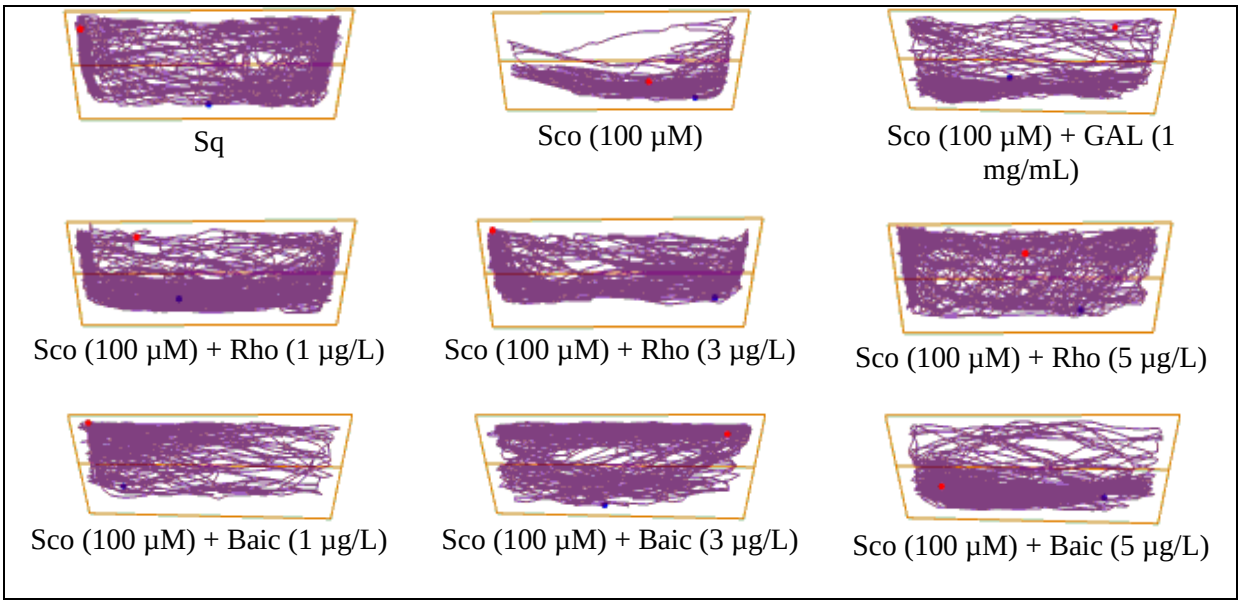
The study evaluated the neuro-pharmacological effects of the natural flavonoids Rho, Baic and Fab on neurocognitive performance with the aim of improving memory and addressing AD symptoms. Although these compounds are known for their beneficial effects at low doses and without side effects, their impact on AD symptoms has not been studied so far. The study used the zebrafish as an animal model for dementia induced by a muscarinic cholinergic (Sco) receptor blocker to evaluate the neurocognitive potential of these flavonoids. Zebrafish proves to be a promising model in biomedical research and neuroactive drug testing (195). The three natural flavonoids tested in this study were chronically

administered by immersion to zebrafish once a day, for 20 days, in concentrations of 1, 3 and 5 µg/L. As a positive control, in this study we used galantamine (GAL, 1mg/ml). GAL, being one of the current drugs widely used in the treatment of behavioral and neurocognitive disorders in human AD patients.

V.8.1 Effects of roifoline, baicalein 5,6-dimethyl ether and agatisflavone on scopolamine-induced anxiety-like behavior in the novel maze test (NTT)

In NTT, representative patterns of locomotion tracking highlight the multiple swimming variations of the cohorts of animals tested (Figure 5.8). Thus, in the fish from the control group (Ctr) there was a tendency to travel a greater distance in the upper zone of the aquarium, highlighting the normal swimming of zebrafish in the NTT test, while the group of fish treated with Sco (100 µM) showed an increased preference for the lower area, indicating a high level of fish anxiety and the anxiogenic profile of Sco (196,197).

An improvement in locomotor activity in the upper zone of the aquarium was observed in animals that were subjected to chronic treatment with Rho (1, 3 and 5 µg/L), Baic (1, 3 and 5 µg/L) and Fab (1, 3 and 5 µg/L), especially in the concentration of 5 µg/L, in a manner similar to GAL-treated fish. During testing, fish from all twelve batches preferred to first exploit the lower area of the aquarium, as close as possible to the base / wall, then gradually, depending on the anxiety level of each animal, began to swim in one direction vertically (196,197).



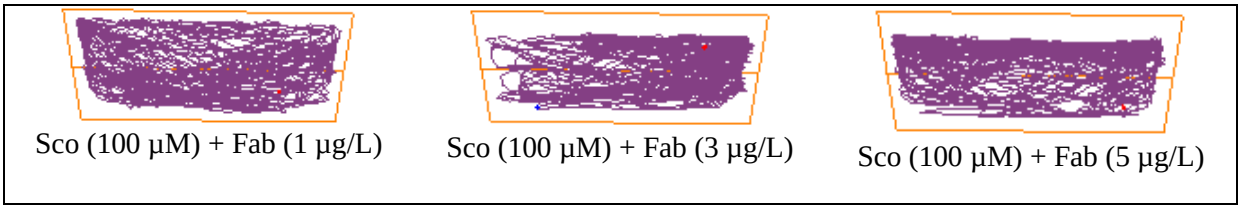


Figure 5.8 Graphic representation of zebrafish swimming in the NTT test; The test consists of a trapezoidal glass aquarium. The aquarium was virtually divided into two equal zones (upper and lower). The beginning of the zebrafish trail is represented by the blue dot, and the end of the fish path is represented by the red dot.

According to the obtained data, in our experiments, as can be seen in Figure 5.9, in the NTT test, the administration of Sco treatment (100 µM), zebrafish, generated a strong anxiogenic response quantified by significantly increasing the latency period required for the fish to begin vertical exploration of the aquarium ($p < 0.0001$; Figure 5.9 A, D and G), by significantly reducing the time assessed in seconds (s) spent by fish in the upper zone of the aquarium and by significantly increasing the period of time spent by fish in the lower zone of the aquarium compared to the control group ($p < 0.0001$; Figure 5.9 B, D and H) and by decreasing the ratio between the distance swimming distance of zebrafish in the upper / lower zone of the aquarium compared to the control group ($p < 0.0001$; Figure 5.9 C, F and I) (196, 197) administration after Rho treatment had a beneficial effect on the latency period required to explore the upper zone, significantly decreasing in all three concentrations tested (1, 3 and 5 µg/L) the time required to start exploring the upper zone, compared to the group treated only with Sco ($p < 0.0001$), in a similar way to fish treated with GAL (1 mg/mL) (Figure 5.9 A). Also, Rho treatment increased in a dose-dependent manner the ratio of time spent by fish in the upper/lower zone of the aquarium, especially in the concentration of 3 µg/L ($p < 0.001$) and 5 µg/L ($p < 0.0001$), but it did not show any effect from a statistical point of view in the concentration of 1 µg/L (Figure 5.9). Moreover, the treatment with Rho managed to restore the ratio between the distance traveled by the fish in the upper/lower zone of the aquarium, which was diminished following the administration of the treatment with Sco (100 µM), showing a statistical significance for the concentrations of 3 and 5 µg/L ($p < 0.0001$) (Figure 5.9 C) (196, 197). Regarding the treatment with Baic, it was observed that this flavonoid showed a strong anxiogenic effect in the NTT test, managing to restore in all three tested concentrations (1, 3 and 5 µg/L) ($p < 0.0001$), the period of latency, necessary to explore the upper zone of the aquarium (Figure 5.9 D). Also, treatment with Baic at concentrations of 3 and 5 µg/L was able to restore exploration time ($p < 0.01$ for Baic at concentration 3 µg/L and $p < 0.001$ for treatment with Baic at concentration 5 µg /L; Figure 5.9 E) and the distance traveled ($p < 0.05$ for Baic in the concentration of 3 µg/L and $p <$

0.0001 for the treatment with Baic in the concentration of 5 µg/L; Figure 5.9 F) in the upper zone of the test aquarium (196, 197).

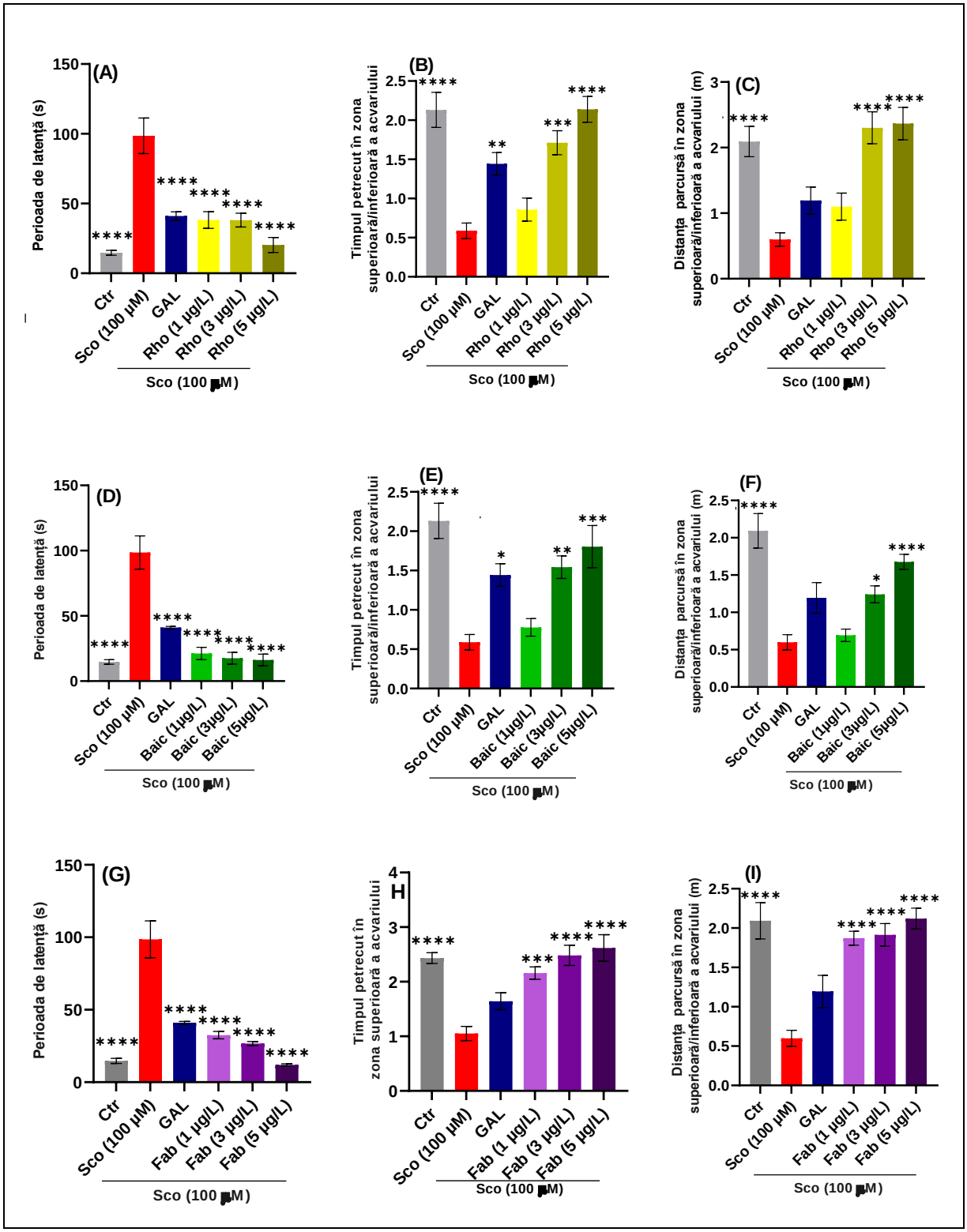


Figure 5.9 Effects of treatment with Rho (1, 3 and 5 µg/L) (A, B, C), Baic (1, 3 and 5 µg/L) (D, E, F) and Fab (1, 3 and 5 µg /L) (G, H, I) on the latency period required for the fish to start exploring the upper area of the aquarium (A, D, G), the time spent by the fish in the upper area of the aquarium (B, E, H) and on the ratio between the distance traveled by the fish in the upper and lower areas of the aquarium (C, F, I). Values are expressed as means ± SEM, (n = 10 animals per group). ANOVA revealed a significant effect of treatment on latency (A) ($F(5, 54) = 20.24$, $p < 0.0001$); (D) ($F(5, 54) = 28.19$, $p < 0.0001$); (G) ($F(5, 54) = 34.83$, $p <$

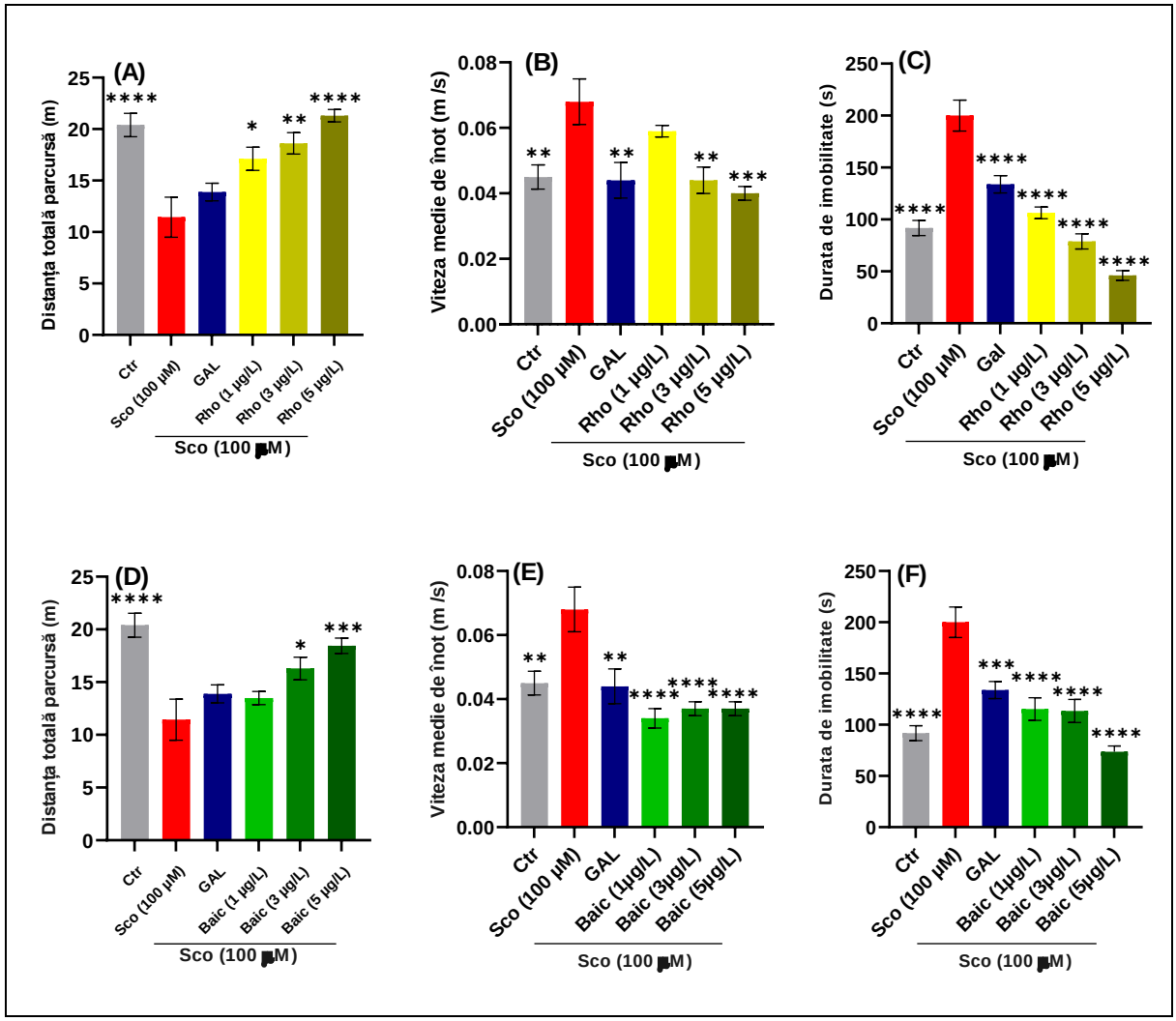
0.0001); the time spent in the upper zone of the aquarium (B) ($F(5, 54) = 16.47$, $p < 0.0001$); (E) ($F(5, 54) = 11.42$, $p < 0.0001$); (H) ($F(5, 54) = 34.32$, $p < 0.0001$); and on the ratio between the distance traveled in the upper and lower areas of the aquarium (C) ($F(5, 54) = 12.21$, $p < 0.0001$); (F) ($F(5, 54) = 14.37$, $p < 0.0001$ (I) ($F(5, 54) = 14.57$, $p < 0.0001$); For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

A robust anxiolytic effect in the NTT test was also demonstrated by Fab treatment, which significantly reduced the time required to initiate upper area exploration in all three concentrations tested (1, 3 and 5 μg/L), ($p < 0.0001$; Figure 5.9 G). Likewise, Fab treatment significantly restored the ratio of time spent in the upper/lower zone of the aquarium, where it showed a more modest significance for the concentration of 1 μg/L ($p < 0.001$), but showed a robust significance statistically significant for concentrations of 3 and 5 μg/L ($p < 0.0001$; Figure 5.9 H). Moreover, treatment with Fab proved to be able to restore in all three tested concentrations ($p < 0.0001$) the ratio between the distance traveled in the upper/lower zone by zebrafish that were previously exposed to the acute treatment with Sco (100 μM) (Figure 5.9 I) (196, 197).

In this study, administration of Sco treatment (100 μM) to zebrafish produced a hypolocomotor effect, significantly reducing the total distance traveled ($p < 0.0001$; Figure 5.10 A, D, G), suggesting its anxiogenic effect on the test animals. Simultaneously, the exposure of the fish to the Sco treatment significantly increased the average swimming speed ($p < 0.01$; Figure 5.10 B, D, H) and the immobility duration of the fish during the 6-minute test ($p < 0.0001$; Figure 5.10 C, F, I), suggesting the degradation of the locomotor activity of the tested animals following the administration of Sco in the concentration of 100 μM (196, 197).

In the NTT test, chronic administration of Rho treatment (1, 3 and 5 μg/L) to amnesic fish significantly restored the total distance traveled by the fish in the test tank,especially inconcentration of 5 μg/L ($p < 0.0001$), but showed more modest statistical significance at concentrations of 1 ($p < 0.05$) and 3 μg/L ($p < 0.01$; Figure 5.10 A). Regarding the effects of Rho treatment on average swimming speed, a differential pattern of efficiency was observed. Thus, it was observed that the treatment with Rho in the concentration of 1 μg/L did not show any effect from a statistical point of view on the average swimming speed, while the treatment with Rho in the concentrations of 3 and 5 μg/L succeeded to significantly restore the mean swimming speed of zebrafish ($p < 0.01$, $p < 0.001$), in a manner similar to that of GAL treatment ($p < 0.01$; Figure 5.10 B). Also, Rho treatment significantly decreased the immobility duration of amnesic animals, in all three concentrations tested (1, 3 and 5 μg/L, $p < 0.0001$), in a manner similar to that of treatment with GAL ($p < 0.0001$; Figure 5.10 C),

suggesting the beneficial effects of Rho treatment on the spontaneous unconditioned behavior of zebrafish, by restoring the locomotor deficit induced by Sco treatment (196, 197). The treatment with Baic in the concentration of 3 μg/L, presented a dose-dependent effect, managing to significantly increase the total distance traveled by the fish in the NTT ($p < 0.1$). A similar effect on the total distance was presented by the treatment with Baic in the concentration of 5 μg/L ($p < 0.001$; Figure 5.10 D), while the treatment with Baic in the concentration of 1 μg/L proved to be ineffective (Figure 5.10D). However, Baic treatment was able to restore the mean swimming speed of fish, in all three concentrations tested ($p < 0.0001$), in a similar manner to that of GAL treatment ($p < 0.01$; Figure 5.10 E). Also, administration of the treatment with Baic had the effect of significantly decreasing the average duration of immobility of the amnesic fish, in all three concentrations tested ($p < 0.0001$; Figure 5.10F) (196, 197).



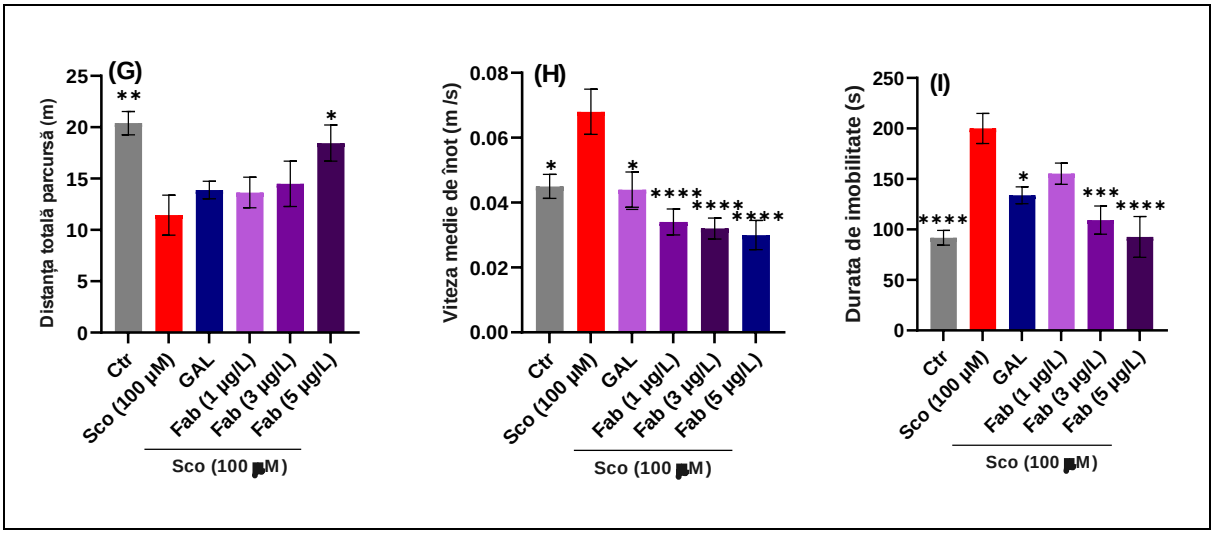


Figure 5.10 Effects of treatment with Rho (1, 3 and 5 µg/L) (A, B, C), Baic (1, 3 and 5 µg/L) (D, E, F) and Fab (1, 3 and 5 µg /L) (G, H, I) on total distance traveled (A, D, G), average swimming speed (B, E, H) and total immobility time of zebrafish in NTT (C, F, I). Values are expressed as means ± SEM, (n = 10 animals per group). ANOVA, revealed a significant effect of treatment on total distance traveled (A) ($F(5, 54) = 10.30$, $p < 0.0001$); (D) ($F(5, 54) = 8.559$); (G) ($F(5, 54) = 4.219$, $p=0.0026$); of average swimming speed (B) ($F(5, 54) = 6.264$, $p < 0.0001$); (E) ($F(5, 54) = 8.444$, $p < 0.0001$); (H) ($F(5, 54) = 8.647$, $p < 0.0001$); and of the total duration of immobility (C) ($F(5, 54) = 37.52$, $p < 0.0001$); (F) ($F(5, 54) = 19.73$, $p < 0.0001$); (I) ($F(5, 54) = 10.03$, $p < 0.0001$);For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

Regarding Fab treatment, a significant effect of treatment on total distance traveled was observed only in the concentration of 5 µg/L ($p < 0.1$), and treatment with Fab in concentrations of 1 and 3 µg/L proved to be ineffective in restoring the total distance traveled by zebrafish in the NTT test (Figure 5.10 G). Regarding the effects of Fab treatment on average swimming speed, it was observed that Fab treatment was able to restore the average swimming speed of fish in all three concentrations tested ($p < 0.0001$), in a manner similar to that of treatment with GAL ($p < 0.01$) (Figure 5.10 H). Simultaneously, Fab treatment decreased the immobility duration of zebrafish during the test period in the NTT test, where it showed a significant effect in the concentration of 3 µg/L ($p < 0.001$) and in the concentration of 5 µg/L ($p < 0.0001$), at a level close to that of GAL treatment ($p < 0.1$), while Fab treatment in the concentration of 1 µg/L proved to be statistically ineffective (Figure 5.10 I) (196, 197).

Thus, exposure of zebrafish to Sco treatment (100 µM) for 30 minutes produced a hypolocomotor effect, significantly reducing the total distance traveled in the NTT test ($p < 0.0001$), suggesting the degradation of the dopaminergic system of zebra fish. Furthermore, in the NTT test, administration of Sco (100 µM) treatment to zebrafish generated a strong anxiogenic response, exemplified by a significant increase in the latency period required for the fish to begin vertical exploration of the aquarium ($p < 0.0001$) by reducing significant

time of exploration of the upper zone of the aquarium, evaluated in seconds (s) and by increasing thigmotaxis compared to the control group ($p < 0.0001$) (196, 197).

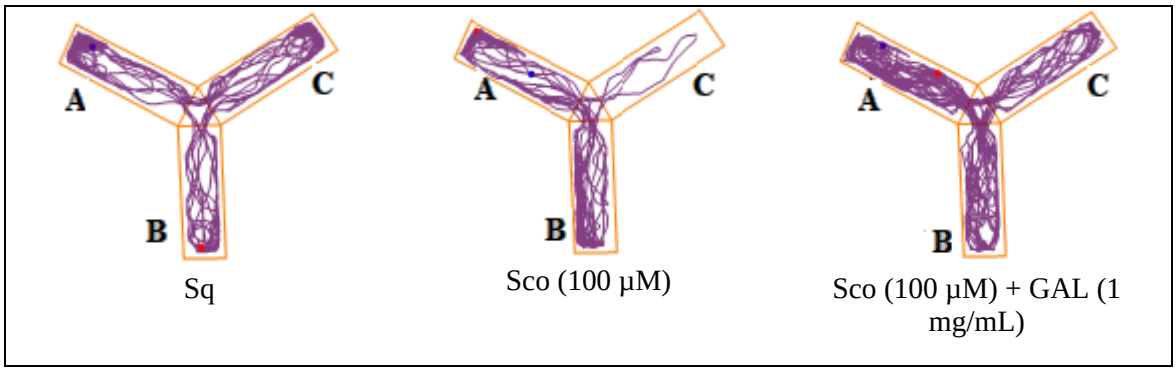
Further exposure of the laboratory animals to the treatments with the three natural flavonoids had the effect of decreasing the time required to start exploring the upper zone, compared to the group treated only with Sco ($p < 0.0001$), restored the total distance traveled by the fish in the 6 minutes test and significantly decreased the immobility duration of amnesic animals in a dose-dependent manner, exerting the most pronounced effect at the concentration of 5 µg/L ($p < 0.0001$) in a manner similar to that of treatment with GAL ($p < 0.001$) (196, 197).

V.8.2 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on zebrafish spatial memory assessed in the Y-maze test

In Figure 5.11, representative tracking patterns of zebrafish locomotion and their preference for one of the three arms within the Y-maze are illustrated. It was observed that during the second full 5 min session in the control fish there was a tendency to travel an approximately equal distance in the start arm (A) and the new arm (C) at the expense of the other arm (B).

Sco (100 µM) treated groups showed decreased locomotor activity and increased preference for the start arm (A) and arm (B), exploring to a lesser extent the novel arm (C), suggesting memory degradation fish following the administration of Sco treatment.

Impairment of zebrafish locomotion triggered by Sco administration in the novel arm was subsequently ameliorated by Rho, Baic and Fab treatment in a manner similar to GAL treatment (196, 197).



Sq | Sco (100 µM) | Sco (100 µM) + GAL (1 mg/mL)

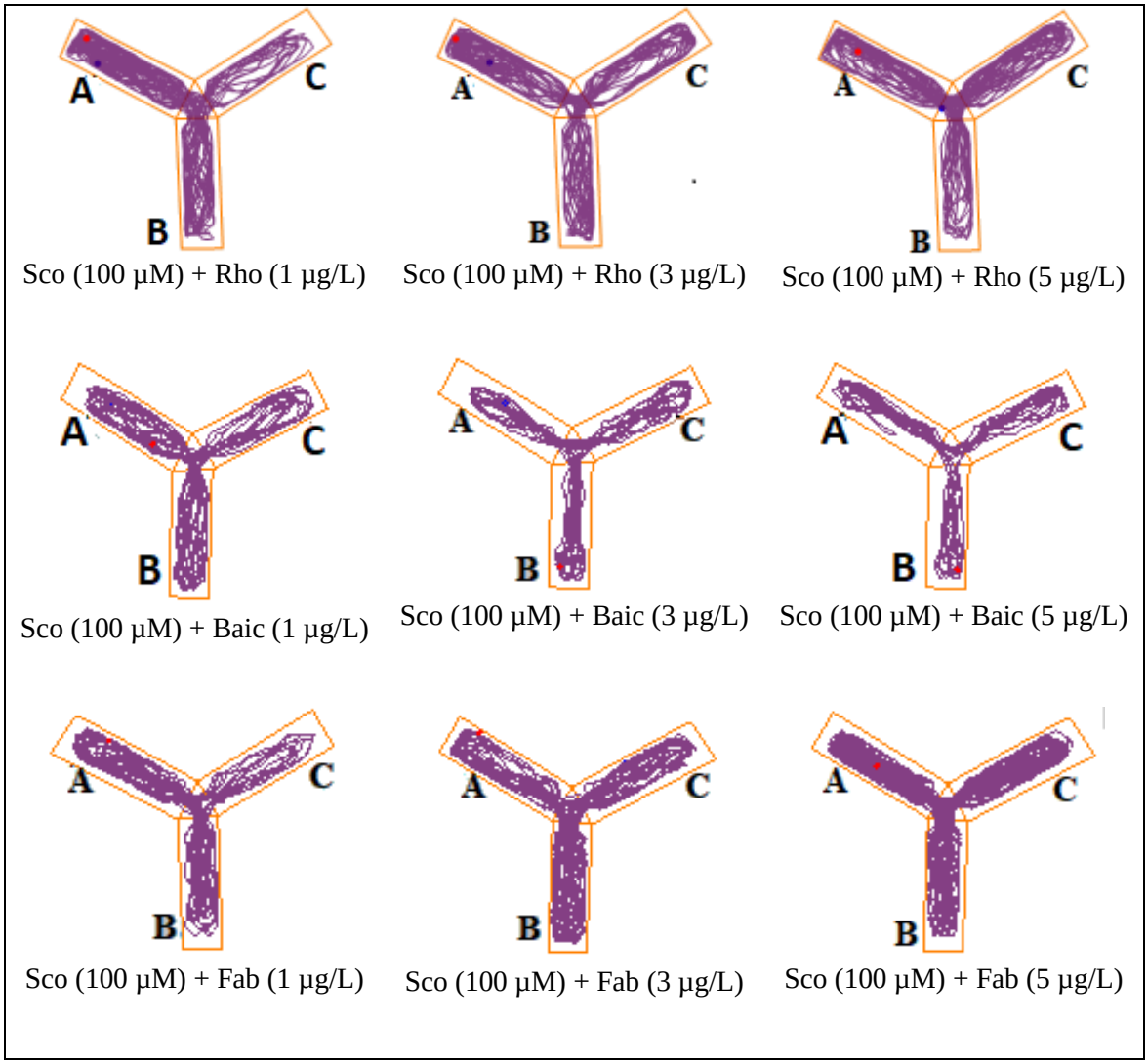
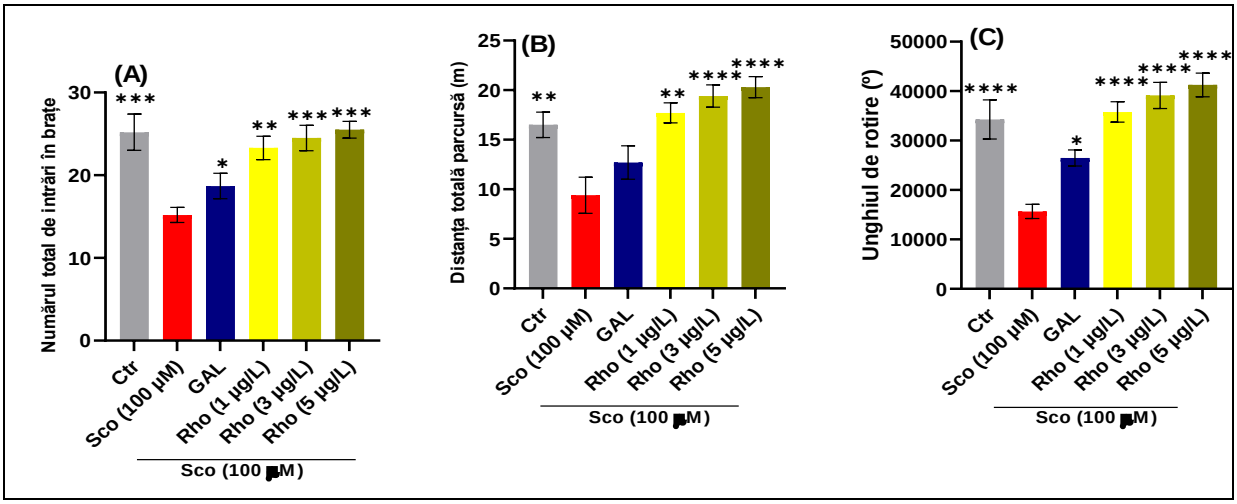


Figure 5.11 Graphical representation of the route taken by zebrafish during the second session of the Y-maze.The beginning of the zebrafish trail is represented by the blue dot, and the end of the fish path is represented by the red dot.

Based on the hypothesis that motor disability interacts with cognitive function, the study investigated the association between the locomotor activity of zebrafish and the effect of three natural flavonoids on cognitive processes in the animal model of dementia induced by the administration of Sco (100 µM).

As can be seen in Figure 5.12, in the Y-maze test, the administration of Sco treatment at a concentration of 100 µM, had a hypolocomotor effect, evaluated by the significant reduction in the number of crossed lines ($p < 0.001$; Figure 5.12 A), ($p < 0.01$; Figure 5.12 D), ($p < 0.05$; Figure 5.12 G), by the obvious decrease of the total distance traveled ($p < 0.05$; Figure 5.12 B), ($p < 0.01$; Figure 5.12 E), ($p < 0.05$; Figure 5.12 H) and by reducing the angle of rotation (°) made by the fish during the test session compared to the Control group ($p < 0.0001$; Figure 5.12C), ($p < 0.0001$; Figure 5.12F) and ($p < 0.0001$; Figure 5.12

I). Subsequent administration of Rho treatment had the effect of preventing Sco-induced locomotor deficit, showing a significant dose-dependent effect in all three concentrations tested, thus, for the concentration of 1 µg/L, ANOVA revealed a significant effect of treatment on the number of arm entries ($p < 0.01$; Figure 5.12 A), the total distance covered by swimming ($p < 0.01$; Figure 5.12 B) and the turning angle (º) ($p < 0.0001$; Figure 5.12 C), for concentrations of 3 and µg/L, ANOVA revealed a significant effect of treatment on the number of arm entries ($p < 0.001$; Figure 5.12 A), the total distance traveled by swimming ($p < 0.0001$; Figure 5.12 B) and the angle of rotation (º) ($p < 0.0001$; Figure 5.12 C), compared to the group of fish that received only the treatment with Sco (100 µM) (196, 197). Regarding the effect of Baic on the locomotor activity of zebrafish in the Y-maze test, it was observed that the administration of Baic in the concentrations of 1, 3 and 5 µg/L, was able to significantly increase both the total number of crossed lines (Figure 5.12 D) as well as the total distance traveled by the fish during the test session (Figure 5.12 E), showing the most pronounced effect in the concentration of 5 µg/L ($p < 0.0001$)(196,197). However, regarding the effects of treatment with Baic, on the angle of rotation (º), a different pattern of action is observed, in a dose-dependent manner, manifesting the most pronounced effect in the concentration of 5 µg/L ($p < 0.0001$; Figure 5.12 F). However, ANOVA revealed a significant effect of Baic treatment on turning angle (º) in both 3 µg/L ($p < 0.001$) and 1 µg/L ($p < 0.01$; Figure 5.12 F).



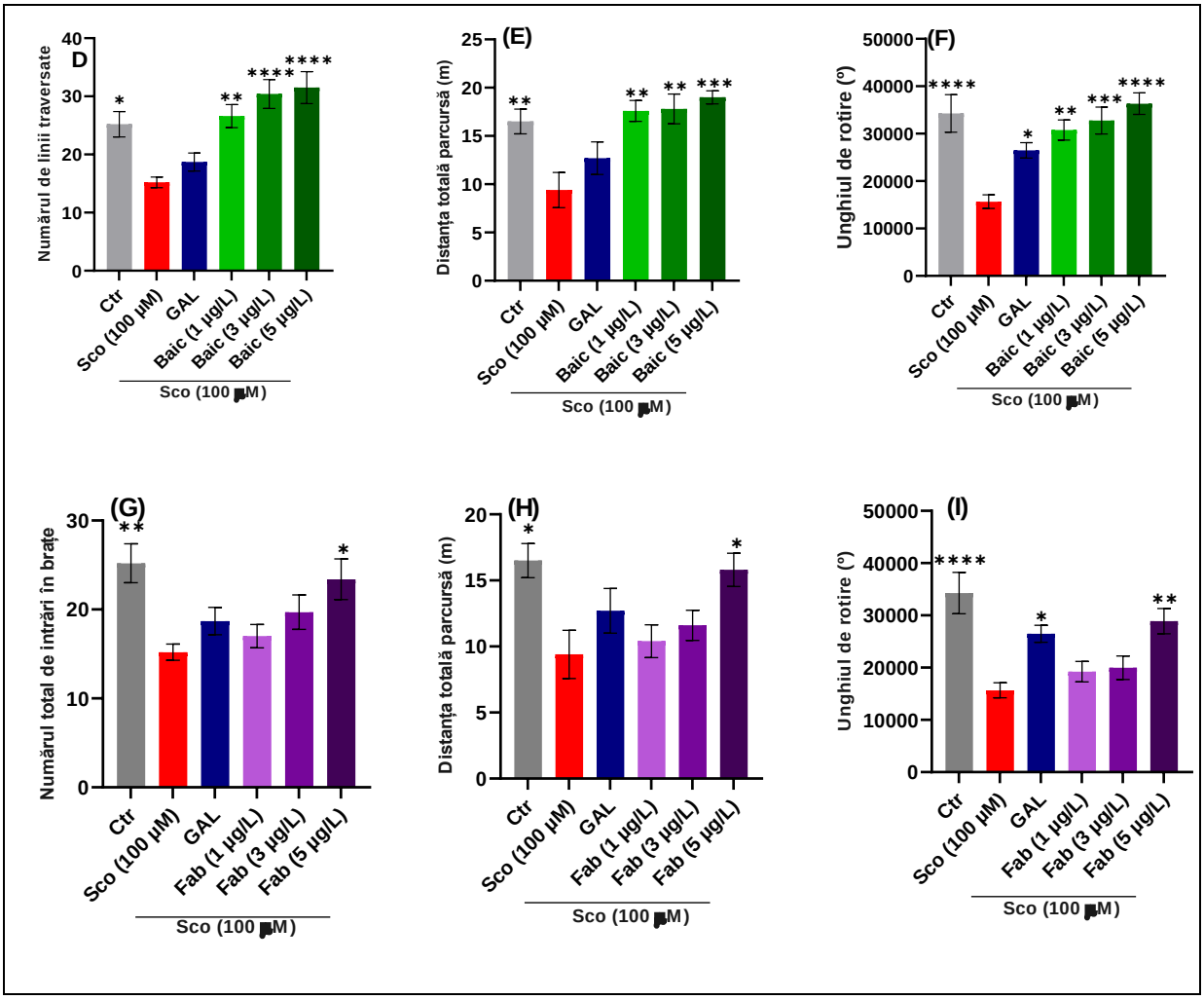


Figure 5.12 Effects of treatment with Rho (1, 3 and 5 µg/L) (A, B, C), Baic (1, 3 and 5 µg/L) (D, E, F) and Fab (1, 3 and 5 µg /L) (G, H, I) on the number of turns in the arms (A, D, G), the total distance traveled (B, E, H) and the angle of rotation (C, F, I). Values are expressed as means ± SEM, (n = 10 animals per group). ANOVA revealed a significant effect of treatment on the number of arm entries (A) ($F(5, 54) = 7.862$, $p < 0.0001$); (D) ($F(5, 54) = 9.786$, $p < 0.0001$); (G) ($F(5, 54) = 4.644$, $p=0.0013$); on the total distance traveled (B) ($F(5, 54) = 9.357$), $p < 0.0001$); (E) ($F(5, 54) = 6.895$, $p < 0.0001$); (H) ($F(5, 54) = 4.048$, $p=0.0034$); and on the rotation angle (C) ($F(5, 54) = 14.56$, $p < 0.0001$); (F) ($F(5, 54) = 8.909$, $p < 0.0001$); (I) ($F(5, 54) = 8.355$, $p < 0.0001$);For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

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Treatment with Fab proved to be less effective in restoring the locomotor activity of amnesic zebrafish compared to the effects of the two previously mentioned flavonoids. Thus, a statistically significant effect of Fab treatment was observed on the total number of lines crossed, on the total distance traveled and on the angle of rotation (º) only in the concentration of 5 µg/L, ($p < 0.05$; Figure 5.12G; Figure 5.12H and Figure 5.12 I), in a manner similar to that of GAL treatment (Figure 5.12 I).

In this study, the administrationtreatment with Sco (100 µM) of zebrafish had the effect of altering the learning and memory capacities of laboratory animals in the Y-maze test, evaluated by means of the percentage of spontaneous alternation (%) ($p < 0.05$; Figure

5.13A), ($p < 0.01$; Figure 5.13B) and ($p < 0.01$; Figure 5.13C). Treatment with Rho (1, 3 and 5 µg/L) restored in all three tested concentrations the percentage of spontaneous alternation (%), showing the most significant effect at the concentration of 5 µg/L ($p < 0.001$), followed by of the concentration of 3 and 1 µg/L ($p < 0.01$; Figure 5.13A). Therefore, Rho could ameliorate Sco-induced cognitive dysfunction by improving learning and memory processes.

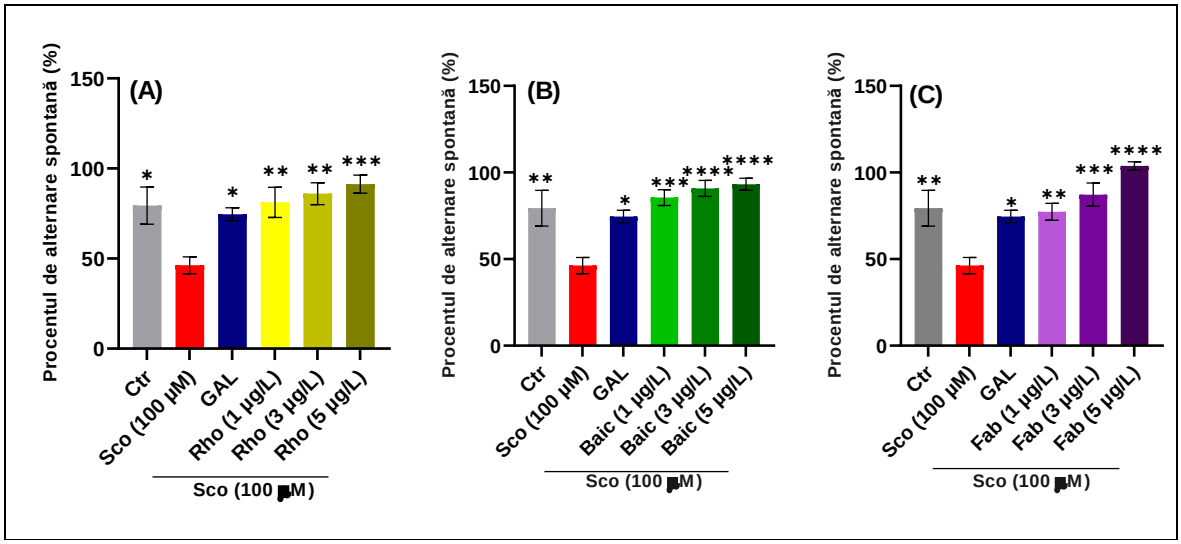


Figure 5.13 Effects of treatment with Rho (1, 3 and 5 µg/L) (A), Baic (1, 3 and 5 µg/L) (B) and Fab (1, 3 and 5 µg/L) (C) on the percentage of spontaneous alternation (%) (A, B, C). Values are expressed as means ± SEM, (n=10 animals per group). ANOVA revealed a significant effect of treatment on percent spontaneous alternation (%) (A) ($F(5, 54) = 5.507$, $p=0.0004$); (B) ($F(5, 54) = 9.054$, $p < 0.0001$); (C) ($F(5, 54) = 9.857$, $p < 0.0001$);For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

Simultaneously, the treatment with Baic proved to mark a more remarkable action potential in the concentrations of 3 and 5 µg/L, in which it caused a significant increase in the percentage of spontaneous alternation (%), compared to the group of animals in which administered only Sco ($p < 0.0001$; Figure 5.13 B), while treatment with Baic in the concentration of 1 µg/L caused a significantly more moderate increase in the percentage of spontaneous alternation (%) ($p < 0.001$; Figure 5.13 B)(196,197). Also, treatment with Fab, in the concentration of (1 µg/L), proved to be the least effectivein restorationthe learning and memorizing capacity of zebrafish, managing to increase only to a small extent the percentage of spontaneous alternation (%) ($p < 0.01$; Figure 5.13 C). A more significant increase in the percentage of spontaneous alternation (%), was observed in the concentration of 3 µg/L ($p < 0.001$) and in the concentration of µg/L ($p < 0.0001$; Figure 5.13 C). Thus, Fab treatment is able to improve the learning and memory capacity of amnesic zebrafish.

In addition, zebrafish treated with Sco (100 µM), showed a reduced number of crossed lines ($p < 0.001$; Figure 5.14A), ($p < 0.01$; Figure 5.14C and E.) and a decrease in

time spent in the novel arm of the maze ($p < 0.001$; Figure 5.14B. D and F), compared to the control group, indicating memory impairment in Sco animals.

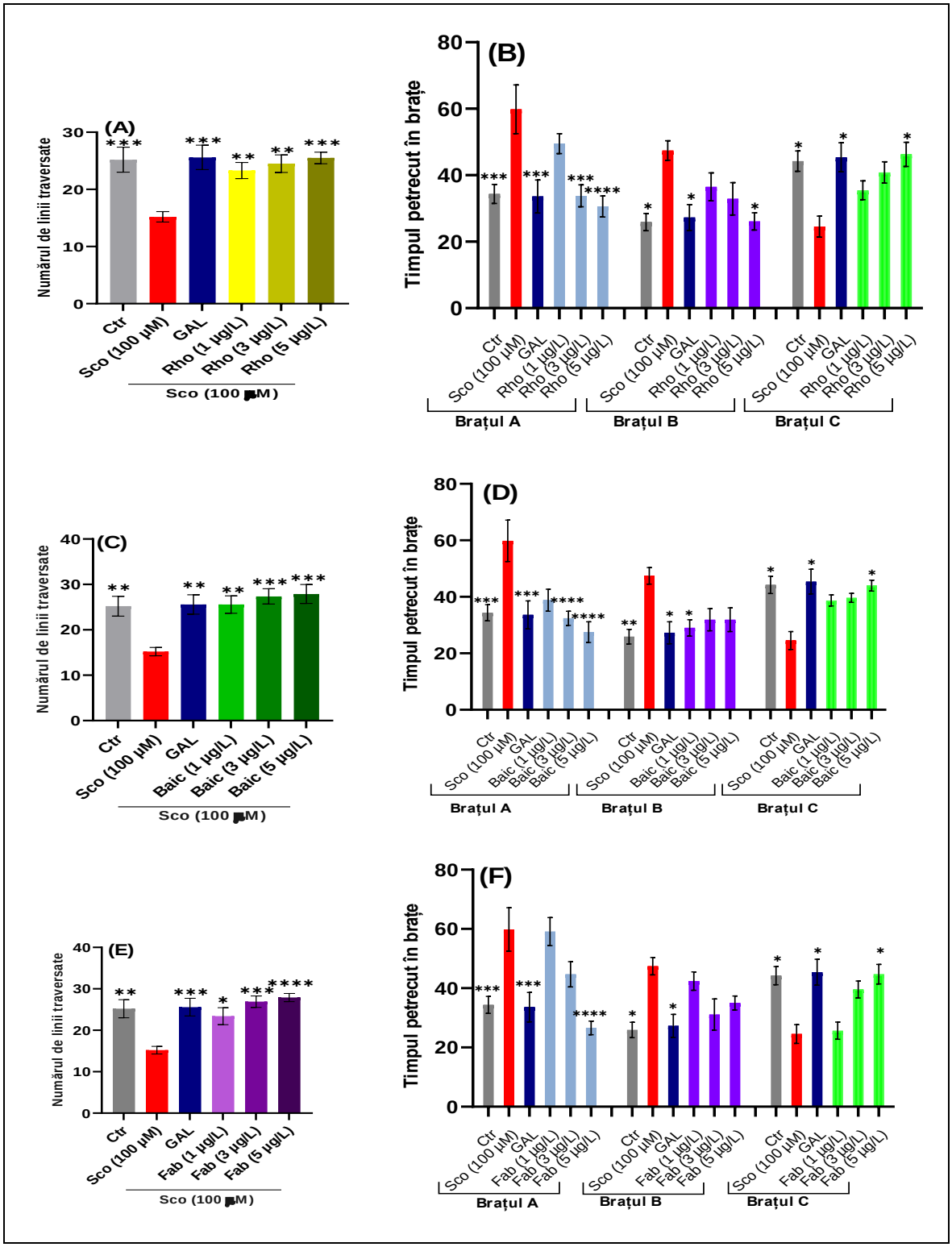


Figure 5.14 Effects of treatment administration with Rho (1, 3 and 5 µg/L) (A, B), Baic (1, 3 and 5 µg/L) (C, D) and Fab (1, 3 and 5 µg/L) (E, F) on the number of crossed lines (A, C, E) and on the time spent in the three arms of the Y maze (A, D, F). Values are expressed as means ± SEM, (n = 10 animals per group). ANOVA revealed a significant effect of treatment on spontaneous alternation percentage (%) (A) ($F (5, 54) = 6.235$, $p=0.0001$); (B) ($F (5, 54) = 6.353$, $p < 0.0001$); (C) ($F (5, 54) = 6.238$, $p=0.0001$); (D) ($F (5, 54) = 6.244$, $p <$

0.0001); (E) (F (5, 54) = 7.347, p < 0.0001); (F) (F (5, 54) = 7.894, p < 0.0001);For Tukey's post hoc analyses: *p < 0.01, ** p < 0.001, *** p < 0.0001 and **** p < 0.00001.

Cognitive deficits induced by Sco treatment in zebrafish were attenuated by administration of the three natural flavonoids. Thus, treatment with Rho showed a beneficial effect on the spatial memory of zebrafish, managing to restore in all three tested concentrations (1, 3 and 5 μg/L) the number of crossed lines. Post hoc analysis showed that the administration of Rho treatment in the concentration of 1 and 3 μg/L reversed the memory deficit caused by the acute administration of Sco ($p < 0.01$). Moreover, administration of Rho at the concentration of 5 μg/L was able to restore the number of lines crossed by the zebrafish during the test session in the Y-maze test ($p < 0.001$; Figure 5.14 A). Also, treatment with Rho at a concentration of 5 μg/L restored the time fish spent in the novel arm of the maze ($p < 0.05$; Figure 5.14 B), to a level close to that of GAL treatment (1 mg/ mL, $p < 0.1$). Therefore, the obtained results suggest that Rho presents a memory-stimulating profile, preventing the negative effects on cognitive processes induced by Sco. Moreover, Rho treatment, especially at the dose of 5 μg/L improved locomotor activity, learning and memory abilities. Concomitantly, Rho treatment has the potential to improve and sustain spatial and differentiation memory in zebrafish and to enhance the anxiogenic effects induced by Sco in the Y-maze test by restoring the number of lines crossed and the time to explore the new arm (196 ,197).

A similar effect on the spatial cognition of amnesic zebrafish was exerted by the treatment with Baic. Thus, the administration of Baic treatment in all three concentrations (1, 3 and 5 μg/L) significantly increased the number of lines crossed by fish within the Y-maze, especially in the concentrations of 3 and 5 μg/L L ($p < 0.001$; Figure 5.14 C). Regarding the effects of Baic treatment on the time spent by the fish in the three arms of the Y-maze, it is observed that it is able to significantly restore the exploration time of the new arm only in the concentration of 5 μg/L ($p < 0.05$; Figure 5.14 C)(196,197). In contrast to Fab treatment, it was shown to show a significant dose-dependent effect. Thus, Tukey's post hoc analysis showed that chronic administration of Fab treatment at 1 μg/L has a modest effect in ameliorating the amnesic effects induced by Sco (100 μM) in zebrafish ($p < 0.1$). While treatment with Fab in the concentration of 3 μg/L, is much more effective in ameliorating the cognitive deficits induced by Sco (100 μM) ($p < 0.001$). On the other hand, administration of Fab treatment at a concentration of 5 μg/L proved to be the most effective, managing to increase in a statistically significant way ($p < 0.0001$), the number of crossed

lines of fish within the Y maze (Figure 5.14 E). Moreover, treatment with Fab at a concentration of 5 µg/L was able to effectively and significantly restore the time fish spent in the novel arm of the maze ($p < 0.05$; Figure 5.14 F). The findings from this study therefore suggest that Fab exhibits a spatial memory-enhancing and differentiation profile within the Y-maze.

Therefore, zebrafish that were subjected to treatment with Sco (100 µM) presented a significant degradation of the behavior assessed by quantifying locomotor activity parameters ($p < 0.0001$), learning and memory abilities, assessed by recording the percentage of alternation spontaneous (%) ($p < 0.01$), as well as by a significant decrease in spatial memory ($p < 0.001$). In contrast, fish treated with Sco (100 µM) and subjected to Rho, Baic and Fab treatment at the concentrations of 1, 3 µg/L and 5 µg/L showed a significant increase in locomotor activity, in a dose-dependent manner, exerting the most pronounced effect in the concentration of 5 µg/L ($p < 0.0001$ for Rho and Baic and $p < 0.01$ for Fab). Simultaneously, the three natural flavonoids were able to improve the ability to learn and memorize new information as well as their consolidation and storage in the zebrafish model of AD that was induced by the acute administration of Sco (100 µM), by significantly increasing the percentage of spontaneous alternation (%) ($p < 0.001$ for Rho and $p < 0.0001$ for Baic and Fab)(196,197). Also, the results obtained suggest that the three flavonoids present a memory-stimulating profile, preventing the negative effects on cognitive processes induced by Sco, by restoring the number of lines crossed and by increasing the time to explore the new arm during the Y-maze task ($p < 0.001$ for Rho and Baic and $p < 0.0001$ for Fab).

V.8.3 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on the reference memory of zebrafish evaluated in the NOR test

In Figure 5.15, representative tracking patterns of zebrafish locomotion in the NOR test and their preference for one of the two objects (OF and ON) are illustrated. During the 10-min session, there was a tendency for control fish to spend a greater amount of time exploring the ON area at the expense of the OF area, while the Sco-treated group (100 µM) showed a preference for the OF to the detriment of ON. The groups treated with Rho, Baic and Fab in concentrations of (1, 3 and 5 µg/L) were distinguished by an increase in the exploration time of the ON at the expense of the OF, at a level close to that of the control group and the treated group with GAL (1 mg/mL) (196, 197).

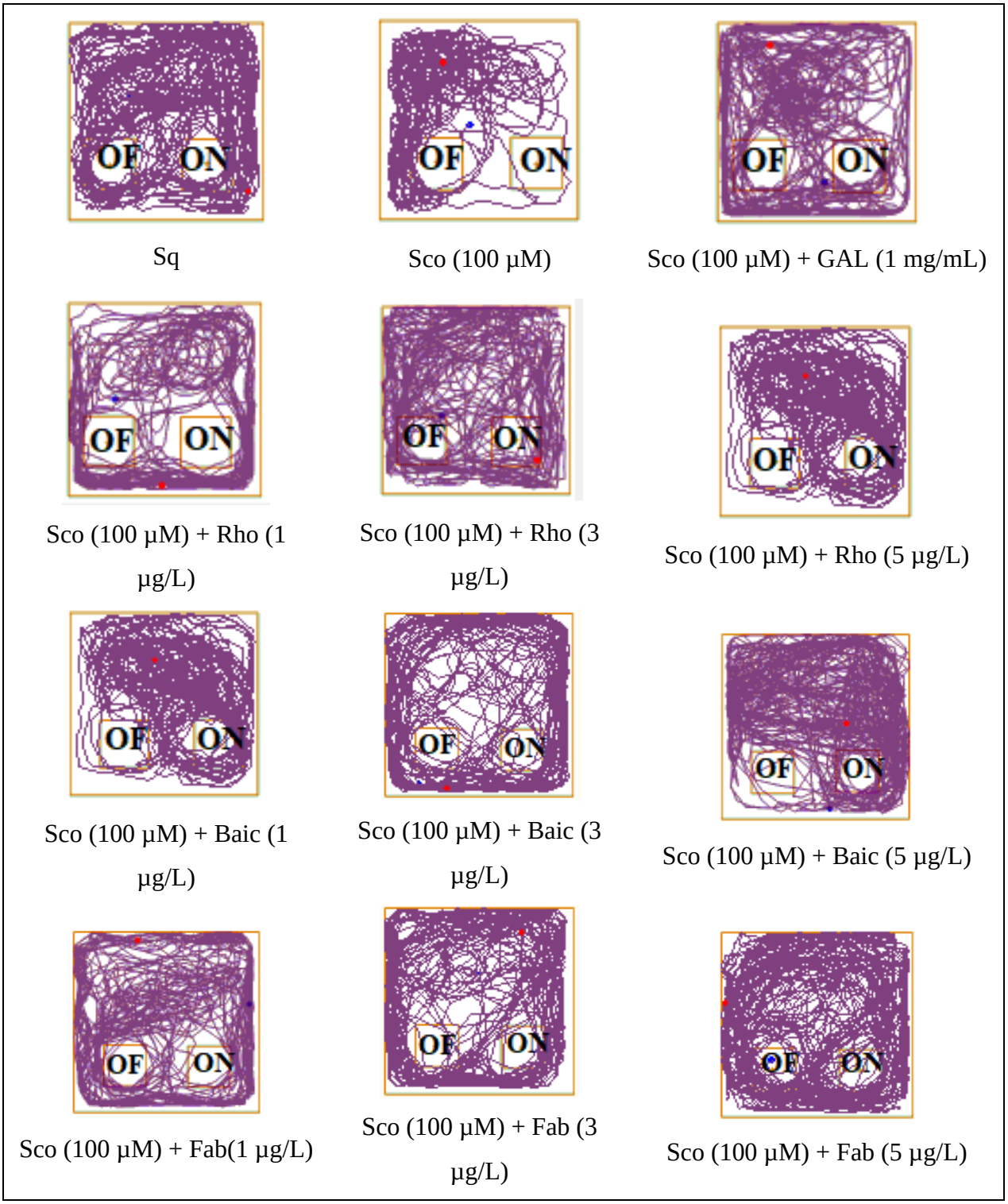
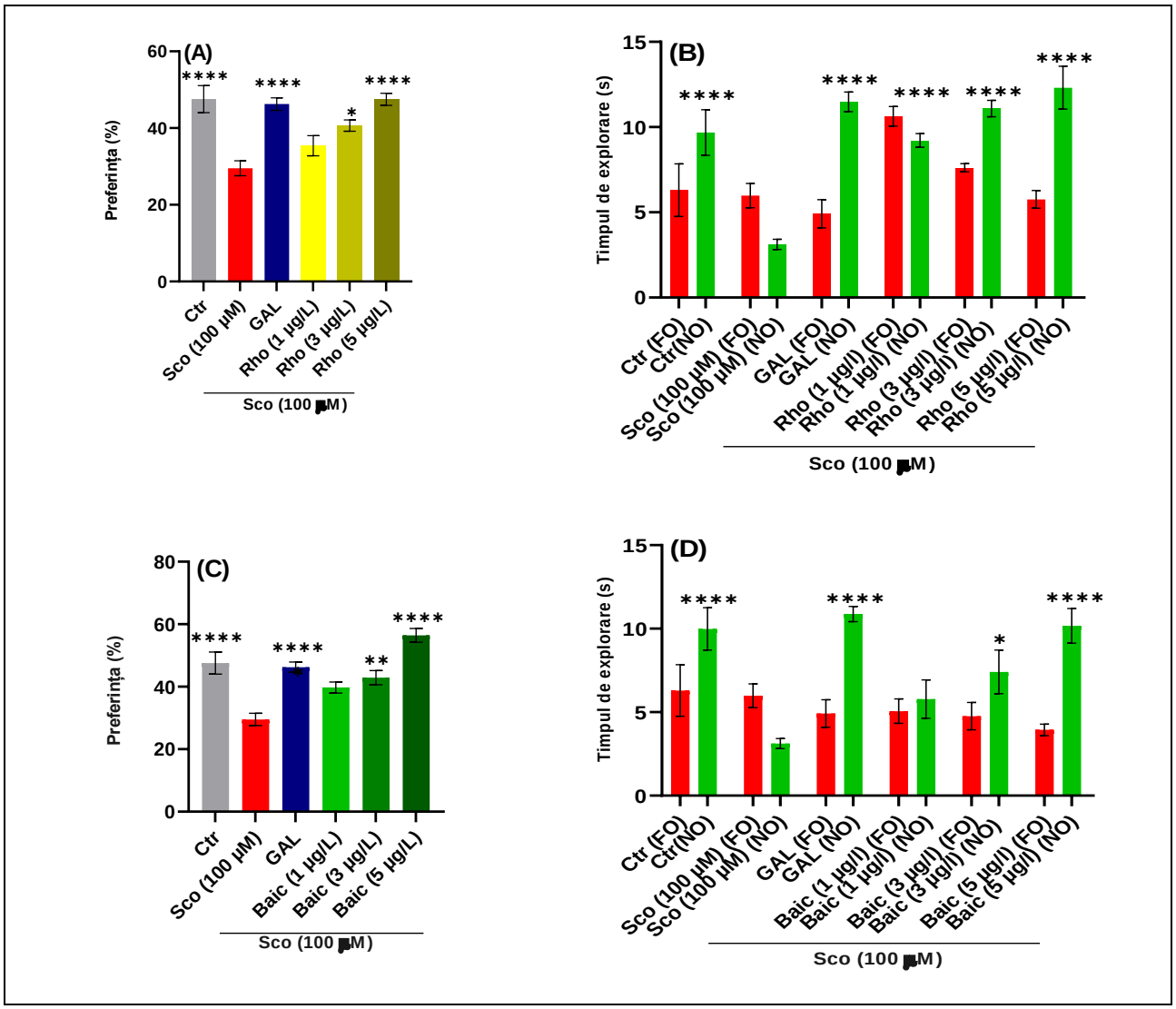


Figure 5.15 Graphical representation of zebrafish trajectory during the test session of the NOR test.The beginning of the zebrafish trail is represented by the blue dot, and the end of the fish path is represented by the red dot.The familiar object area was denoted by the initials (OF) and the novel object area was denoted by the initials (ON).

In the NOR test, one-way ANOVA revealed a significant effect of the three natural flavonoid treatments on preference percentages (Rho: $F(5, 54) = 10.85$, $p < 0.0001$; Figure 5.16 A), (Baic : $F(5, 54) = 14.97$, $p < 0.0001$; Figure 5.16 C), (Fab: $F(5, 54) = 8.087$, $p <$

0.0001; Figure 5.16 E) and on exploration time a ON (Rho: F (5, 54) = 107.7, p < 0.0001; Figure 5.16 B), (Baic: F (5, 54) = 46.43, p < 0.0001; Figure 5.16 D), (Fab: F (5, 54) = 79.74, p < 0.0001; Figure 5.16 F). In this test it was observed that fish in the control group showed a high preference for ON exploration and a reduced one for OF exploration (p < 0.0001; Figure 5.16 A, C and E). Also the fish in the control group spent alonger time exploring the ON area than that of the OF (Figure 5.16 B, D and F). In contrast, fish treated with Sco (100 µM) showed a significant decrease in preference percentages (p < 0.0001; Figure 5.16 A, C and E) and ON exploration time (p < 0.0001; Figure 5.16 B, D and F), suggesting the onset of memory deficits induced by Sco treatment (196, 197).



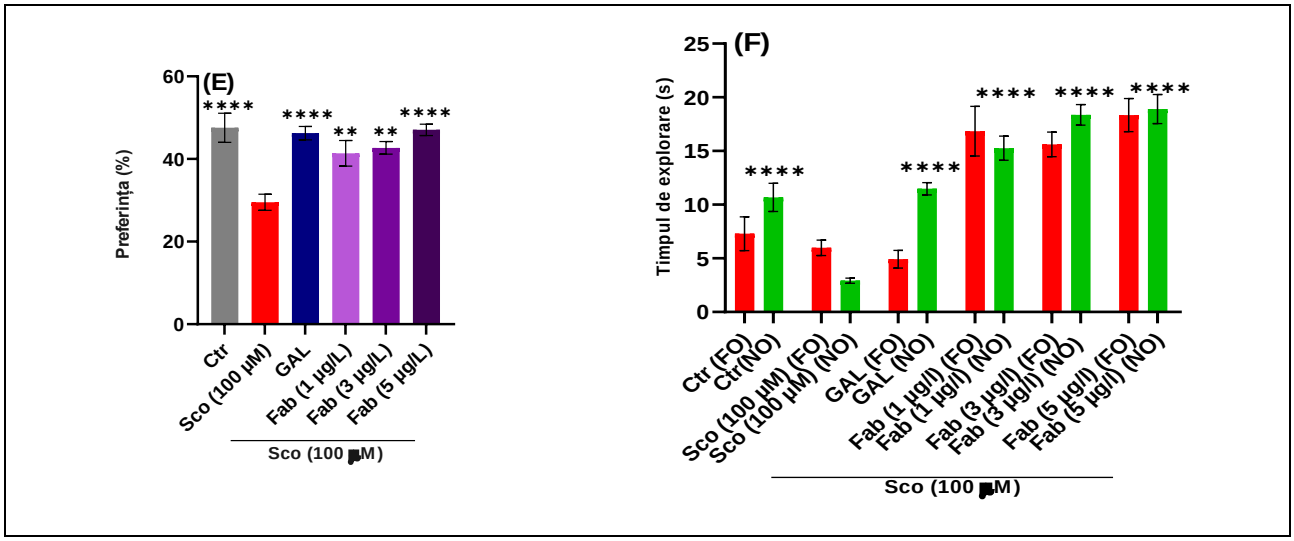


Figure 5.16 Effect of treatment with Rho (1, 3 and 5 µg/L) (A, B), Baic (1, 3 and 5 µg/L) (C, D) and Fab (1, 3 and 5 µg/L) (E, F) on the investigation of reference memory performance in zebrafish during the 10-min session, assessed by the fish's preference (%) for the familiar object or for the novel object (A, C, E) and object exploration time new (s) (B, D, F). Values are expressed as means ± SEM, (n = 10 animals per group). In the NOR test, ANOVAOne Wayrevealed a significant effect of treatment on preference for one of the two objects in the NOR test: (A) ($F(5, 54) = 10.85$, $p < 0.0001$); (C) ($F(5, 54) = 14.97$, $p < 0.0001$); (E) ($F(5, 54) = 8.087$, $p < 0.0001$); and (B) ($F(5, 54) = 107.7$, $p < 0.0001$); (D) ($F(5, 54) = 46.43$, $p < 0.0001$); (F) ($F(5, 54) = 79.74$, $p < 0.0001$).For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

The subsequent administration of Rho treatment in the concentration of 1 µg/L, was found to be ineffective in increasing the preference (%) of the fish towards ON (Figure 5.16A). However, there was a significant improvement in reference memory in zebrafish chronically treated with Rho at concentrations of 3 and 5 µg/L, by restoring fish preference at both 3 µg/L ($p < 0$ .05), as well as in that of 5 µg/L ($p < 0.0001$; Figure 5.16A). Moreover, chronic treatment with Rho succeeded in all three tested concentrations to restore ON exploration time ($p < 0.0001$; Figure 5.16B), to a level close to that of GAL treatment (1 mg/ mL), suggesting the beneficial effects of Rho treatment on learning and memory processes in zebrafish, especially reference memory.

An effect similar to that of Rho treatment on preference percentages in the NOR test was also observed with Baic treatment. Thus, the fish treated with Baic, in the concentration of 1 µg/L, did not show a significant preference for either of the two objects. However, a significant increase in fish preference (%), was evident starting at the concentration of 3 µg/L ($p < 0.01$; Figure 5.16 C). Moreover, treatment with Baic at the concentration of 5 µg/L was able to significantly restore fish preference (%) to ON ($p < 0.0001$) during the test session of the NOR test, in - a way similar to that of the treatment with GAL (1 mg/mL) (Figure 5.16C). Also, regarding the effects of Baic treatment on ON exploration time, a significant effect was observed only within the concentrations of 3 ($p < 0.1$) and 5 µg/L ($p < 0.0001$; Figure

5.16 D) at a level close to that of GAL (1 mg/mL) ($p < 0.0001$), suggesting the beneficial effects of Baic treatment especially in the concentration of 5 µg/L on the reference memory of zebrafish (196, 197).

Alternatively, Fab treatment was found to be effective in restoring reference memory in amnesic fish at all three concentrations tested. Thus, administration of Fab treatment in concentrations of 1 and 3 µg/L was shown to have a similar significant effect on preference percentages ($p < 0.01$; Figure 5.16 E), while Fab treatment in a concentration of 5 µg /L showed a more pronounced effect ($p < 0.0001$; Figure 5.16 E). Moreover, Fab treatment also showed a significant effect on ON exploration time (s) in all three concentrations studied ($p < 0.0001$; Figure 5.16 F), in a similar way to the fish group treated with GAL ($p < 0.0001$), suggesting beneficial effects of Fab treatment on reference memory in the NOR test. The results from this study are also supported by the data of a meta-analysis carried out by(198), where the authors described, Fab, as a natural biflavonoid showing promising characteristics to act as an adjunctive neuroprotective agent for the treatment of neurodegenerative diseases.

Zebrafish treated with Sco (100 µM) showed a decrease in preference percentages ($p < 0.0001$) and ON exploration time ($p < 0.0001$), suggesting the degradation of cognitive processes and the establishment of a profile amnesiac Subsequent administration of treatments with the three natural flavonoids resulted in a significant improvement in the zebrafish's reference memory. A more pronounced effect on cognitive processes and especially on reference memory was observed following the administration of Rho and Baic treatment at the concentrations of 3 and 5 µg/L and Fab treatment at all three tested concentrations 1, 3 and 5 µg/L, by restoring fish preference for ON and ON exploration time, especially for the 5 µg/L concentration ($p < 0.0001$), to a level close to that of GAL treatment (1 mg/ mL).

V.9 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on oxidative stress in the zebrafish model of Alzheimer's disease

V.9.1 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on superoxide dismutase activity

In this study, administration of Sco treatment (100 µM) for 30 minutes to zebrafish showed a significant decrease in SOD activity ($p < 0.0001$; Figure 5.17 A, B and C) compared to the control group. In contrast, the administration of Rho treatment (1, 3, 5 µg/L) caused the increase of SOD activity, especially in the concentration of 5 µg/L ($p < 0.0001$;

Figure 5.17 A), but showed a significant effect statistically also in the lower concentrations ($p < 0.01$ for the 3 µg/L concentration and $p < 0.05$ for the 1 µg/L concentration; Figure 5.17 A).

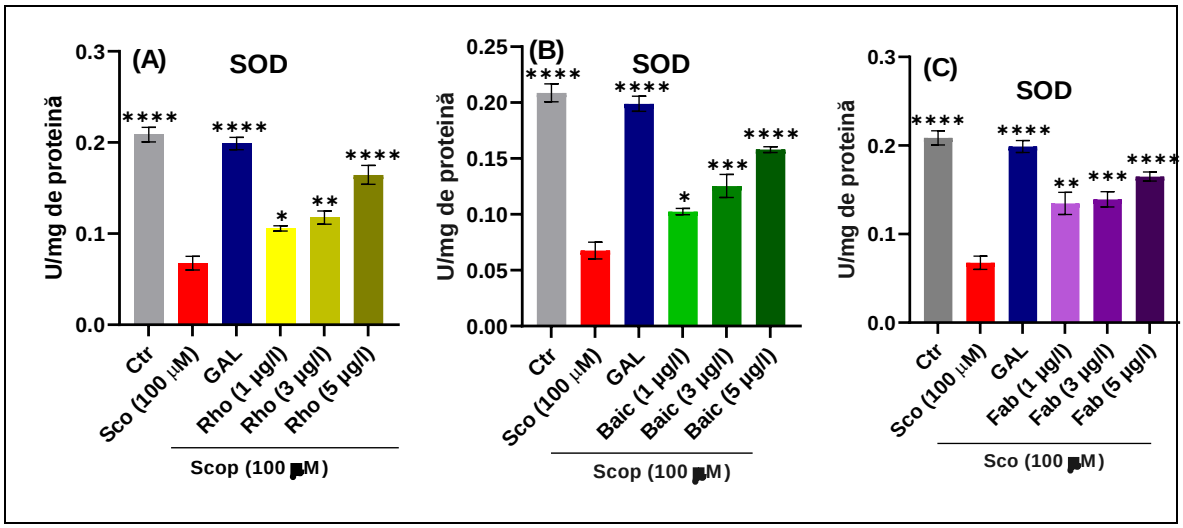


Figure 5.17 Effects of treatment with Rho (1, 3 and 5 µg/L) (A), Baic (1, 3 and 5 µg/L) (B) and Fab (1, 3 and 5 µg/L) (C) on activity SOD in zebrafish model of AD induced by administration of 100 µM Sco. Values are expressed as means ± SEM (n = 10 animals per group). ANOVA revealed a significant effect of treatment on specific SOD activity. (A) ($F(5, 12) = 55.84$, $p < 0.0001$) and (B) ($F(5, 12) = 64.20$, $p < 0.0001$) and (C) ($F(5, 12) = 37.26$, $p < 0.0001$). For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

Regarding the treatment with Baic (1, 3, 5 µg/L), it was observed that this natural flavonoid also significantly increased the specific activity of SOD ($p < 0.05$ for the concentration of 1 µg/ L, $p < 0.01$ for the concentration of 3 µg/L and $p < 0.0001$ for the concentration of 5 µg/L; Figure 5.17 B) (196,197). Also, as seen in Figure 5.17C Fab treatment remarkably up-regulated SOD activity in a concentration-dependent manner. Thus, for the concentration of 1 µg/L the statistical significance is $p < 0.01$, for the concentration of 3 µg/L the statistical significance is $p < 0.001$, and for the concentration of 5 µg/L the statistical significance is $p < 0, 0001$.

V.9.2 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on catalase activity

In this study, Tukey's Post hoc test showed a significant difference between the control group and the Sco treated zebrafish. Thus, in fish treated with Sco, a significant decrease in the specific activity of CAT was observed, compared to the control group ($p < 0.001$; Figure 5.17 A, B and C), suggesting the establishment of OS and the generation of oxidative changes in DNA- of protein and lipids in zebrafish brain tissue(196,197).

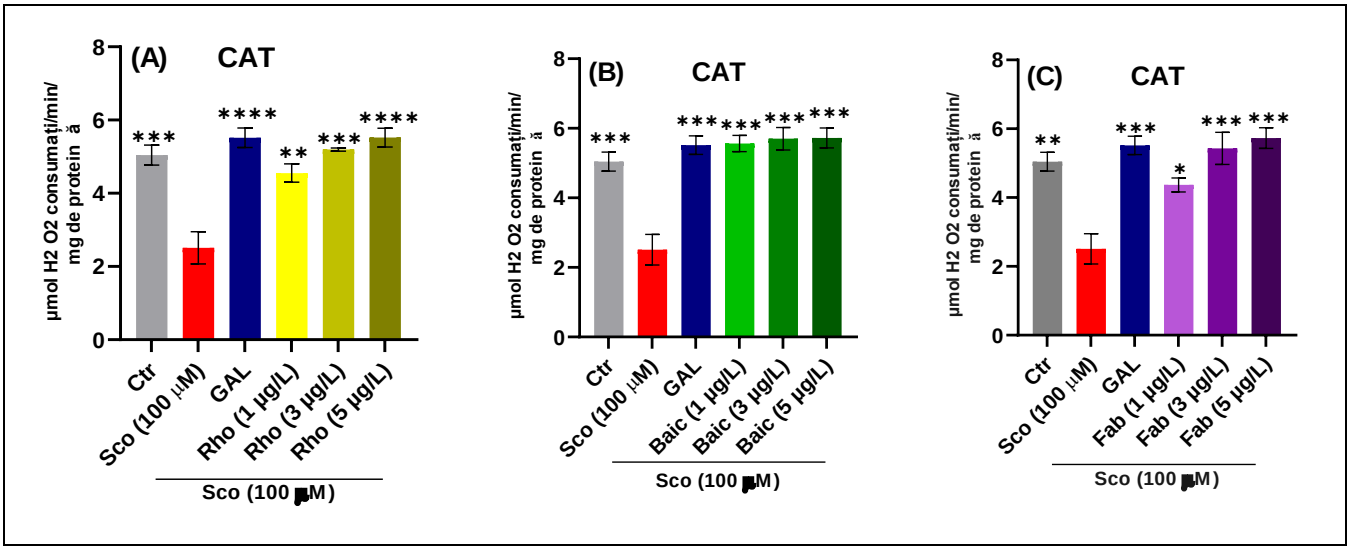


Figure 5.18 Effects of treatment with Rho (1, 3 and 5 µg/L) (A), Baic (1, 3 and 5 µg/L) (B) and Fab (1, 3 and 5 µg/L) (C) on activity CAT in the zebrafish model of AD induced by administration of 100 µM Sco. Values are expressed as means ± SEM, (n = 10 animals per group). ANOVA revealed a significant effect of treatment on the specific activity of CAT. (A) ($F(5, 12) = 16.56$, $p < 0.0001$) and (B) ($F(5, 12) = 16.09$, $p < 0.0001$) and (C) ($F(5, 12) = 12.57$, $p=0.0002$). For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

Treatments with Rho, Baic and Fab (1, 3, 5 µg/L) showed a significant effect on CAT activity, significantly increasing the specific activity of CAT. Thus for Rho treatment Tukey's Post hoc test showed a significant difference in a dose-dependent manner between the group of fish treated with Sco and the group of fish treated with Rho in the concentration of 1 µg/L ($p < 0, 01$), between the group of fish treated with Sco and the group of fish treated with Rho in the concentration of 3 µg/L ($p < 0.001$) and between the group of fish treated with Sco and the group of fish treated with Rho in the concentration of 5 µg/L L ($p < 0.0001$), in a similar manner to the GAL-treated group ($p < 0.0001$; Figure 5.18 A)(196,197). Regarding the effects of Baic treatment, Tukey's Post hoc test showed that chronic treatment with Baic was able to restore in a similar manner for all 3 tested concentrations (1, 3, 5 µg/L) the specific activity of CAT from zebrafish brain tissue ($p < 0.0001$), at a level close to that of GAL ($p < 0.001$; Figure 5.18 B) (196, 197).

Alternatively, the administration of Fab treatment at concentrations of 1, 3, 5 µg/L to laboratory animals that had previously undergone acute treatment with Sco, was able to exert a significant effect on CAT activity, especially in concentrations of 3 and 5 µg/L ($p < 0.001$) at a level close to that of GAL ($p < 0.001$; Figure 5.18 C). However, it was noted that treatment with Fab at a concentration of 1 µg/L showed little effect on the modulation of CAT activity (196, 197).

V.9.3 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on glutathione peroxidase activity

Regarding the effects of treatments with the three natural flavonoids on GPX activity, Tukey's Post hoc test showed that Rho treatment (1, 3, 5 µg/L) significantly up-regulated GPx level only in dose of 5 µg/L ($p < 0.05$; Figure 5.19 A)(196,197). Baic treatment (1, 3, 5 µg/L) was found to be more effective in regulating the specific activity of GPX than Rho treatment. Thus, the treatment with Baic in the concentration of 3 µg/L managed to exert a significant effect on GPX activity ($p < 0.01$), at a level close to that of GAL ($p < 0.001$; Figure 5.19 B). Furthermore, treatment with Baic at the concentration of 5 µg/L was able to restore the specific activity of GPX in the zebrafish brain ($p < 0.0001$).

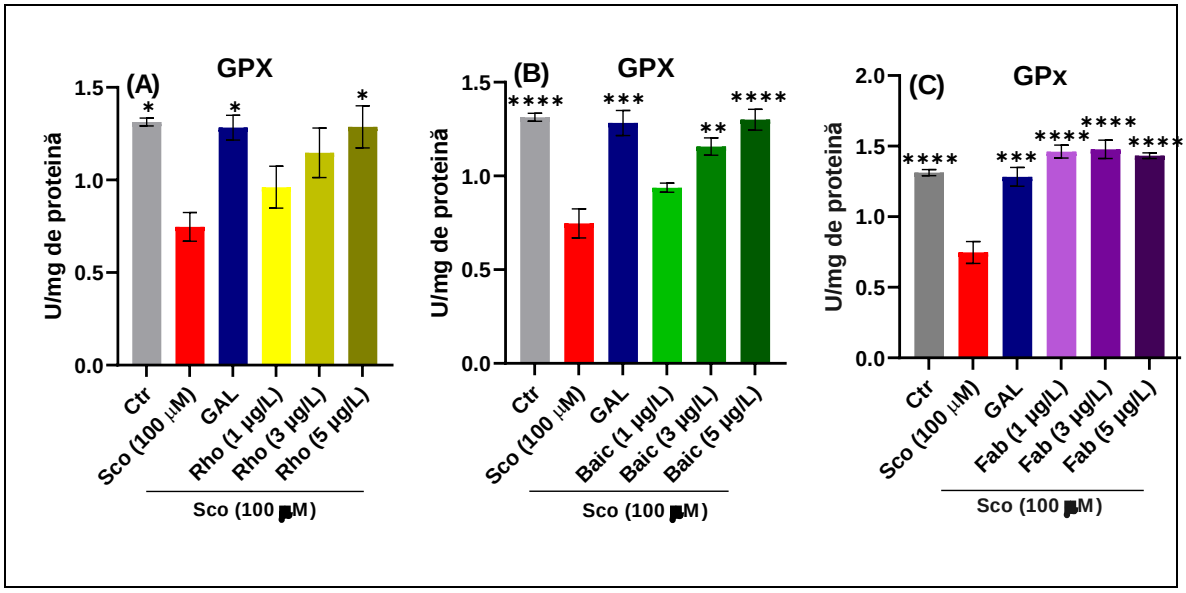


Figure 5.12. Effects of treatment with Rho (1, 3 and 5 µg/L) (A), Baic (1, 3 and 5 µg/L) (B) and Fab (1, 3 and 5 µg/L) (C) on activity GPX in the zebrafish model of AD induced by administration of 100 µM Sco. Values are expressed as means ± SEM (n = 10 animals per group). ANOVA revealed a significant effect of treatment on the specific activity of GPX. (A) ($F(5, 12) = 5.636$, $p=0.0067$) and (B) ($F(5, 12) = 19.33$, $p < 0.0001$) and (C) ($F(5, 12) = 25.71$, $p < 0.0001$).For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

Regarding Fab treatment (1, 3, 5 µg/L), a more pronounced increase in GPX activity is observed in all three tested concentrations ($p < 0.0001$; Figure 5.19 C), in a similar manner with that of GAL treatment ($p < 0.001$) (196, 197).

V.9.4 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on total reduced glutathione content

Treatment with Sco (100 µM) reduced the level of GSH in the brain of zebrafish compared to the group without Sco ($p < 0.01$; Figure 5.20 A), ($p < 0.001$; Figure 5.20 B), ($p < 0.0001$; Figure 5.20 C).

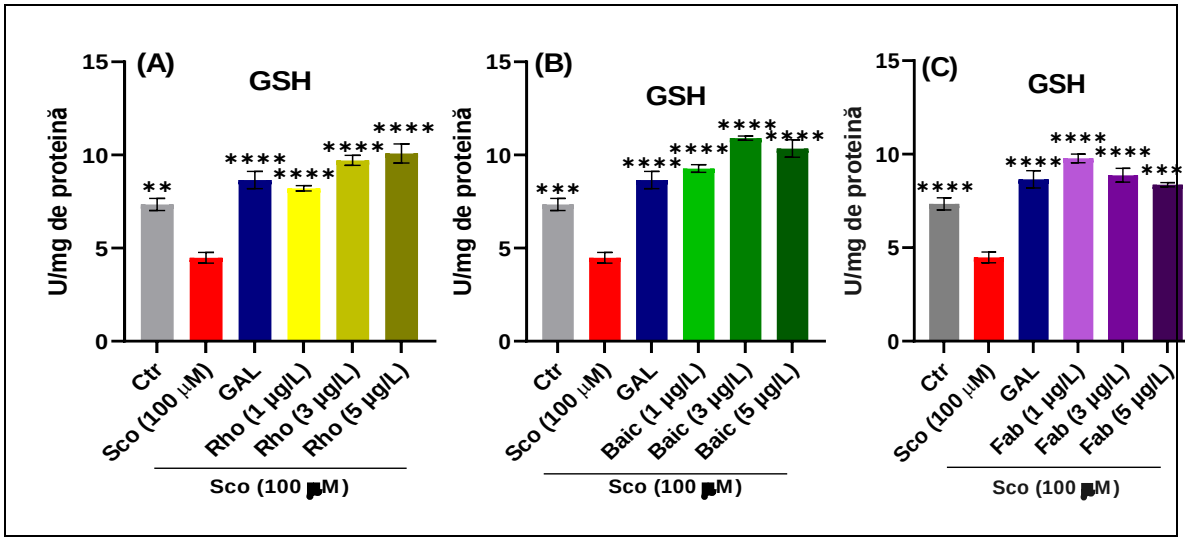


Figure 5.20 Effects of treatment with Rho (1, 3 and 5 µg/L) (A), Baic (1, 3 and 5 µg/L) (B) and Fab (1, 3 and 5 µg/L) (C) on activity GSH in the zebrafish model of AD induced by administration of 100 µM Sco. Values are expressed as means ± SEM, (n = 10 animals per group). ANOVA revealed a significant effect of treatment on specific GSH activity. (A) ($F(5, 12) = 32.53$, $p < 0.0001$) and (B) ($F(5, 12) = 48.57$, $p < 0.0001$) and (C) ($F(5, 12) = 34.44$, $p < 0.0001$). For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

Subsequent administration of Rho, Baic, and Fab treatments resulted in a significant increase in GSH levels in a similar manner at all three concentrations tested (1, 3, and 5 µg/L) ($p < 0.0001$; Figure 5.20 A, B and C), in a manner similar to that of the GAL-treated group ($p < 0.0001$) (196, 197).

V.9.5 Effects of roifoline, baicalein 5,6-dimethyl ether and agatisflavone on malondialdehyde level

Acute administration of Sco (100 µM) caused significant increases in the level of MDA compared to the control group ($p < 0.0001$; Figure 5.21) (196, 197). Chronic administration of Rho treatment (1, 3, and 5 µg/L) to amnesic animals significantly restored the antioxidant status in the zebrafish brain, but a differential pattern of efficacy was observed. Thus, it was observed that treatment with Rho in the concentrations of (1.3 µg/L) showed a more modest potential in ameliorating the amnesic effects induced by Sco (100 µM) ($p < 0.001$; Figure 5.21 A), compared to the concentration of (5 µg/L) which showed a higher potency ($p < 0.0001$), in a similar way to the GAL-treated group ($p < 0.0001$; (Figure 5.21A).

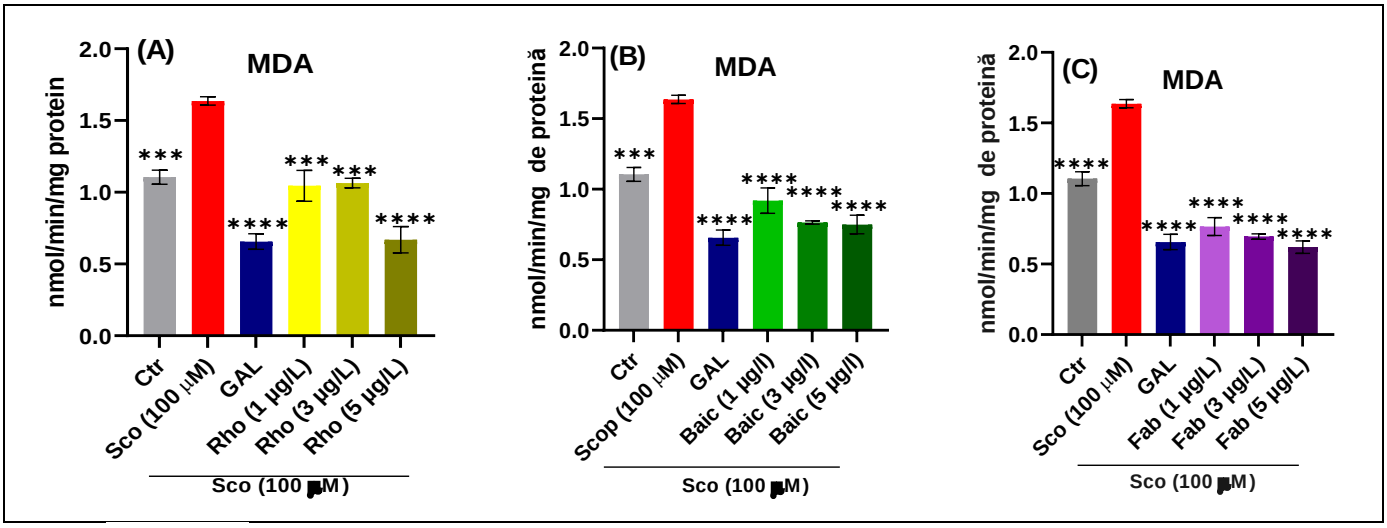


Figure 5.21 Effects of treatment with Rho (1, 3 and 5 µg/L) (A), Baic (1, 3 and 5 µg/L) (B) and Fab (1, 3 and 5 µg/L) (C) on activity MDA in zebrafish model of AD induced by administration of 100 µM Sco. Values are expressed as means ± SEM, (n = 10 animals per group). ANOVA revealed a significant effect of treatment on specific MDA activity. (A) ($F(5, 12) = 28.33$, $p < 0.0001$) and (B) ($F(5, 12) = 41.93$, $p < 0.0001$) and (C) ($F(5, 12) = 75.07$, $p < 0.0001$).For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

Regarding the treatment with Baic and FAB, it was observed that the two natural flavonoids showed a similar efficiency in all three concentrations (1, 3 and 5 µg/L) in regulating MDA levels, in the brain of zebrafish ($p < 0.0001$), in a similar manner to the GAL-treated group ($p < 0.0001$; Figure 5.21 B and C) (196, 197).

V.9.6 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on the level of carbonylated proteins

Following the quantification of the levels of carbonylated proteins in the brains of zebrafish, it was observed that the chronic administration of treatment with Sco (100 µM) had the effect of significantly increasing the level of carbonylated proteins, compared to untreated fish ($p<0.0001$) (Figure 5.15) (196, 197).Administration of Rho treatment at the concentration of 1 µg/L caused a decrease in the level of carbonylated proteins compared to the group treated with Sco alone (100 µM), but caused a statistically insignificant decrease (Figure 5.22 A).Regarding the treatment with Rho in the concentration of 3 µg/L it was observed that in this concentration Rho is able to decrease in a significant way, although modestly, the level of carbonylated proteins compared to the group of fish treated only with Sco (100 µM) ($p < 0.05$; Figure 5.22 A). A more obvious statistical significance of the treatment with Rho compared to the group subjected only to the treatment with Sco (100 µM), was observed in the concentration of 5 µg/L ($p < 0.001$), at a level close to that of the treatment with GAL ($p < 0.0001$; Figure 5.22 A) (196, 197).

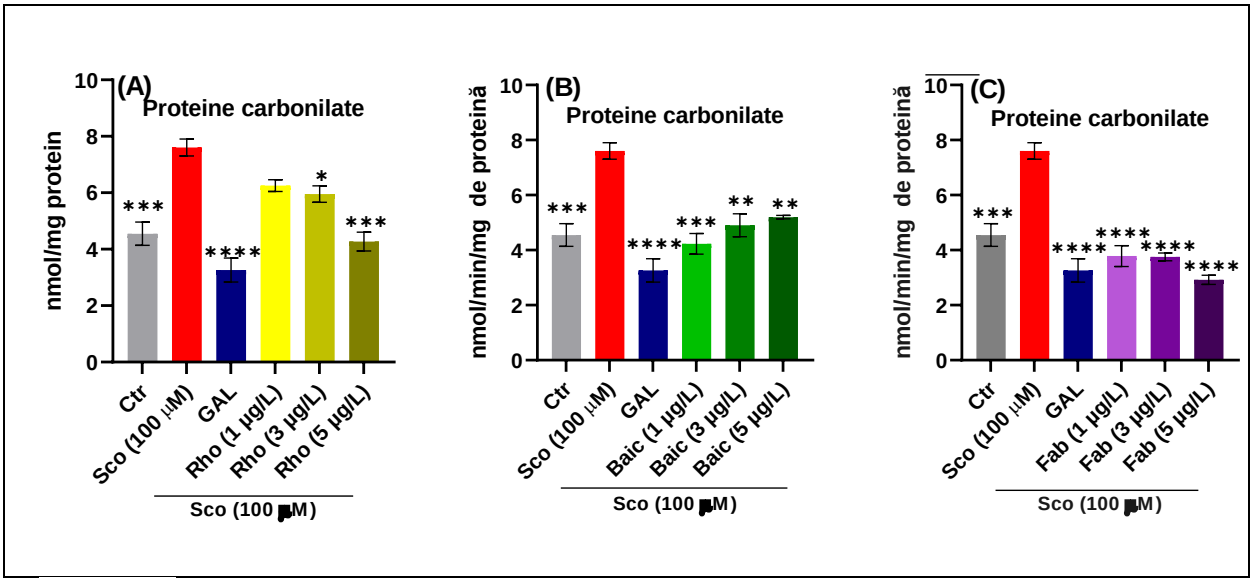


Figure 5.22 Effects of treatment with Rho (1, 3 and 5 µg/L) (A), Baic (1, 3 and 5 µg/L) (B) and Fab (1, 3 and 5 µg/L) (C) on activity carbonylated proteins in the zebrafish model of AD induced by administration of 100 µM Sco. Values are expressed as means ± SEM, (n=10 animals per group). ANOVA revealed a significant effect of treatment on the specific activity of carbonylated proteins. (A) ($F(5, 12) = 21.75$, $p < 0.0001$) and (B) ($F(5, 12) = 16.68$, $p < 0.0001$) and (C) ($F(5, 12) = 27.42$, $p < 0.0001$).For Tukey's post hoc analyses: *p < 0.01, ** p < 0.001, *** p < 0.0001 and **** p < 0.00001.

The treatment with Baic proved to be more effective in the lowest concentration tested, namely in that of 1 µg/L,where it was able to significantly regulate the level of carbonylated proteins ($p<0.001$; Figure 5.22 B), while treatment with Baic at concentrations of 3 and 5 µg/L caused a more modest significant decrease in protein content carbonylated ($p<0.001$; Figure 5.22B). In contrast, chronic administration of Fab treatment to animals with an amnesic model of dementia revealed a significant decrease in the level of carbonylated proteins in all three tested concentrations 1, 3 and 5 µg/L ($p < 0.0001$), in -a similar way to GAL treatment ($p < 0.0001$; Figure 5.22 C) (196, 197).Therefore, the data from this study suggest that Sco induces remarkable suppression of the protective functions of the endogenous antioxidant enzymes SOD, CAT, GPX and GSH leading to high levels of accumulation of free radicals in the cell, such as ROS. Alternatively, treatment with Rho, Baic and Fab effectively restored the antioxidant defense mechanism by regulating the activity of the antioxidant enzymes SOD, CAT, GPX and GSH. Also, the treatments with the three natural flavonoids were able to stop the oxidation of proteins and lipids and decrease the level of carbonylated proteins and the level of MDA.

V.10 Effects of roifolin baicalein 5,6-dimethyl ether and agatisflavone on acetylcholinesterase activity

In this study, treatment with Sco (100 µM) for 30 minutes significantly increased AChE activity in zebrafish compared to the control group ($p<0.01$) (Figure 5.23) (196,197).

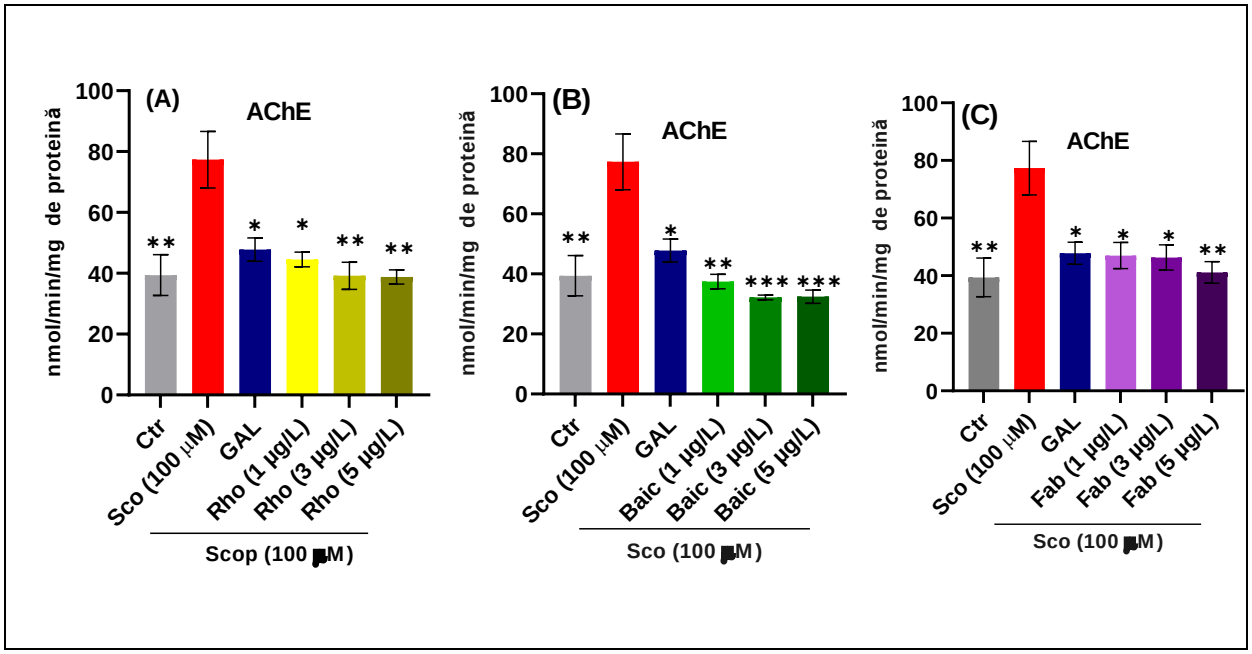


Figure 5.23 Effects of treatment with Rho (1, 3 and 5 µg/L) (A), Baic (1, 3 and 5 µg/L) (B) and Fab (1, 3 and 5 µg/L) (C) on activity AChE in the zebrafish model of AD induced by administration of 100 µM Sco. Values are expressed as means ± SEM (n = 10 animals per group). ANOVA revealed a significant effect of treatment on specific AChE activity: (A) ($F(5, 12) = 7.524$, $p=0.0021$) and (B) ($F(5, 12) = 11.19$, $p =0.0003$) and (C) ($F(5, 12) = 5.803$, $p=0.0060$).For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

Subsequent administration of chronic Rho treatment (1, 3, 5 µg/L) resulted in a decrease in AChE activity, particularly at concentrations of 3 and 5 µg/L ($p < 0.01$). In contrast, treatment with Rho at a concentration of 1 µg/L was able to exert a significant effect on AChE activity, acting in a manner similar to that of GAL treatment ($p < 0.05$) compared to animals treated with only Sco (100 µM) (Figure 5.23 A) (196,197).Regarding the treatment with Baic (1, 3, 5 µg/L), it was observed that it showed a significant decrease in the specific activity of AChE in a dose-dependent manner ($p < 0.01$ for 1 µg/L and $p < 0.001$ for 3 and 5 µg/L), compared to fish treated with Sco alone (100 µM). Groups of animals treated with Fab (1, 3, 5 µg/L), showed an effect similar to that of Rho and Baic, indicating a significant decrease in the specific activity of AChE in a dose-dependent manner ($p < 0.05$ for 1 µg/L and 3 µg/L and $p< 0.01$ for 5 µg/L) compared to fish treated with Sco alone (100 µM) (Figure 5.23C) (196, 197).Therefore, based on these results, we could suggest that Rho, Baic, and Fab show the potential to improve cognitive dysfunction in the Sco-induced amnesic zebrafish model by inhibiting AChE activity and ameliorating cholinergic deficits induced by Sco treatment administration. This is also correlated with the improvement of

memory parameters, as evidenced in behavioral approaches (NTT, Y-maze and NOR) and by the effective restoration of the antioxidant defense mechanism).

V.11 Effectsof roifolin, baicalein 5,6-dimethyl ether and agatisflavone on gene expression

We evaluated the neuropharmacological effects of Rho, Baic and Fab on *bdnf*, *npy*, *egr-1*, *nrf2a* and *creb1* gene expression

V.11.1 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on brain-derived neurotrophic factor (bdnf) expression.

The results of the study indicate that the administration of Sco treatment (100 µM) for 30 minutes to zebrafish showed a significant decrease in the number of mRNA copies of the *bdnf* gene in the brain of the fish, compared to the group of fish that underwent only the treatment with Sco (100 µM) ($p < 0.01$; Figure 5.17).

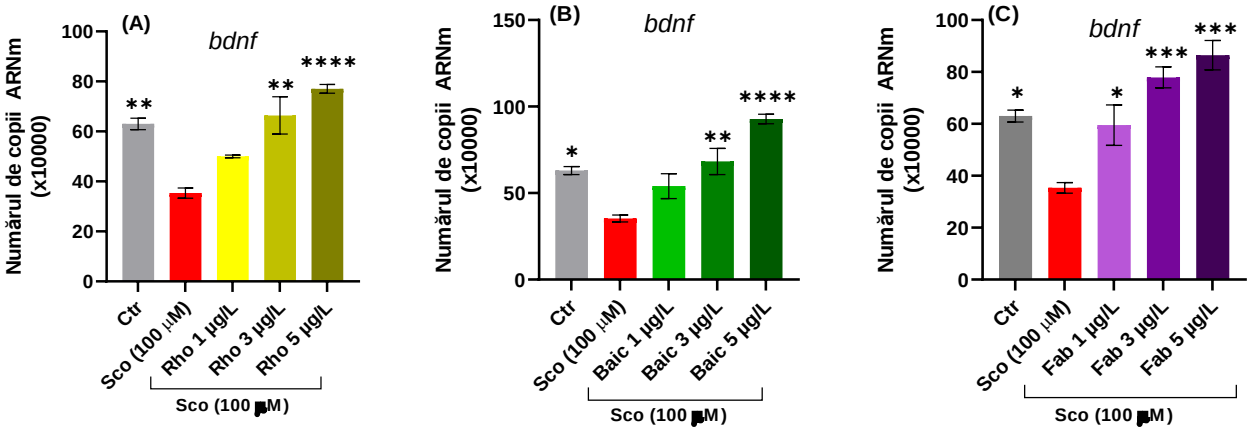


Figure 5.24 Effects of Rho (1, 3 and 5 µg/L), Baic (1, 3 and 5 µg/L) and Fab (1, 3 and 5 µg/L) treatment administration on *bdnf* gene mRNA copy number in model brain of AD zebrafish induced by administration of 100 µM Sco. Values are expressed as means ± SEM, (n = 3 animals per group). ANOVA revealed a significant effect of treatment on bdnf gene mRNA copy number. (A) ($F(4, 10) = 18.94$, $p=0.0001$); (b) ($F(4, 10) = 17.65$, $p=0.0002$); (C) ($F(4, 10) = 16.19$, $p=0.0002$).For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

Regarding the effects of the 3 flavonoids on *bdnf* gene expression in zebrafish brain, Rho and Baic were observed to have a similar effect on *bdnf* gene expression. Thus, both Rho and Baic treatment determined in a significant way increased mRNA copy number of *bdnf* gene in zebrafish brain at 3 and 5 µg/L concentrations ($p < 0.01$ for 3 µg/L and $p < 0.0001$ for 5 µg/L), while Rho treatments and Baic in the concentration of 1 µg/L, were found to be ineffective in modulating *bdnf* gene expression (Figure 5.24 A and B). Also, as seen in Figure 5.24C, Fab treatment remarkably increased *bdnf* gene mRNA copy number in a dose-

dependent manner, exhibiting a significant effect at all three concentrations tested ($p < 0.1$ for the concentration of 1 µg/L and $p < 0.001$ for the concentrations of 3 and 5 µg/L).

V.11.2 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on neuropeptide Y (NPY) expression

In this study, administration of Sco treatment at a concentration of (100 µM) induced a significant decrease in *npy* gene mRNA copy number in the zebrafish brain compared to the control group ($p < 0.0001$; Figure 5.25).

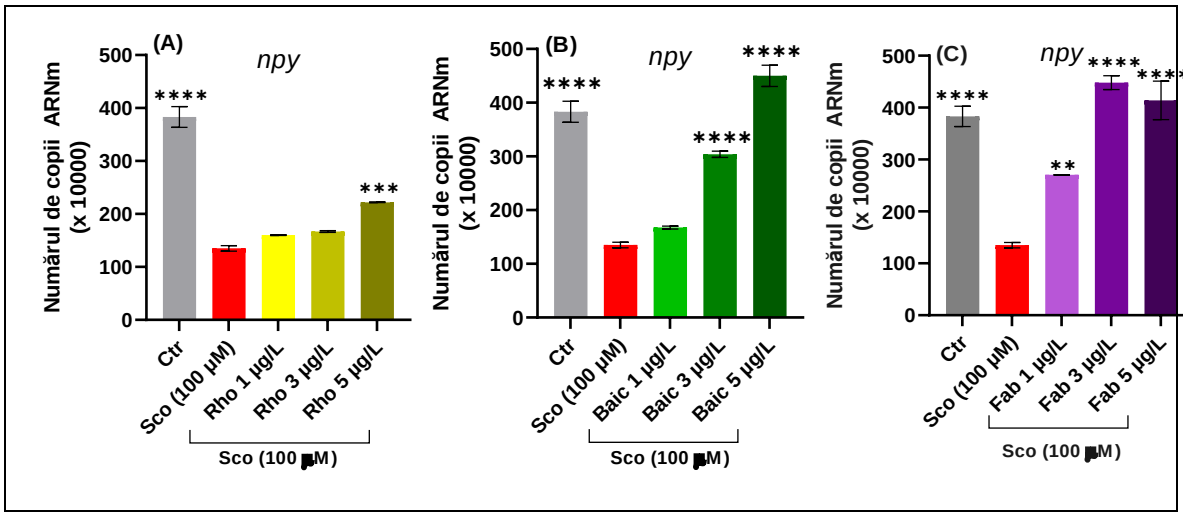


Figure 5.25 Effects of Rho (1, 3 and 5 µg/L), Baic (1, 3 and 5 µg/L) and Fab (1, 3 and 5 µg/L) treatment administration on *npy* gene mRNA copy number in model brain of AD zebrafish induced by administration of 100 µM Sco. Values are expressed as means ± SEM, (n = 3 animals per group). ANOVA revealed a significant effect of treatment on npy gene mRNA copy number: (A) ($F (4, 10) = 120.2$, $p < 0.0001$); (b) ($F (4, 10) = 109.6$, $p < 0.0001$); (C) ($F (4, 10) = 41.81$, $p < 0.0001$).For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

In the case of treatment with Rho (1, 3, 5 µg/L) it was observed that it was able to determine a statistically significant intensification of *npy* gene expression only in the concentration of 5 µg/L ($p < 0.001$) (Figure 5.25). In contrast, treatment with Baic proved to be effective in regulating the gene expression of the *npy* gene, both in the concentration of 3 and in that of 5 µg/L, intensifying in a significant way ($p < 0.0001$) the number of *npy* gene mRNA copies. Regarding treatment with Fab (1, 3, 5 µg/L) it was observed that it remarkably increased the mRNA copy number of the *npy* gene, showing a statistically significant effect in all 3 concentrations tested ($p < 0.01$ for concentration of 1 µg/L and $p < 0.0001$for concentrations of 3 and 5 µg/L; Figure 5.25C).

V.11.3 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on early growth response protein 1 (egr1) gene expression

Based on the results we obtained in this study, it can be seen that the administration of Sco treatment in a concentration of (100 µM) suppressed the expression of the *egr-1* gene compared to the animals in the control group, which, in turn, caused memory impairment ($p < 0.0001$; Figure 5.26).

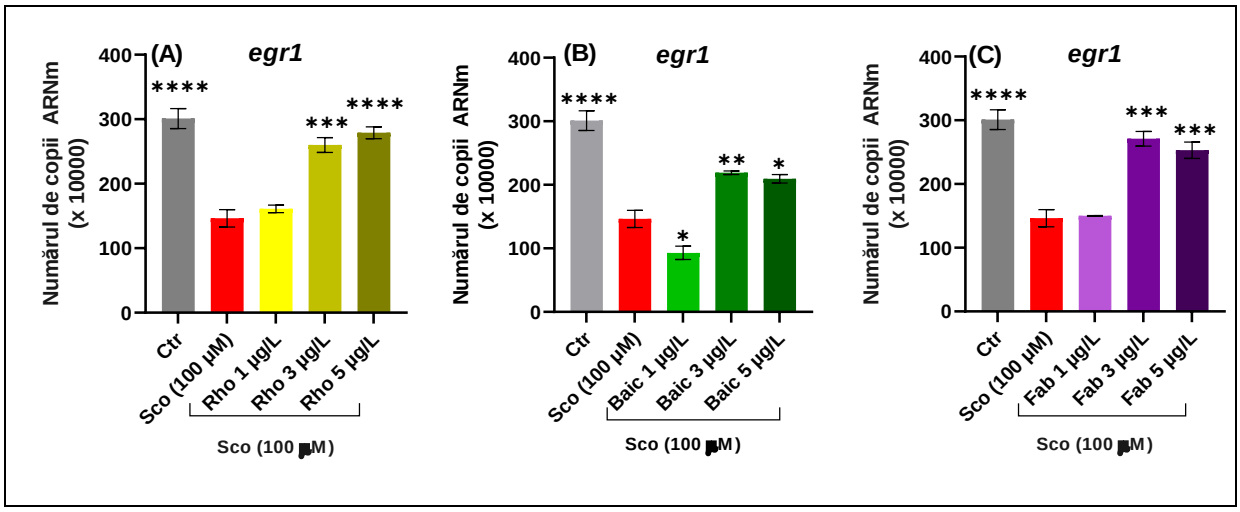


Figure 5.26 Effects of Rho (1, 3 and 5 µg/L), Baic (1, 3 and 5 µg/L) and Fab (1, 3 and 5 µg/L) treatment administration on *egr1* gene mRNA copy number in model brain of AD zebrafish induced by administration of 100 µM Sco. Values are expressed as means ± SEM, (n=3 animals per group). ANOVA revealed a significant effect of treatment on egr-1 gene mRNA copy number: (A) ($F(4, 10) = 37.33$, $p < 0.0001$); (b) ($F(4, 10) = 53.25$, $p < 0.0001$); (C) ($F(4, 10) = 35.76$, $p < 0.0001$).For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

Subsequent administration of Rho, Baic and Fab treatments were able to significantly restore the number of copies *egr-1* gene mRNA in zebrafish brain (Figure 5.26 A, B and C). Tukey's multiple comparison tests showed that both Rho and Fab treatment showed similar efficiency in ameliorating the negative effects of Sco on egr-1 expression for the concentration of 3 µg/L ($p < 0.001$). But at the concentration of 5 µg/L, a different pattern of action was observed. Thus, Rho treatment was found to be more effective in restoring *egr-1* gene mRNA copy number in the zebrafish brain compared to Fab treatment ($p < 0.0001$, for Rho treatment at the concentration of 5 µg/L and $p< 0.001$, for treatment with Fab in the concentration of 5 µg/L; Figure 5.26 A and C). In contrast, treatment with Baic showed a more modest effect in terms of restoring *egr-1* gene mRNA copy number in the zebrafish brain ($p < 0.05$ for the 5 µg/L concentration and $p < 0.05$ for the of 3 µg/L) (Figure 5.26 B). However, regarding the concentration of 1 µg/L, it was observed that none of the flavonoids were able to induce the regulation of *egr-1* gene expression.

V.11.4 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on nuclear factor erythroid 2α-related factor 2 (NRF2) gene expression

In this study, acute exposure of zebrafish to Sco treatment at a concentration of (100 µM) resulted in a significant (p< 0.0001) decrease in *NRF2* gene mRNA copy number in the brain of zebrafish compared to control fish from the control group (Figure 5.27A, B and C). Chronic administration of Rho treatment (1, 3 and 5 µg/L) to amnesic animals resulted in a significant increase in mRNA copy number of the gene *nrf2* ($p < 0.01$, for the 3 µg/L concentration and $p < 0.001$, for the 5 µg/L dose; Figure 5.27A).

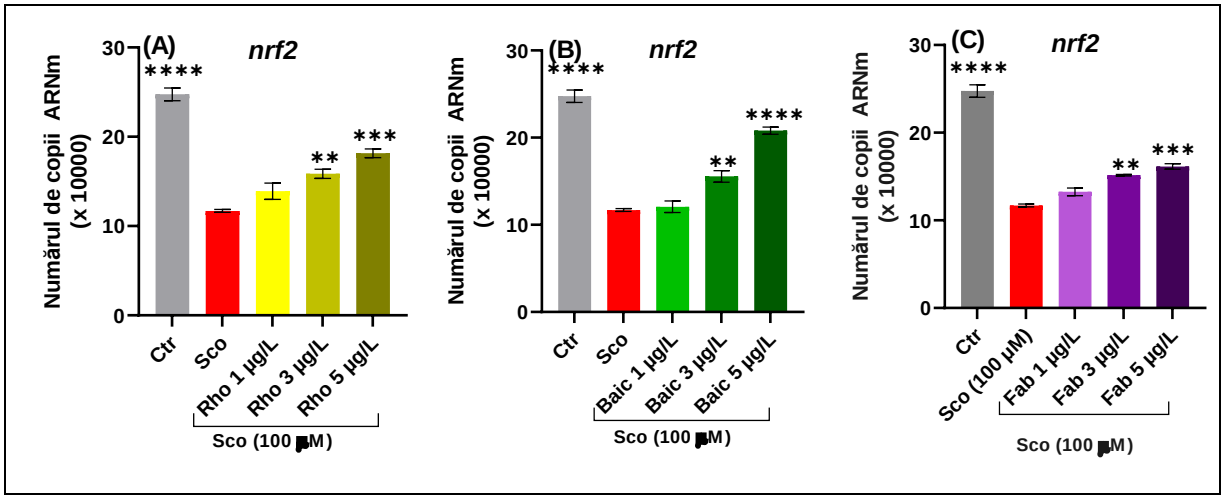


Figure 5.27 Effects of Rho (1, 3 and 5 µg/L), Baic (1, 3 and 5 µg/L) and Fab (1, 3 and 5 µg/L) treatment on gene mRNA copy number *nrf2* in the brain of the zebrafish model of AD induced by administration of 100 µM Sco. Values are expressed as means ± SEM, (n = 3 animals per group). ANOVA revealed a significant effect of treatment on mRNA copy number of the gene*nrf2*: (A) ($F(4, 10) = 67.11$, $p < 0.0001$); (b) ($F(4, 10) = 101.5$, $p < 0.0001$); (C) ($F(4, 10) = 153.5$, $p < 0.0001$).For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

Regarding the effects of Baic treatment, Tukey's Post hoc test showed that chronic treatment with Baicwas able to significantly restore gene expression *nrf2* ,however, a different pattern of efficiency was observed. Thus, it was observed that the treatment with Baic in concentrations of 1 µg/L did not have any effect onthe number of mRNA copies of the gene *nrf2*. Regarding the treatment with Baic in the concentration of 3 µg/L, it showed a more modest potential to ameliorate the effects induced by Sco (100 µM), still managing to increase in a statistically significant manner the number of mRNA copies of the gene *nrf2* ($p < 0.05$), compared to the group treated with Sco (100 µM) (Figure 5.27 B). In contrast, treatment with Baic at a concentration of 5 µg/L was able to significantly restore the mRNA copy number of the *nrf2*α gene ($p < 0.0001$), which was diminished following the administration of Sco treatment (100 µM) (Figure 5.27B).

Also, as seen in Figure 5.27 C, Fab treatment remarkably enhanced the mRNA copy number of the *nrf2* gene in a dose-dependent manner. Thus, Tukey's Post hoc test showed that chronic treatment with Fab in the concentration of 1µg/L showed no effect on *nrf2* mRNA copy number in zebrafish brain, while Fab treatments at higher concentrations of 3 and 5 µg/L, were able to restore in a significant manner mRNA copy number of the *nrf2* gene ($p < 0.01$ for the 3 µg/L concentration and $p < 0.001$ for the 5 µg/L concentration; Figure 5.27C).

V.11.5 Effects of roifolin, baicalin and agatisflavone on the expression of the cAMP response element binding gene (creb1)

The administration of Sco treatment at a concentration of (100 µM) induced a significant decrease in the number of mRNA copies of the *creb1* gene in the brain of zebrafish compared to the control group ($p < 0.0001$; Figure 5.28). Subsequent administration of Rho treatment was able to significantly restore the mRNA copy number of *creb1* gene in the zebrafish brain, which was decreased as a consequence of the administration of Sco treatment, both at the concentration of 3 µg/L ($p < 0.1$) as well asin the concentration of 5 µg/L ($p < 0.0001$; Figure 5.28 A).

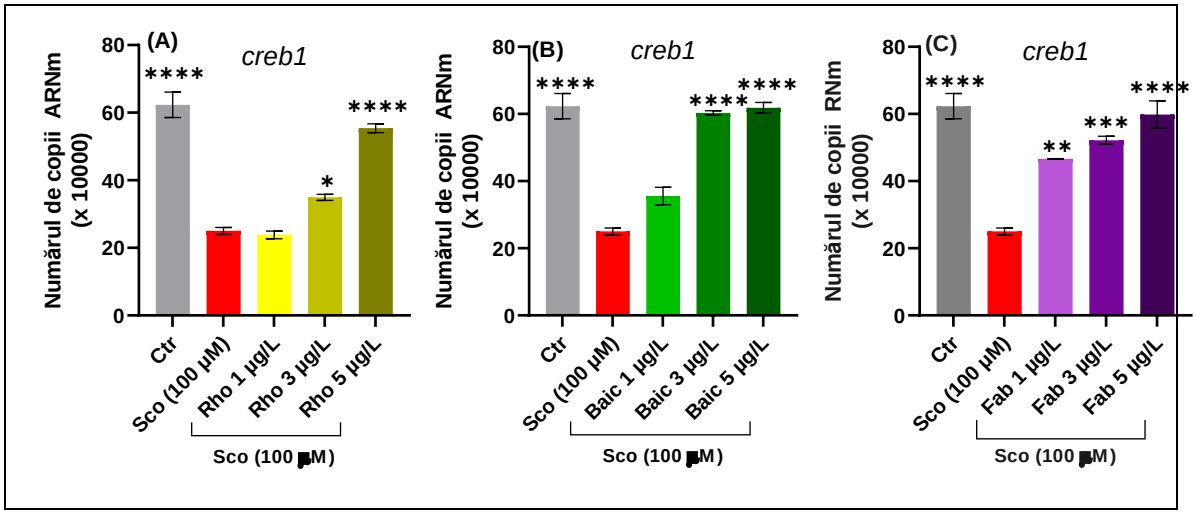


Figure 5.28. Effects of Rho (1, 3 and 5 µg/L), Baic (1, 3 and 5 µg/L) and Fab (1, 3 and 5 µg/L) treatment administration on *creb1* gene mRNA copy number in model brain of AD zebrafish induced by administration of 100 µM Sco. Values are expressed as means ± SEM, (n = 3 animals per group). ANOVA revealed a significant effect of treatment on creb1 mRNA copy number: (A) ($F(4, 10) = 81.45$, $p < 0.0001$); (B) ($F(4, 10) = 92.87$, $p < 0.0001$); (C) ($F(4, 10) = 33.31$, $p < 0.0001$). For Tukey's post hoc analyses: *$p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ and **** $p < 0.00001$.

Regarding the treatment with Baic, it was observed that this natural flavonoid was able to restore *creb1* gene expression in the brain of fish treated with Sco, only in the concentrations of 3 and 5 µg/L ($p < 0.0001$; Figure 5.28 B). Regarding the effects of Fab

treatments on *creb1* gene expression in the zebrafish brain, it was observed that it showed a different pattern of action. Thus, the treatment with Fab, in the concentration of 1 µg/L, managed to intensify in a rather modest way the expression of the *creb1* gene ($p < 0.05$). In contrast, a greater influence of Fab treatment on *creb1* gene expression was observed at concentrations of 3 ($p < 0.001$) and 5 µg/L ($p < 0.0001$; Figure 5 .28 C).

V.12 Effects of roifoline, baicalein 5,6-dimethyl ether and agatisflavone on the level of poteins of interest

V.12.1 Effects of roifolin, baicalein 5,6-dimethyl ether and agatisflavone on Creb1 protein level

The results of the study show that the administration of Sco (100 µM) treatment for 30 minutes to zebrafish induced a significant decrease in Creb1 protein level relative to GAPDH, compared to the control group (Figure 5.29).

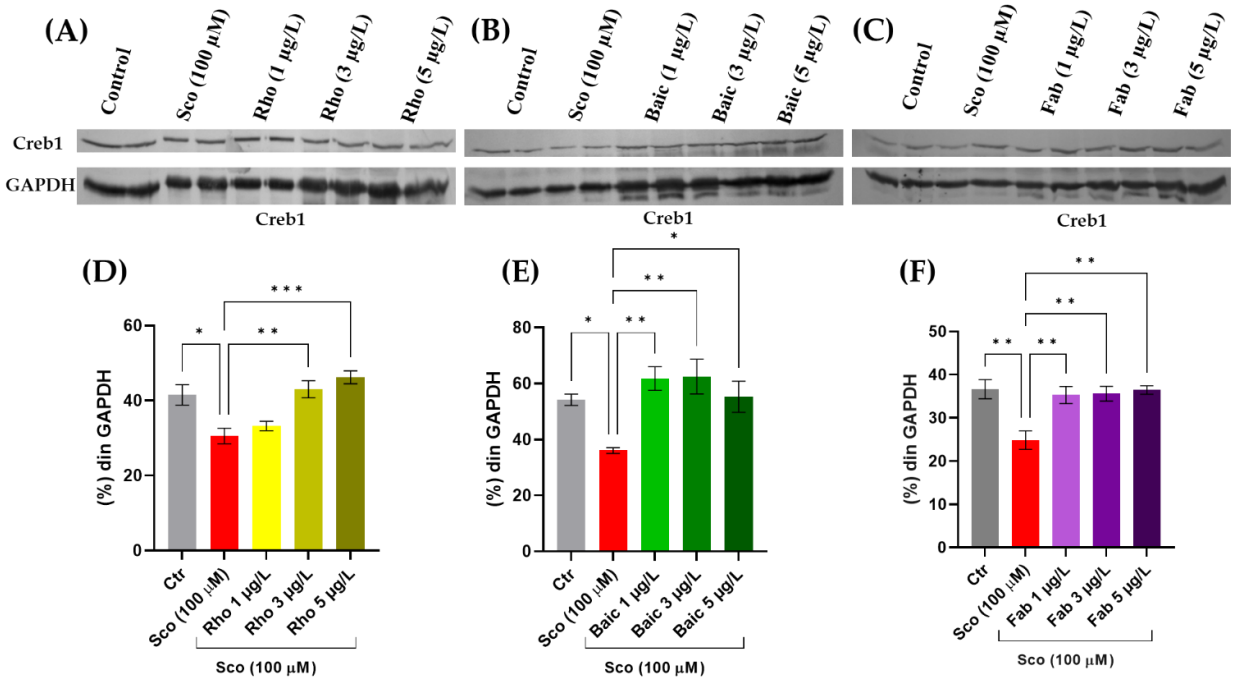


Figure 5.29 Representative bands of CREB1 and GAPDH protein levels in zebrafish brain tissue obtained by immunoblotting (**A, B, C**) and the effects of Rho (1, 3 and 5 µg/L), Baic (1, 3 and 5 µg/L) and Fab (1, 3, and 5 µg/L) on Creb1 protein level in the brain of zebrafish model of AD induced by administration of 100 µM Sco **(D, E, F**). Creb1 protein abundance was calculated by its ratio to GAPDH. Values are expressed as means ± SEM, (n=6 samples per group). ANOVA revealed a significant effect of treatment on Creb1 protein level. **D)** (F (4, 15) = 10.43, p=0.0003); **E)** (F (4, 25) = 5.539); **F)** (F (4, 25) = 7.233, p=0.0005).For Tukey's post hoc analyses: *p < 0.01, ** p < 0.001, *** p < 0.0001 and **** p < 0.00001.

Administration of Rho treatment significantly up-regulated the expression levels of Creb1 protein in the brain of Sco-treated zebrafish, especially at the concentrations of 3 µg/L ($p < 0.01$) and 5 µg/L ($p < 0.001$; Figure 5.29 D). In contrast, the treatment with Baic, proved

to be more effective in the concentration of 1 μg/L ($p < 0.01$) and 3 μg/L ($p < 0.01$), where it managed to regulate in a way significantly the Creb1 protein level, while in the concentration of Baic 5 μg/L, it proved to have a more modest effect ($p < 0.05$; Figure 5.29 E). Regarding Fab treatment, it was observed that this flavonoid was able to regulate Creb1 protein activity in all three concentrations tested ($p < 0.01$, for concentrations of 1, 3 and 5 μg/L; Figure 5.29 F).

V.13 Pearson correlations between behavioral, biochemical and molecular parameters

The Pearson correlation coefficient (r) was calculated to assess the statistical correlation between behavioral and biochemical parameters in zebrafish, including exploration latency, time spent in different areas of the aquarium, performance in memory tests, and levels of AChE and MDA (Figure 5.30). Data are expressed as follows: Latency period (s), time spent in the upper/lower zone of the aquarium (s), time spent in the novel arm (% of total time), percentage of spontaneous alternation (%), exploration time a ON (s), preference (%), AChE (nmol ATCh/min/mg protein), MDA (nmol/mg protein). The latency period (Figure 5.30 A) showed a significant positive correlation with AChE ($r = 0.8752$), in contrast, the ratio of the time spent in the upper zone to the time spent in the lower zone of the aquarium showed a negative correlation, significant from statistical point of view with MDA ($r = - 0.6424$; Figure 5.30 B). At the same time, a significant negative correlation was identified between the time spent in the new arm and AChE ($r = - 0.5263$; Figure 5.30 C), as well as between the percentage of spontaneous alternation and MDA ($r = - 0.5759$; Figure 5.30 D). A significant negative correlation was also observed between ON exploration time with AChE ($r = - 0.4362$; Figure 5.30 E) and between exploration preference and MDA ($r = - 0.6298$; Figure 5.29F).

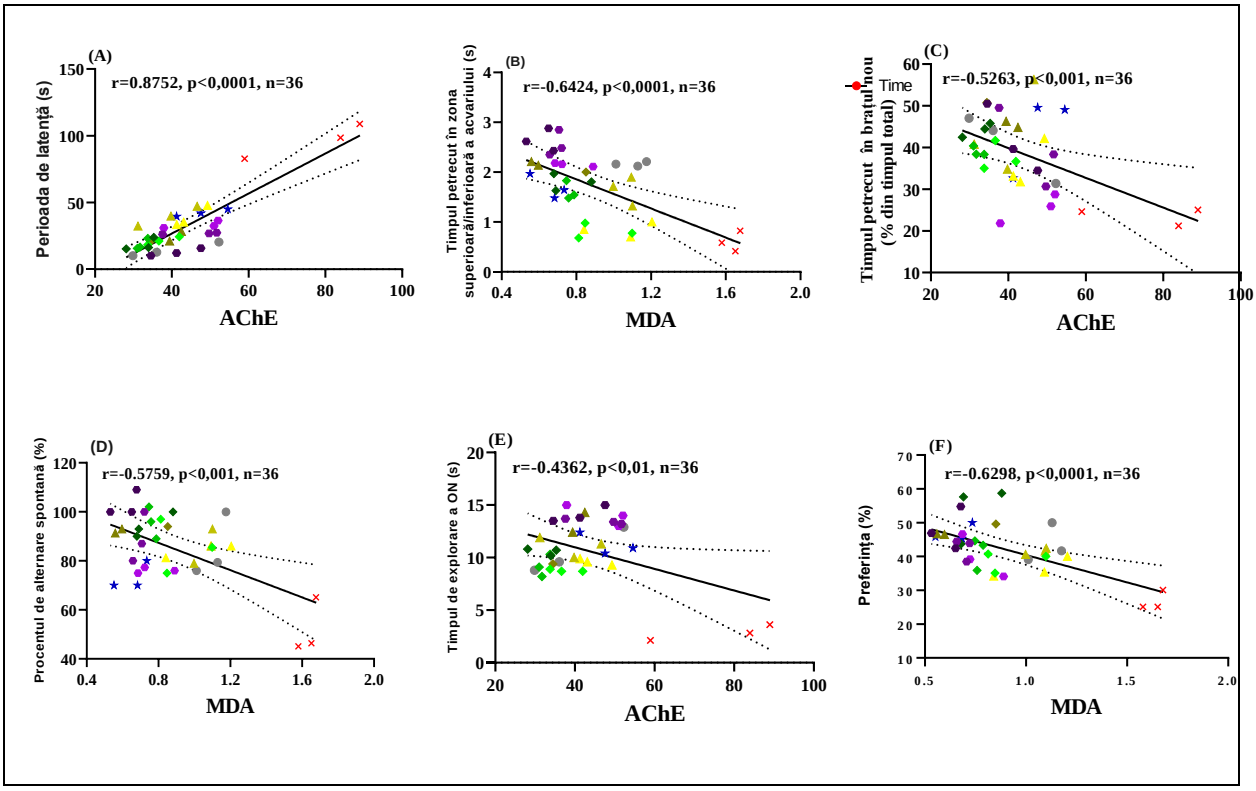


Figure 5.30 Pearson correlation coefficient between behavioral and biochemical parameters.Values are expressed as means ± SEM, (n=3 animals per group). Linear regression analysis indicated a significant positive correlation between:

(A) latency period and AChE level($r = 0.8752$, $p < 0.0001$); **(B)** the ratio of time spent in the upper zone to time spent in the lower zone of the aquarium and MDA ($r = - 0.6424$, $p < 0.0001$);**(C)**time spent in the new arm and AChE level ($r = - 0.5263$, $p < 0.001$); **(D)** the percentage of spontaneous alternation and the level of AChE ($r = - 0.5759$, $p < 0.001$); **(E)** ON and MDA exploration time ($r = - 0.4362$, $p < 0.01$);
(F) exploration preference and level of MDA($r = - 0.6298$, $p < 0.0001$).

- Sq, Sco (100µM), GAL (1mg/mL), Rho 1 (µg/L), Rho (3µg/L), Rho (5µg/L), Bike (1µg/L), Bike (3µg/L), Bike (5µg/L), Fab (1µg/L), Fab (3µg/L), Fab (5µg/L);
-

We also performed correlation analyzes between enzyme activities, lipid peroxidation and gene expression, including SOD, CAT, GPX, AChE activity, GSH level, carbonylated proteins and gene expression of *bdnf, nrf2* and *creb1* genes (Figure 5.31).

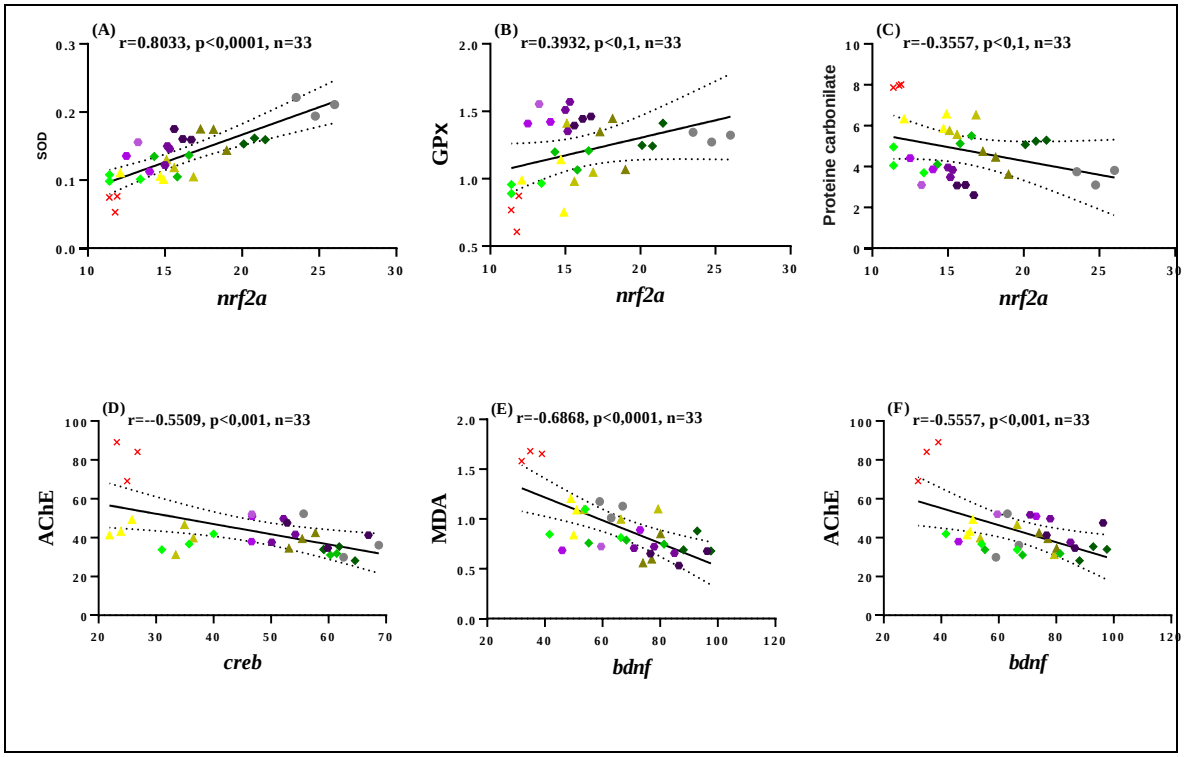


Figure 5.31 Pearson correlation coefficient (r) between enzyme activities, lipid peroxidation and absolute expression of genes of interest.Values are expressed as means ± SEM, (n = 3 animals per group). Linear regression analysis indicated a significant positive correlation between:
(A)SOD activity andnrf2 gene mRNA copy number (r = 0.8033, p < 0.0001);**(B)**GPx activity andnrf2 gene mRNA copy number (r = 0.3932, p < 0.05);(**C**)the level of carbonylated proteins andnrf2 gene mRNA copy number (r = - 0.3557, p < 0.05);(**D**)AChE activity andcreb1 gene mRNA copy number (r = - 0.5509, p < 0.001); **(E**)the level of MDA andbdnf gene mRNA copy number (r = - 0.686, p < 0.0001);(**F**)AChE activity andbdnf gene mRNA copy number (r = - 0.557, p < 0.001).

Sq, Sco (100µM), Rho 1 (µg/L), Rho (3µg/L), Rho (5µg/L), Bike (1µg/L), Bike (3µg/L), Bike (5µg/L), Fab (1µg/L), Fab (3µg/L), Fab (5µg/L).

Data are expressed as follows: SOD (U/mg protein), GPX (U/mg protein), carbonylated proteins (nmol/min/mg protein), AChE (nmol ATCh/min/mg protein), MDA (nmol/mg protein), bdnf (mRNA copy number, ÷ 10,000), nrf2 (mRNA copy number, ÷ 10,000), creb1 (mRNA copy number, ÷ 10,000).

SOD activity (Figure 5.33 A) and GPx activity (Figure 5.33 B) showed a significant positive correlation with *nrf2* gene mRNA copy number with r of 0.8033 (Figure 5.31 A) and with r of 0.3932 (Figure 5.31 B), in contrast to the level of carbonylated proteins which showed a significant negative correlation with *nrf2* gene mRNA copy number, with r of - 0.3557 (Figure 5.31 C). A significant negative correlation was also observed between the level of MDA and AChE activity (Figure 5.31 F) with the number of mRNA copies of the bdnf gene, with r of - 0.6868 (Figure 5.31 E) and r of - 0.5557 (Figure 5.31 F). Similarly, a

significant negative correlation was also identified between AChE activity and creb1 gene mRNA copy number with r of -0.5509 (Figure 5.31 D).

Taken together, these results revealed, for the first time, that Rho, Baic and Fab exert cognitive-enhancing effects in Sco-induced amnesia and suggested that they might be potential candidate compounds for regulating neuropsychiatric symptoms, maintaining and/or improving processes cognitive as well as in the treatment of neurodegenerative diseases such as AD.

CONCLUSIONS

We conducted this study to evaluate the neuropharmacological effects of Rho, Biac and Fab, onneurocognitive performance, anxiety-like state, oxidative and cholinergic status, mRNA expression of brain-derived neurotrophic factor (*bdnf*), neuropeptide Y (*npy*), early growth response protein 1 (*egr-1*), of nuclear factor erythroid factor 2-related 2 (*nrf2*) and cAMP response element-binding protein (*creb1*), Creb1 protein level, as well as possible memory improvement in immersion-induced AD zebrafish model in Sco. Considering the results of this study, we conclude that:

1) Baic complies with all five pharmacokinetic filters, suggesting it is a promising candidate for drug development. In contrast, Rho and Fab violate these rules, suggesting moderate bioavailability. Baic shows PAINS and Brenk alerts, indicating a potentially problematic pharmacological structure, which Rho and Fab do not. All three compounds have low oral solubility, with varying BBB permeability and different plasma distribution profiles.
2) Chronic administration of Rho, Baic, and Fab treatments in the zebrafish model of AD resulted in significant improvements in locomotor activity, anxiolytic behavior, and cognitive processes, demonstrating the potential of these flavonoids in restoring memory and impaired cognitive functions.
3) Rho, Baic and Fab showed neuroprotective effects by increasing the expression of genes involved in cognitive processes and antioxidant defense, such as *bdnf, npy, egr-1, nrf2* and *creb1*, ameliorated oxidative stress and mitochondrial dysfunction. It also demonstrated anti-acetylcholinesterase effects and protected brain proteins and lipids from oxidative degradation, thus suggesting their neuroprotective potential
4) Although the studied flavonoids show a promising neuroprotective profile and can restore memory degradation in AD animal models, there are still limitations and discrepancies in their bioavailability between zebrafish and mammalian models. Further research is needed to better understand the applicability of these compounds in ameliorating the memory degradation associated with AD and to optimize their pharmacological profile for their possible clinical use.

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SCIENTIFIC ACTIVITY

ISI articles published in full from the doctoral thesis

1. Brinza I., Abd-Alkhlek AM, El-Raey MA, Boiangiu RS, Eldahshan, OA and Hritcu L., 2020, Ameliorative effects of rhoifolin in scopolamine-induced amnesic zebrafish (Danio rerio) model. Antioxidants, 9(7): 580.https://doi.org/10.3390/antiox9070580(IF 6.313, AIS 0.910, Q1)

2. Brinza I., Ayoub IM, Eldahshan OA, Hritcu, L., 2021, Baicalein 5,6-dimethyl ether prevents memory deficits in the scopolamine zebrafish model by regulating cholinergic and antioxidant systems. Plants, 10(6), 1245.https://doi.org/10.3390/PLANTS10061245(IF 4.658, AIS 0.654, Q1)

Other published ISI articles

1. Boiangiu RS; Brinza I.; Hancianu M.; Erdogan Orhan I.; Eren G.; Gündüz E.; Ertas H.; Hritcu L.; Cioanca, O., 2020, Cognitive facilitation and antioxidant effects of an essential oil mix on scopolamine-induced amnesia in rats: molecular modeling of in vitro and in vivo approaches. Molecules 25 (7), 1519.https://doi.org/10.3390/molecules25071519(IF 4.412, AIS 0.694, Q2)

2. Brinza I.; Boiangiu RS; Hancianu M.; Cioanca O.; Erdogan Orhan I.; Hritcu L., 2021, Bay leaf (Laurus Nobilis L.) incense improved scopolamine-induced amnesic rats by restoring cholinergic dysfunction and brain antioxidant status. Antioxidants, 10(2), 259.https://doi.org/10.3390/antiox10020259(IF 7.675, AIS 0.921, Q1)

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5. Pecio Ł.; Kozachok S.; Brinza I.; Boiangiu RS; Hritcu L.; Mircea C.; Bachelor AF; Cioanca O.; Hancianu M.; Wronikowska-Denysiuk O.; et al., 2022, Neuroprotective effect of Yucca schidigera Roezl ex Ortgies bark phenolic fractions, yuccaol B and gloriosaol A on scopolamine-induced memory deficits in zebrafish. Molecules, 27(12), 3692.https://doi.org/10.3390/molecules27123692. (IF 4.6 AIS 0.660, Q2)

6. Brinza I.; Boiangiu RS; Cioanca O.; Hancianu M.; Dumitru G.; Hritcu L.; Birsan G.-C.; Todirascu-Ciornea E., 2023, Direct evidence for using Coriandrum sativum var. microcarpum essential oil to ameliorate scopolamine-induced memory impairment and brain oxidative stress in the zebrafish model. Antioxidants, 12(8), 1534.https://doi.org/10.3390/antiox12081534. (IF 7 AIS 0.946, Q1)

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Participation in scientific events

a)Nation

1. Brînza Ion, Hritcu Lucian - Evaluation of the effects of roifolin, baicalin and agatisflavone on cognitive performance and oxidative status, in the zebrafish model of Alzheimer's disease, SSBF, May, Iași, Romania 2021;

2. Brînza Ion, Hrițcu Lucian, Omayama Eldahshan - Sweroside can prevent scopolamine-induced memory impairment and brain oxidative stress in zebrafish (Danio rerio) SSFB, October 28-29, Iași, Romania 2021;

3. Brînza Ion, Hrițcu Lucian, Omayama Eldahshan, Impact of rhoifolin, baicalin and agathisflavone on scopolamine-induced memory decline and brain oxidative stress in a zebrafish model (Danio rerio) "Student Scientific Communications Session, XIXth Edition, 25 - November 26, Arad, Romania, online", 2021;

4. Brînza Ion, Stache A. Bogdan, Omayma Eldahshan, Gorgan D. Lucian, Mihășan Marius, Hrițcu Lucian - Sweroside supports memory function in the scopolamine-induced zebrafish model (Danio rerio) of Alzheimer disease, RSBMB, 21st-23rd, October Cluj-Napoca, Romania, 2022;

5. Bîrsan Gheorghe-Ciprian, Dumitru Gabriela, Boiangiu Răzvan Ștefan, Brînza Ion, Hrițcu Lucian - Coriandrum sativum linn. Attenuated scopolamine – induced anxiety and oxidative stress in the amnestic zebrafish model "Student Scientific Communications Session, XXI Edition, May 29-31, Arad, Romania, 2023.

b) international

1. Brînza Ion, Razvan Stefan Boiangiu, Omayma Eldahshan, Lucian Hritcu, Abdel Nasser Singab - Impact of baicalin and rhoifolin on scopolamine-induced memory decline and brain oxidative stress in a zebrafish model (Danio rerio). The 12th FENS, Forum of Neuroscience, abstract submission system, Galsgow, UK, July 11-15, 2020;

2. Brînza Ion, Răzvan Stefan Boiangiu, Omayma Eldahshan, Lucian Hritcu, Abdel Nasser Singab - Baicalin and Rhoifolin prevents scopolamine-induced memory impairment and brain oxidative stress in zebrafish (Danio rerio), FEBS OPEN BIO, 4-9 July 2021;

3. Brînza Ion, Hrițcu Lucian, Omayama Eldahshan - Evaluation of the effects of sweroside on cognitive performance and oxidative status in the zebrafish (Danio rerio) model of Alzheimer's disease, FENS Regional Meeting, Kraków, Poland, August 25-27, 2021;

4. Brînza Ion, Stache A. Bogdan, Omayma Eldahshan, Gorgan D. Lucian, Mihășan Marius, Hrițcu Lucian - Sweroside supports memory function by increasing mRNA expression of bdnf, creb, npy and decreasing AChE activity and brain oxidative stress in the scopolamine ¬ induced zebrafish model (Danio rerio), YSF congress, Vimeiro, Portugal, 6-49 July 2022;

5. Brînza Ion, Stache A. Bogdan, Omayma Eldahshan, Gorgan D. Lucian, Mihășan Marius, Hrițcu Lucian - Sweroside supports memory function by increasing mRNA expression of bdnf, creb, npy and decreasing AChE activity and brain oxidative stress in the scopolamine ¬ induced zebrafish model (Danio rerio), IUBMB-FEBS-PABMB congress, Lisbon, Portugal, July 9-14, 2022;

6. Brînza Ion, Hritcu Lucian – The impact of agatisflavone on memory processes. Studies on an animal model of dementia;- Life sciences in the dialogue of generations: Connections between Universities, Academia and Business community and International Scientific Symposium Advanced Biotechnologies – Achievements and Prospects (Vth Edition), Chisinau, Republic of Moldova, October 21-22, 2019 ;

7. Brînza Ion, Hritcu Lucian - Quantification of neurotrophin expression in the hippocampus of an oil-treated dementia model volatile, Ith International Congress of Geneticists and Breeders from the Republic of Moldova, June 2021.