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PHARMACOLOGICAL STUDY OF BEHAVIORAL EFFECTS OF BIOLOGICLLY ACTIVE SUBSTANCES OF PLANT ORIGIN IN EXPERIMENTAL ANIMAL MODELS OF DEPRESSION

EXTENDED ABSTRACT OF A PHD THESIS

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I. INTRODUCTION

Neurodegenerative diseases and the often accompanying affective disorders are a significant medico-social and financial burden for societies worldwide.

The World Health Organization reports that in 2018, people affected by affective disorders worldwide are over 264 million (GBD, Disease and Injury Incidence and Prevalence Collaborators, 2018). More recent analyzes (COVID-19 Mental Disorders Collaborators; The Lancet, 2021) show that the COVID-19 pandemic led in 2020 to a sharp increase in the total number of depressive and anxiety disorders worldwide. The growth of anxiety disorders increased by an additional 53.2 million cases, and the number of people diagnosed with major depressive disorder (MDD) increased by an additional 76.2 million (COVID-19 Mental Disorders Collaborators; The Lancet, 2021).

Depression is an affective disorder with great health, economic and social significance (World Health Organization, 2020). According to the WHO, depression is the second most common cause of suicide among young people between the ages of 15 and 29 (Bernaras et al., 2017). Major depressive disorder is a highly debilitating condition expected to become the second most common illness after chronic diseases such as coronary heart disease and diabetes mellitus (WHO, 2020).

Despite the widespread use of antidepressants, up to 30% of patients do not respond to them (Guan et al., 2016) or show a partial response, coupled with functional impairment, poor quality of life, suicide attempts, self-injurious behavior and a high rate of relapse (Al-Harbi, 2012). Furthermore, depression is often associated with chronic illness (Katon, 2011) or other mood disorders (Klein, 1993) due to the inadequacy of conventional treatment (Birmaher et al., 1998). Most antidepressants require administration for several weeks to achieve a clinical effect, but their use is associated with numerous adverse drug reactions (Santarsieri et al., 2015).

On the other hand, a number of neurodegenerative diseases are often accompanied by affective problems that worsen their course. They can worsen patients' social and functional status (Kessler et Greenberg, 2002). Zhao et al (2016) reported that 40% of Alzheimer's disease cases have comorbid clinical depression. Meanwhile, a significant percentage of patients diagnosed with Parkinson's disease have a high frequency of comorbid anxiety disorders, eating disorders (Kessler and Greenberg, 2002) and persistent/chronic pain symptoms (Lee et al, 2012).

Therefore, in recent years, the need to conduct new studies with therapeutic sources has increased in order to optimize the complex prevention and treatment of affective and anxiety disorders, as well as of various neurodegenerative diseases. The discoveries about the passage of some plant polyphenols through the blood-brain barrier became a prerequisite for the study of their neuroprotective and neuromodulatory function, for the study of their effects in anxiety, depression and neurodegenerative diseases.

A number of biologically active substances of plant origin, among which are polyphenolic compounds, show favorable psychopharmacological effects. It is believed that polyphenols can be involved in the modulation of various signaling pathways, and thus affect the fate of cells (Ďuračkova Z, 2010), including the survival, regeneration, development or death of neuronal cells (Spencer, 2005). Polyphenols and their metabolites act through modulation of various cellular functions that play an important role in neuroprotection and are able to neutralize the effects of oxidative stress, inflammation and apoptosis (Giuliano et al., 2021), as well as show beneficial vascular effects and stimulate the proliferation of nerve cells (Di Meo et al., 2020).

A review of the international database shows that different classes of polyphenolic compounds from the fruit and fruit juice of *Aronia melanocarpa* exhibit neurobiological effects that have been studied in healthy experimental animals as well as in various experimental models of depression/anxiety/amnesia/hypokinesia (Stefanello et al., 2014; Mansouri et al., 2014; Lee et al., 2016; Sandeep et al., 2018). Thanks to the research work of a team of authors from the Department of Pharmacology and Clinical Pharmacology and Therapeutics at the Medical University of Varna, anxiolytic, antidepressant-like and memory-improving effects of *Aronia melanocarpa* fruit juice have been established in young healthy rats (Valcheva-Kuzmanova et al., 2013; Eftimov et al., 2014; Valcheva-Kuzmanova et al., 2014; Valcheva-Kuzmanova et al., 2016), as well as the phenolic acids contained in it (chlorogenic, ferulic and gallic) (Georgieva et al., 2015; Georgieva et al., 2016).

There are insufficient data on the effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on the behavior of ovariectomized rats. There are no data on the effects of phenolic acids - chlorogenic, ferulic and gallic acids on the behavior of rats with bilateral olfactory bulbectomy. Based on the known biologically active substances in *Aronia melanocarpa*, it can be expected that the fruit juice and the rather high concentrations of phenolic acids (chlorogenic,

ferulic and gallic acids) contained in it will show beneficial effects on the behavior of experimental animals in model conditions of surgically induced depression and behavioral disturbances.

The obtained results would expand our knowledge about the pharmacological effects of *Aronia melanocarpa* fruit juice and the phenolic acids contained in it - chlorogenic, ferulic and gallic acids and would contribute to their potential future use with curative and/or prophylactic purposes in diseases that are a challenge to modern medicine, such as anxiety disorders, depressions and dementias.

II. OBJECTIVE AND TASKS

The **objective** of this dissertation is:

Pharmacological study of effects of biologically active substances of plant origin *Aronia melanocarpa* fruit juice and the phenolic acids - chlorogenic, ferulic and gallic in experimental models of behavioral disorders in rats induced by ovariectomy and olfactory bulbectomy.

In order to achieve this objective, we set ourselves the following **TASKS:**

1. To induce an experimental model of depression and other behavioral changes in rats by bilateral ovariectomy and to examine behavioral indices providing information about:
    - 1.1. The locomotor activity;
    - 1.2. The anxiety;
    - 1.3. Depressive-like behaviour;
    - 1.4. Pain sensitivity;
2. In ovariectomized rats, to study the effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on:
    - 2.1. The locomotor activity;
    - 2.2. The anxiety;
    - 2.3. Depressive-like behaviour;
    - 2.4. Pain sensitivity;
3. To induce an experimental model of bilateral olfactory bulbectomy in rats and to study the behavioral indices providing information about:
    - 3.1. Learning and memory;
    - 3.2. The anxiety;
4. In bulbectomized rats, to study the effects of the phenolic acids - chlorogenic, ferulic and gallic on:

4.1 Learning and memory;

4.2 The anxiety.

III. MATERIALS AND METHODS

1. MATERIALS

1.1. Experımental anımals

For the experiment 90 male and 70 female white rats, Wistar breed (200–240 g), were used; they were bred in plastic cages at an average room temperature of 22±2ºC, exposed to 12-hour light /dark cycle, with unlimited access to food and water. All procedures for the treatment of the animals and experiments were conducted in accordance with EU Directive 2010/63/EU and approved by the Bulgarian Food Safety Agency (Document №177/07.07.2017) and in accordance with the rules of the ethics committee at the Institute of Neurobiology, Bulgarian Academy of Sciences (registration FWA 00003059 by the US Department of Health and Human Resources).

1.2. Experimental substances

1.2.1. *Aronia melanocarpa* fruit juice (AMFJ)

For the purposes of the experimental study, *Aronia melanocarpa* fruit juice was used in two different doses. *Aronia melanocarpa* fruit juice was prepared from fresh fruits by grinding, crushing, squeezing and filtering. The resulting juice was filtered and preserved with potassium sorbate (1.0g/l) and stored at 0°C until use.

The composition of polyphenolic components in *Aronia melanocarpa* fruit juice was determined by the team of Valcheva-Kuzmananova (2014). Results revealed a very high content of phenolic substances (5461 mg /gallic acid equivalents per liter of juice). The group of phenolic compounds with the highest concentration in chokeberry berries are procyanidins, followed by anthocyanins and phenolic acids: chlorogenic (3-O-caffeolylquinic acid) and neochlorogenic (5-O-caffeolylquinic acid). In addition, flavonols (quercetin glycosides) (Slimestad et al., 2005) and flavan-3-ols (epicatechin) (Rop et al., 2010) are present in lower concentration.

1.2.2. Other substances used

Chlorogenic acid, ferulic acid and gallic acid from Sigma-Aldrich (Germany); ketamine (Gedeon Richter, Germany); xylazine (Bioveta, Czech Republic) were used.

2. MODELS

2.1. Experimental model of bilateral ovariectomy (OV) in rats

The bilateral ovariectomy (OBX) model induces estrogen deficiency in animals, eliminating the role of endogenous ovarian steroids in the control of normal brain plasticity. Subsequently, behavioral changes largely similar to postmenopausal symptoms in women are observed (Li et al., 2014).

Surgical procedures were performed according to the method described by Lasota et al. (2004). Experimental animals (aged 4 months) were anesthetized intraperitoneally with Ketamine 30 mg/kg and Xylazine 30 mg/kg and then fixed. Abdominal hair was removed and skin was disinfected using iodine. Ovaries were isolated, fallopian tubes were clamped and a thread was tightly tied around the oviduct including blood vessels. After closing the abdominal wall, a 5-day antibacterial prophylactic dose of cefazolin (200 mg/kg) was administered intraperitoneally.. The rats underwent a two-week postoperative recovery period.

2.2.Experimental model of bilateral olfactory bulbectomy (OB) in rats

In recent years, bilateral olfactory bulbectomy in rats has been accepted as the model that could be extrapolated to major depressive disorder in humans, also leading to cognitive impairment.

Bilateral olfactory bulbectomy is based on the method described by Kelly et al (1997). Experimental animals were anesthetized with Calypsol (50 mg/kg, i.p.), then fixed in a stereotaxic apparatus (Stoelting Co, USA). The soft tissues on the head and periosteum were removed, and a puncture of the skull bones was drilled, left and right to the mean line. The coordinates of olfactory bulbs were determined by the Atlas of Pellegrino and Cushman (1967) for rats Wistar breed. The bulbectomy itself was performed by aspiration of bulbi olfactorii with a stainless steel needle, attached to a water pump. A hemostatic agent (Gelaspon) was placed in the holes after aspiration. The animals were provided with a 15-day recovery period after the end of the surgical intervention, during which they were treated daily with antibiotics - locally (with Nemybacin for 7 days) and intraperitoneally (with Gentamycin for 5 days). In order to adapt to the conditions of the experiment during the recovery period, the animals were handled (handled) daily for ten minutes for 5 days. Macroscopic verification of the bilateral bulbectomy was performed after completion of the behavioral experiments. When incomplete (< 80%) destruction

of the bulbs was found, the data obtained from the behavioral tests were excluded from the processing of the results.

3. BEHAVIORAL METHODS

3.1. Method for examination of locomotor activity – open field test (OFT)

The open field test is a well-established method for determining locomotor activity and exploratory behavior in rodents in an unfamiliar environment. The test was performed in a walled field (100 x 100 x 40 cm), painted all white and marked with 6 mm thick blue lines dividing the floor into equal (20 x 20 cm) squares. The animal was placed in the center of the field and its behavior was examined within 5 min. The number of squares crossed (horizontal movements) and the number of rearing (vertical movements) were recorded as measures of locomotor activity. by the animal with all four paws and the straightening of the hind paws.

3.2. Methods to evaluate learning and memory

3.2.1. Two-way active avoidance test – shuttle box

The test was conducted in a Shuttle box apparatus according to the method of Buserova and Bures (1983), modified by Petkov et al. (1993). The apparatus had two identical sectors, connected by a centrally located round opening for the passage of the rat from one sector to the other. The floor of the chamber was a swinging metal grid connected to the electrical system of the apparatus. The unconditioned stimulus was an alternating electric current (0.5 mA, 50 Hz). An artificial light (21 W light bulb) was used as a conditioned stimulus, which was switched on in the free sector of the apparatus, while the animal remained in the dark sector. The conditioned stimulus (light) preceded the application of the unconditioned stimulus (electric current) for 9 seconds. Two training sessions were held over 2 consecutive days. Each session consisted of 50 training sessions per rat. The test for retention (memory) was performed on the 24th hour, after the second day of training. The light stimulus was applied for 9 seconds, followed by the electric current just for 2 seconds (like "reminder"). The number of the avoidances for each training session (1-st, 2-nd day) and the memory test were registered as an index of learning and memory.

3.2.2. One-way passive avoidance test – step through

The passive avoidance test was conducted after the method of Buresova and Bures (1963). The apparatus had two chambers: one enlightened and a dark closed one. The learning included a single training session. The animal was set on the platform in the enlighted chamber

with an open door. After the animal entered the dark chamber, the door was closed and the electric current was switched on the floor net. The memory test was performed on the 3rd and 24th hour after the learning session when each animal was again set in the enlighted chamber with an open door; the time of the rat, staying there was registered in seconds. As a criterion of achieved learning, the stay of the animal in the enlightened chamber should be not less than 180 seconds.

3.3. Methods for assessment of anxiety

3.3.1. Elevated plus maze test (EPM)

The elevated plus maze test was introduced by Pellow and File (1986). The test was carried out in an apparatus with two open and two closed arms elevated at 50 cm above the floor. Rat behaviour was observed for 5 min. The rat was put in the center of the maze and the number of open and closed arms and the total arm entries was recorded, as well as time spent in the open and in the closed arms. The index of open- vs. total arm entries was calculated as well. This behavioural test gives information about the level of anxiety in rodents because it is their natural behaviour to seek safety by preferring the closed arms of the maze. Anxiety levels are inversely proportional to the number of open-arm entries and the time spent there

3.3.2. Social interaction test (SIT)

The test was conducted according to the method of File and Hyde (1978). Social interaction test was performed in the same square arena used for open field test. Two unfamiliar rats with similar weights were released in the opposite corners of the box. Their behaviour was recorded for 5 min and the time spent in social interaction was measured. Sniffing, following, wrestling, crawling under or over the other rat were considered an interaction, while passive contact such as lying or sitting over, under or next to the other animal were not. This test is used to assess anxiety in rodents, as levels of anxiety are inversely proportional to the time spent in interaction.

3.4. Method for examination of depressive behavior – forced swim test (FST)

The forced swim test (FST), called also Porsolt test, is widely used to assess behavioral despair in rodents. It was performed in a glass cylinder filled with water. The rodent was put inside it for 5 min and was thus forced to swim. There was a training session and on the next day immobility time was measured as a marker of depressive behaviour. After swimming, the animals were wiped and dried before they returned to their home cages.

3.5. Method for studying pain sensitivity – hot plate test (HPT)

Hot plate test is widely used to encounter effects on thermal nociception in rodents. The test was performed according to the method described by Eddy and Leimbach (1953). HPT was carried out on a heated (52°C) surface enclosed by a glass cylinder with a diameter of 24cm (Ugo Basile S. R. L., Italy). Time latency before shaking or licking paw, or before jumping was measured and taken as an index for nociceptive pain sensitivity. Animals were removed from the plate immediately after responding or after a cut-off time (45 sec) to prevent tissue damage. Three consecutive measures at an interval of 2 hours were performed and the mean value was calculated for each animal

4. STATISTICAL METHODS

The processing and analysis of the data were performed with the statistical software GraphPad Prism (Version 5.00, GraphPad Software), using one-way variation analysis (one-way ANOVA) and unpaired two-tailed Student's t-test. ANOVA analyses were followed by Dunnett's multiple comparisons post-test. Results are presented as mean ± standard error (mean ± SEM). Statistical reliability was assumed at a value of $p < 0.05$.

IV. RESULTS AND DISCUSSION

1. Effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on the behavior of rats with bilateral ovariectomy

The present studies were conducted on 70 female Wistar rats, divided into five groups, each of 14 animals, marked as: Control (SO), ovariectomized (OVX), OVX+AMFJ5, OV+AMFJ10 and OVX+CGA. Rats from OVX, OVX+AMFJ5, OV+AMFJ10 and OVX+CGA were ovariectomized, control animals were sham operated. After a 14-day recovery from the operation, animals were treated once daily by rigid orogastric gavage. Control (SO) and OVX received distilled water at a dose of 10 ml/kg. AMFJ was administered in a dose of 5 ml/kg, diluted with distilled water to 10 ml/kg (for OVX+ AMFJ5 group) and 10 ml/kg (for OVX+AMFJ10 group), and chlorogenic acid (for OVX+CGA group) in a dose of 20 mg/kg in the form of a solution in a volume of 10 ml/kg.

Behavioral tests were performed after the 30^{th} and 75^{th} day from the start of treatment.

The tests after day 30 were conducted in the following sequence:

- Day 30: an open field test was performed 60 minutes after the last treatment, between 8 a.m. and 4 p.m.;
- Day 31: a social interaction test was conducted 60 minutes after the last treatment, between 8 a.m. and 4 p.m.;
- Day 32: there was a forced swim test training session was conducted 60 minutes after the last treatment between 8 a.m. and 4 p.m.;
- Day 33: a forced swim test was performed 60 minutes after the last treatment, between 8 a.m. and 4 p.m.

The sequence of tests after 75 days of treatment was as follows:

- Day 75: an open field test was performed 60 minutes after the last treatment, between 8 a.m. and 4 p.m.;
- Day 76: a social interaction test was conducted 60 minutes after the last treatment, between 8 a.m. and 4 p.m.;
- Day 77: there was a forced swim test training session 60 minutes after the last treatment, between 8 a.m. and 4 p.m.;
- Day 78: a forced swim test was performed 60 minutes after the last treatment, between 8 a.m. and 4 p.m.;
- Day 79: a hot plate test was performed 60 minutes after the last treatment, between 8 a.m. and 4 p.m.

1.1. Effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on locomotor activity in open field test

1.1.1. Effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on motor activity after 30 days of treatment

After statistical analysis of the results they showed that the horizontal and vertical activity of OVX rats was decreased, but not significantly compared to those of the Control (SO) group. Horizontal activity, reduced by OVX, is further reduced by AMFJ. In the animals of the OVX+AMFJ5 group, the horizontal activity was significantly reduced compared to the Control (SO) ($p<0.001$) and the OVX group ($p<0.01$). In the animals of the OVX+ AMFJ10 group, the horizontal activity was significantly lower than that of the Control (SO) ($p<0.01$) and the OVX

group ($p<0.05$). The horizontal activity of the OVX+CGA group was significantly lower than that of the Control (SO) ($p<0.05$) and did not differ from the OVX group.

The vertical activity of OVX rats was reduced but not significantly compared to those of the Control (SO) group. Vertical activity, reduced in OVX, was further reduced by AMFJ at a dose of 10 ml/kg, being significantly different from the Control (SO) ($p<0.05$), but not reaching statistical significance compared to that of the OVX group. In the animals of the OVX+AMFJ5 group, the vertical activity did not differ significantly from the Control and the OVX group. The vertical activity of the OVX+CGA group was not significantly different from that of the Control (SO) and OVX animals. The results show that after 30 days of administration, *Aronia melanocarpa* fruit juice in doses of 5 ml/kg and 10 ml/kg significantly reduced the locomotor activity of the ovariectomized animals, possibly due to a sedative effect. Chlorogenic acid at a dose of 20 mg/kg after 30 days of administration suppressed the locomotor activity of ovariectomized rats to the extent that it was significantly lower than that of sham-operated animals.

1.1.2. Effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on motor activity after 75 days of treatment

The results recorded during the test show that the horizontal and vertical activity of OVX rats was decreased, but not significantly compared to those of the Control (SO) group.

Horizontal activity, reduced in OVX, is further reduced by AMFJ. In the animals of the OVX+ AMFJ5 group, the horizontal activity was significantly reduced compared to the Control (FO) ($p<0.01$) and the OV group ($p<0.01$). The horizontal activity of OVX+ AMFJ10 group was significantly lower than that of Control (SO) ($p<0.01$) and OVX group ($p<0.01$). Chlorogenic acid significantly reduced the horizontal activity of animals from the OVX+CGA group compared to the Control (SO) ($p<0.05$) and was not significantly different from the OVX group.

The vertical activity of OVX rats was reduced but not significantly compared to those of the Control (SO) group. Vertical activity, reduced in OVX, was further reduced by AMFJ at a dose of 10 ml/kg, being significantly different from the Control (SO) ($p<0.01$) and from the OVX group ($p<0.01$). In the animals of the OVX+ AMFJ5 group, the vertical activity did not differ significantly from the Control and the OVX group. The vertical activity of the OVX+CGA group was significantly lower than that of the Control (SO) ($p<0.05$) and was not significantly different from that of the OVX. The conducted research shows that after 75 days of administration, *Aronia*

melanocarpa fruit juice in doses of 5 ml/kg and 10 ml/kg significantly reduces the motor activity of the ovariectomized, probably due to a sedative effect. Chlorogenic acid at a dose of 20 mg/kg after 75 days of administration suppressed the locomotor activity of ovariectomized animals to the extent that it was significantly lower than that of sham-operated animals.

1.2.3. Discussion

Sex hormones exert a wide range of effects in the human body, and their role is not limited to regulating reproductive behavior. The participation of estrogen hormones in the processes of sexual differentiation, emotions, memory, neuronal survival, as well as in the perception of somatosensory stimuli is key (Amandusson and Blomqvist, 2013; Frizell and Dumas, 2018). The bilateral ovariectomy (OBX) model induces estrogen deficiency in animals, eliminating the role of endogenous ovarian steroids in the control of normal brain plasticity. Subsequently, behavioral changes are observed, largely similar to postmenopausal symptoms in women (Diaz Brinton, 2012; Li et al., 2014).

The present study aimed to test the effects of Aronia melanocarpa fruit juice and chlorogenic acid on the locomotor activity of ovariectomy-induced estrogen-deficient rats in an open field test.

Open field test is a well-established method for determining locomotor activity and exploratory behavior in rodents in an unfamiliar environment (Gould et al., 2009). The usual behavior of animals is to initially explore the new environment and subsequently habituate to the environment (Bolivar et al., 2000). Exploratory behavior is defined as active exploration (e.g. movement) that may result in the animal obtaining information about its environment (Lynn and Brown, 2009). Activity over a short period of time indicates exploratory behavior (Gould et al., 2009). The number of squares crossed (horizontal activity) and the number of rearing (vertical activity) were recorded as measures of the locomotor activity (Walsh and Cummin, 1976).

The present study showed that *Aronia melanocarpa* fruit juice administered at doses of 5 ml/kg and 10 ml/kg for a period of 30 and 75 days significantly reduced the horizontal locomotor activity of ovariectomized rats. *Aronia melanocarpa* fruit juice in a dose of 10 ml/kg, after 75 days of administration, statistically reliably reduces the number of vertical movements of OVX animals. Chlorogenic acid decreased horizontal activity in ovariectomized animals after 30 and 75 days of treatment and vertical activity after 75 days of administration, but without reaching

statistical significance compared to the OVX group. The research conducted shows that after 30- and 75-day administration, *Aronia melanocarpa* fruit juice reduces the locomotor activity of ovariectomized rats, which is most likely due to a sedative effect. A similar effect was found by Valcheva-Kuzmanova's team (2014) in healthy rats treated with *Aronia melanocarpa* fruit juice at a dose of 10 ml/kg for a period longer than three weeks.

The observed effects of *Aronia melanocarpa* fruit juice are probably the result of the action of the biologically active polyphenolic compounds – flavonoids contained in it mainly from the subclass of anthocyanins, procyanidins and phenolic acids (Valcheva-Kuzmanova, 2014). Decreased spontaneous motor activity (walking and standing) may result from reduced CNS excitability and sedation (Prut & Belzung, 2003). The brain's GABA-ergic system is known to be responsible for sedation (Gottesmann, 2002). Ligands that act on GABA-A receptors possessing α2 and/or α3 subunits can convey an anxiolytic effect, while the sedative-hypnotic effect results from activation of the α1 subunit of GABA-A receptors (Rudolph and Möhler, 2006). The sedative effect of *Aronia melanocarpa* fruit juice observed in the present experiment was also found in previous studies (Eftimov et al., 2013). It is likely that the influence of GABA-A receptors containing the α1 subunit will continue due to the accumulation of polyphenols in the CNS with prolonged consumption of the juice.

A partial allosteric modulatory action on the GABA-A receptor complex has been found for a number of flavonoids (Fernandez et al., 2009). Literature data show that natural extracts containing procyanidins, phenolic acids and other polyphenols possess affinity to bind to GABA-A receptors (Fernandez et al., 2009; Wang et al., 2008) and can induce sedative, anxiolytic and anticonvulsant effect (Jäger and Saaby, 2011; Wasowski and Marder, 2012). According to a study by Tu and colleagues (2012), ferulic acid, which is present in the composition of *Aronia melanocarpa* fruit juice, dose-dependently (± 15mg/kg) suppresses locomotor activity and causes sedation in intact mice. The observed sedative-hypnotic effect of ferulic acid is also confirmed in other studies (Deng et al., 2018) and is most likely due to the effect of the acid on central GABA neurotransmission.

It has been found that flavonoids can have anxiolytic and sedative effects by binding to BDZ-receptors or at sites other than benzodiazepine receptors (de Carvalho et al., 2011). Gallic acid and quercetin demonstrate a sedative effect associated with BDZ-receptor activation (Mansouri et al., 2014). Activation of opioid receptors can also be suggested for quercetin

(Anjaneyulu and Choptra, 2003). According to the present experiment, chlorogenic acid at a dose of 20 mg/kg after 30- and 75-day administration suppressed the locomotor activity of ovariectomized animals to the extent that it was significantly lower than that of sham-operated animals. Literature analysis revealed divergent effects of chlorogenic acid on locomotor activity in behavioral tests in experimental animals. Administered subchronically in healthy rats, chlorogenic acid at a dose of 20 mg/kg suppressed locomotor activity (Georgieva et al., 2017). There are data indicating that the administration of chlorogenic acid at a dose of 2.8 mmol/kg leads to marked locomotor activation in mice 10 to 60 minutes after its administration (Ohnishi et al., 2006). It is possible that the divergent effects are due not only to the dosage, but also to the duration of administration, the experimental models, and the species of animals tested.

1.2. Effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on anxiety in social interaction test

1.2.1. Effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on anxiety after 31 days of treatment

Using a social interaction test, behavioral changes were tracked after 31-day of treatment. Two unfamiliar rats placed in the apparatus at the same time were given five minutes to become familiar. For each animal, the time of movements associated with active interaction between unfamiliar partners was recorded. An increase in social contact time between experimental animals is taken as an indicator of an anxiolytic effect.

The test results showed that after 31 days of treatment, the social interaction time of OVX animals was not significantly different from that of the control (SO) group. OVX does not lead to anxiety after 31-day of application. The social interaction time of OVX+AMFJ5 and OVX+AMFJ10 was significantly shorter compared to that of the control (SO) ($p<0.05$) and OVX groups ($p<0.01$) (Figure 5). The social interaction time of the OVX+CGA group was not significantly different from that of the control (SO) and OVX group animals.

The research conducted showed that in the social interaction test conducted on day 31, when no development of anxious behavior was reported in ovariectomized animals, AMFJ at doses of 5 ml/kg and 10 ml/kg reduced the time of active social contacts. This effect may be due to the decreased total locomotor activity of these animals, which was demonstrated in the open field test.

1.2.2. Effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on anxiety after 76 days of treatment

The test results show that after 76 days of treatment, the social interaction time of the OVX group was shortened significantly compared to the Control (SO) ($p<0.001$). From which we can conclude that OVX induces anxiety-like behavior. The social interaction time of OVX+AMFJ5 did not differ significantly from the Control (SO) group and was prolonged significantly compared to OVX group ($p<0.05$). AMFJ at a dose of 5 ml/kg increases the time of active social contacts, probably due to an anxiolytic-like effect. The social interaction time of OVX+AMFJ10 was shortened significantly from that of the Control (SO) ($p<0.001$) and did not differ from the OVX group. The lack of effect of AMFJ at a dose of 10 ml/kg may be the result of a more pronounced reduction in locomotor activity reported in the open field test. The social interaction time of OVX+CGA was non-significantly prolonged compared to that of OVX group and not different from that of Control (SO), which gives us reason to assume that CGA counteracts OVX-induced anxiety.

1.2.3. Discussion

Interrelated systems of hormones and neurotransmitters, neurotrophic factors and cytokines are major regulatory mechanisms controlling normal brain plasticity. The dysfunction of these systems is the basis for the development of brain pathologies, including mental illnesses. Fluctuations in female sex hormones during the premenstrual period, postpartum, and perimenopause can impact neurochemical pathways controlling anxiety and depression (Stahl, 2001), as well as learning and memory processes (Fedotova et al., 2017; Mc Ewen, 2013). Hormonal changes can increase or decrease the expression of specific estrogen receptor subtypes, correlating with anxiogenic or anxiolytic effects (Borrow & Handa, 2017). Physiologically, anxiety has an adaptive function and is a normal emotional response to a threat or potential threat (Bouayed et al., 2009). Anxiety is classified as a pathological condition when it is persistent and extreme (Croos et al., 2004). Several literature data reveal a relationship between estrogen deficiency and increased anxiety in ovariectomized animals (Ter et al., 2009).

The present study aimed to test the effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on the alertness of ovariectomy-induced hormone-deficient rats in a social interaction test.

The social interaction test is conducted under conditions of bright light, an unfamiliar arena, and an unfamiliar test partner to create a high level of anxiety (File and Hyde, 1978). Anxiolytic-like behavior is observed when social interaction time is increased while gross locomotor activity remains unchanged. The research conducted shows that in the social interaction test conducted on day 31, when no development of anxious behavior was reported in ovariectomized animals, *Aronia melanocarpa* fruit juice in doses of 5 ml/kg and 10 ml/kg reduced the time of active social contacts. This effect may be due to the reduced gross locomotor activity demonstrated in the same animals in the open field test.

Administered for 76 days, *Aronia melanocarpa* fruit juice at a dose of 5 ml/kg prolonged social interaction time between OVX test animals. These data indicate that *Aronia melanocarpa* fruit juice has an anxiolytic-like effect in OVX rats. The lack of effect of *Aronia melanocarpa* fruit juice in a high dose in the OVX+AMFJ10 group may be the result of a more pronounced reduction in locomotor activity registered in the open field test, possibly due to reduced CNS excitability and sedation. Similar reduced locomotor activity was found in intact rats treated with *Aronia melanocarpa* fruit juice at a dose of 10 ml/kg for a period longer than three weeks in an open field test (Valcheva-Kuzmanova et al., 2014). The social interaction time of the OVX group treated for 76 days with chlorogenic acid was prolonged compared to that of the OVX group and was not different from that of the Control (SO). Chlorogenic acid after 76 days of administration prevents OVX-induced anxiety.

The brain's GABA-ergic system is known to be responsible for sedation (Gottesmann, 2002). The sedative effect of *Aronia melanocarpa* fruit juice observed in the present experiment may be due to the influence of GABA-A receptors containing the α1 subunit, as a result of the accumulation of polyphenols in the CNS upon prolonged intake of the juice. Anxiolytic-like effect was observed after 76 days of treatment with *Aronia melanocarpa* fruit juice at a dose of 5 ml/kg. It is possible that with the lower dose of *Aronia melanocarpa* fruit juice and by extending the treatment period, for a combination of action mechanisms to be achieved, including stimulation of GABA-ergic neurotransmission, reduction of oxidative stress and neurotrophic action, etc. The anxiolytic-like effect of *Aronia melanocarpa* fruit juice is probably due to a complex mechanism of action of polyphenols, which are the main biologically active substances in the juice and which have the ability to pass through the blood-brain barrier. These results are consistent with other

studies demonstrating that flavonoids (Vignes et al., 2016), including anthocyanins (Kumar et al., 2008), have anxiolytic effects in animals.

Pathological chronic anxiety and depression are associated with increased oxidative stress in brain tissue. Reactive oxygen species (ROS) – superoxide anions (O2-), hydroxyl radicals (OH), peroxide radicals (RO2), hypochlorous acid (HOCI), peroxynitrite (ONOO−), cause oxidative stress, lipid peroxidation, protein oxidation and possible DNA damage of nerve cells (Bouayed et al., 2009). The antioxidant activity of polyphenols is mainly due to their redox properties. As donors of electrons or H atoms, polyphenols neutralize reactive oxygen species (ROS). Anthocyanins are thought to reduce oxidative stress on neuronal DNA. Thus, they exhibit neuronal protection, which is assumed to be responsible for their anxiolytic effect (Barros et al., 2006). It has also been established that by inhibiting monoamine oxidases (MAO-A, MAO-B) anthocyanins counteract the decrease in neuronal levels of serotonin, noradrenaline and dopamine. These are neurotransmitters implicated in the pathogenesis of anxiety and mood disorders. (Dreiseitel et al., 2009).

Chronic stress leads to cytokine overproduction that alters growth factor synthesis, reduces neurogenesis and synaptic plasticity in brain regions involved in behavior and cognition (Calabrese et al., 2014 Kim et al., 2016). The reduction of pro-inflammatory cytokines could contribute to the anxiolytic-like effect of *Aronia melanocarpa* fruit juice. In this regard, ferulic acid, which is a component of the juice composition, significantly inhibited the production of TNF-α, IL-6, IL-1β and NO, and reduced COX-2 and iNOS (Wu et al., 2015).

A number of polyphenols are known to owe their anxiolytic properties to modulation of GABA-A and GABA-C receptors (Fernandez et al., 2009; Goutman et al., 2003). The flavonoid epigallocatechin-3-gallate (EGCG) has been reported to produce behavioral responses that have been described as benzodiazepine-like. EGCG is believed to interact with GABA-A receptors and this accounts for its anxiolytic-like properties (Vignes et al., 2006).

Serotonergic neurons project widely to different brain regions and are involved in the regulation of many aspects of brain functions, for example, autonomic nervous system reactivity, mood, aggression, memory, and learning (Rudolph et al., 2016). There is ample evidence for the role of serotonin imbalance in anxiety- and depression-like behaviors in humans and in experimental animal models (Hiroi et al., 2006; Nelson and Bulun, 2001). 5-HT1A receptors for serotonin (5-hydroxytryptamine) activate G i/o/z proteins, which have an inhibitory signaling

function (Barnes and Sharp, 1999). These receptors are known to be involved in the modulation of exploratory and fear-related behavior, and their suppression is associated with increased anxiety (Stiedl et al., 2015). Data indicate that in rats, gallic acid exerts an anxiolytic-like effect similar to that of the 5-HT1A receptor agonist Buspirone, which is blocked using a 5-HT1A receptor antagonist (Mansouri et al., 2014).

Another possible mechanism contributing to the anxiolytic-like effect of *Aronia melanocarpa* fruit juice is an increase in BDNF levels. This neurotrophic factor is a key molecule involved in neuronal survival, differentiation and synaptic plasticity (Patki et al., 2013). In the experiments of Liu et al. (2017), ferulic acid, a polyphenolic compound in *Aronia melanocarpa* fruit juice, increases BDNF levels in the hippocampus and prefrontal cortex, as well as inhibits microglial activation, pro-inflammatory cytokine expression, and NF kB signaling.

In experiments with healthy rats, *Aronia melanocarpa* juice exhibited an anxiolytic-like effect in both a single (Valcheva-Kuzmanova et al., 2009) and subchronic challenge (Efftimov et al., 2013; Valcheva-Kuzmanova et al., 2016). With a single application, this effect is not accompanied by sedation and impairment of working memory (Valcheva-Kuzmanova et al., 2009).

According to the present experiment, chlorogenic acid at a dose of 20 mg/kg after 31 days of administration did not significantly change the social interaction time of ovariectomized rats, in which no anxious behavior developed during this period. Chlorogenic acid at a dose of 20 mg/kg after 76 days of administration prevented ovariectomy-induced anxiety by increasing the time of active social contacts to values not significantly different from those of sham-operated rats. The effect of chlorogenic acid found after 76 days of administration is most likely of a complex mechanism and includes: reduction of oxidative stress, stimulation of neuronal growth and differentiation, maintenance of neuroplasticity, as well as possible influence of GABA-ergic neurotransmission. These are mechanisms discussed by a number of authors when studying the anxiolytic effect of chlorogenic acid under experimental conditions (Bouayed et al., 2007; Rammal et al., 2008; Saitou et al., 2018). A review of the literature revealed positive effects of chlorogenic acid on anxiety in behavioral tests in experimental animals. In a mouse model, chlorogenic acid demonstrated an anxiolytic effect examined in the light/dark box test and in the elevated plus maze test (Bouayed et al., 2007). In an experiment with healthy rats, chlorogenic acid showed an anxiolytic-like effect with subchronic administration (Georgieva et al., 2016).

1.3. Effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on the depressive symptoms in forced swimming test

1.3.1. Effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on depressive symptoms after 33 days of treatmen

In case of 33 days of administration, immobility time of OVX rats was prolonged significantly ($p<0.01$) compared to the Control (SO) group of animals. Treatment with AMFJ at a low dose of 5 ml/kg had no significant effect on immobility time. The immobility time of OVX rats treated with AMFJ at a dose of 10 ml/kg was significantly ($p<0.05$) shorter than that of OVX group and not significantly different from SO. CGA had no significant effect on the immobility time of OVX rats compared to the Control (SO) group of animals. These results show that AMFJ exhibits a dose-dependent effect at a dose of 10 ml/kg and reduces depressive symptomatology in an ovariectomized rat model of depression.

1.3.2. Effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on depressive symptoms after 78 days of treatment

In case of 78 days of administration, immobility time of OVX rats was prolonged significantly ($p< 0.01$) compared to Control (SO) animals. Treatment with AMFJ at a low dose of 5 ml/kg had no significant effect on immobility time. The immobility time of OVX rats treated with AMFJ at a dose of 10 ml/kg was significantly ($p<0.05$) shorter than that of the OVX group. Chlorogenic acid had no significant effect on immobility time. These results show that AMFJ at a dose of 10 ml/kg administered chronically, exhibits an antidepressant-like effect in a model of depression in ovariectomized rats.

1.3.3. Discussion

Depressive disorders affect both the individual's emotional state and also lead to functional changes. The clinical manifestations of depression can vary greatly, from mild states bordering on the norm to severe disorders with hallucinations and delusions. The presence of memory deficits and cognitive disorders are common serious health complications of this disease that worsen the quality of life. Major depressive disorder is a highly debilitating disease, and by 2030 it is expected to become the most common disease after chronic diseases such as coronary heart disease and diabetes mellitus (Malhi and Mann, 2018).

Depression has a multifactorial etiology arising from interrelated psycho-social, genetic, epigenetic and neuroendocrine processes (De la Torre et al., 2018). Despite decades of research efforts, the specific pathogenetic mechanisms remain still incompletely understood. Currently, the integrative importance of monoamines, neuroendocrine and neurotrophic factors in the pathogenesis of depressive disorders is leading. Serotonergic and noradrenergic dysfunctions have long been associated with depression. Cortical and limbic deficits in the levels or functions of serotonin (5-HT), norepinephrine (NA) and dopamine (DA) have been reported to be responsible for the development of depression (Delgado and Moreno, 2000). Serotonin and/or norepinephrine are the target molecules involved in the mechanism of action of most antidepressants.

Data indicate that women are two to three times more likely to be affected by depressive disorders than men (WHO, 2020). Estrogens, through various mechanisms, are involved in the complex and multifactorial regulation of behavior, emotions, and cognitive functions. All of these can be affected during menopause, when estrogen levels begin to decline. A body of literature supports the proposition that fluctuations in female sex hormones during the premenstrual, postpartum, and perimenopausal periods may affect neurochemical pathways controlling anxiety and depression (Stahl, 2001), as well as learning and memory processes (Fedotova et al., 2017). Hormonal changes can increase or decrease the expression of specific estrogen receptor subtypes, correlating with anxiogenic or anxiolytic effects (Borrow & Handa, 2017). Mood disorders are known to be twice as common in women with low plasma estradiol levels than in men (Dalla et al., 2005). The risk of anxiety-depressive disorders increases two- to six fold during menopause (Frizell & Dumas, 2018).

Ovarian steroid hormones and a number of neurotransmitters share common pathways and receptors in the brains of rodents, monkeys and humans, according to scientific evidence. These pathways are located in areas responsible for basic functions of the nervous system such as emotions, behavior, learning and memory – hippocampal formation, amygdala, cerebral cortex (Almey et al., 2015; Frizell & Dumas, 2018). Ovarian steroids affect many neuroregulatory systems involved in the pathophysiology of affective and cognitive disorders (Dotlic et al., 2020). Animal studies have shown that sex hormones regulate basal and stimulated function of the hypothalamic-pituitary-adrenal axis. Studies have shown that administration of estradiol decreases glucocorticoid receptor mRNA production in the thymus and pituitary (Protopopescu et al., 2008). Furthermore, estradiol affects the serotonergic regulation of the hypothalamic-pituitary-adrenal

axis by altering the function of the 5-HT1A and 5-HT2 receptor systems in the cortex and hippocampus.

Direct hippocampal activation of estrogen receptors produces antidepressant and anxiolytic-like effects (Vandegrift et al., 2017). Estrogen receptor activation enhances cell proliferation (McEwen et al., 1997), which increases BDNF expression in ovariectomized rats in a model of poststroke depression (Su et al., 2016). Numerous previous studies have shown that estrogens and BDNF stimulate neurogenesis in the hippocampus (Rossi et al., 2006) and participate in synaptic modification (Cui et al., 2013) to enhance learning and memory processes (Savitz et al., 2009). Other literature sources report that estrogen signaling affects neurotransmitter systems (serotonin-, noradrenaline-, dopamine-, and glutamatergic) involved in the pathogenesis of mental disorders (Bosse & Di Paolo, 1996; Dalla et al., 2005).

There is ample evidence for the role of serotonin imbalance in anxiety- and depression-like behaviors in humans and in experimental animal models (Hiroi & McDevitt, 2006; Nelson & Bulun, 2001). Estrogen increases serotonin (5-HT) postsynaptic effects (Halbreich et al., 1995), facilitates 5-HT synthesis (Dotlic et al., 2020) by increasing tryptophan hydroxylase and tyrosine hydroxylase expression, and decreases monoamine oxidase activity (Chakravorty & Halbreich, 1997). Estradiol acts through multiple mechanisms in brain regions involved in mood regulation. Frey et al. (2004) found a significant reduction in depressive-like behavior in experimental animals after direct injection of estradiol into the amygdala. Changes in the hippocampus due to depletion of estrogen levels during the menopausal transition can be delayed or prevented by exogenous administration of estrogen (Protopopescu et al., 2008).

The present study aimed to test the effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on the depressive-like behavior of ovariectomy-induced hormone-deficient rats in a forced swim test. This is a method that makes it possible to evaluate the behavior of animals in a situation of helplessness from which they cannot escape. Increased immobility time is associated with depressive-like behavior. The results of testing on the 33rd and 78th day showed that *Aronia melanocarpa* fruit juice administered at a dose of 10 ml/kg significantly reduced the immobility time of ovariectomized rats. Treatment with *Aronia melanocarpa* fruit juice, at a dose of 5 ml/kg and chlorogenic acid, had no significant effect on the immobility time of OVX animals. This shows that *Aronia melanocarpa* juice exhibits a dose-dependent effect and reduces depressive symptoms when administered at a dose of 10 ml/kg in an ovariectomized rat model of depression.

In previous research, Valcheva-Kuzmanova's team found that *Aronia melanocarpa* juice reduced depressive symptomatology in both healthy rats and alcohol- and social isolation-induced anxiety-depressive behavior (Eftimov et al, 2013; Eftimov et al, 2015; Valcheva-Kuzmanova et al., 2013, Valcheva-Kuzmanova et al., 2014).

It is possible that the observed antidepressant-like effect in the present experiment is due to the pharmacological properties of polyphenols contained in *Aronia melanocarpa* juice. It is likely that in the higher dose of *Aronia melanocarpa* fruit juice, a combination of mechanisms of action of the biologically active substances of the juice is achieved, contributing to the observed effect. Natural polyphenols found in many plant products show protective effects in various neurological and mental disorders. It is believed that polyphenols may be involved in the modulation of various signaling pathways and thus affect cell fate (Ďuračkova Z, 2010), including the survival, regeneration, development or death of neuronal cells (Spencer, 2005).

Antidepressant polyphenolic signaling is most likely associated with the following neurobiological substrates: hippocampal and prefrontal neurogenesis, BDNF signaling, monoamine neurotransmission, hypothalamic-pituitary-adrenal axis, influencing the synthesis of inflammatory mediators.

Some polyphenols reduce depressive-like behavior by modulating neurotransmitter systems and trophic factors in the brain (Li et al., 2016). In aronia berries, quercetin, quercetin glycosides and epicatechin are present as minor components. Evidence suggests that quercetin increases the availability of serotonin and norepinephrine in the synaptic space, which appears dysregulated in depression (Kahraman et al., 2012). Demir et al (2016) reported antidepressant-like effects of quercetin in rats with induced diabetes. In a study, Noldner and Schotz (2002) revealed that the flavonoid rutin (quercetin-3-O-rutinoside) is essential for the antidepressant effect of the extract of the Hypericum perforatum plant. St. John's wort is currently used as an alternative to traditional antidepressants for the treatment of mild depression (Butterweck and Schmidt, 2007).

An antidepressant effect of proanthocyanidins, which are the main active component in the fruit and fruit juice of *Aronia Melanocarpa*, in animal models was first reported by Xu et al., (2010). Neurochemical studies reveal that proanthocyanids increase levels of serotonin, norepinephrine, dopamine in a number of brain regions, which is consistent with the involvement of the monoamine system in the antidepressant activity of these polyphenolic compounds. Antidepressant effects of red wine anthocyanins were found in a model of depression induced by

light stress in male rats (Varadinova et al., 2007a) and in ovariectomized female rats (Varadinova et al., 2007b). Anthocyanins are one of the main components of PSAM.

Monoamine oxidases (MAOs) are mitochondrial enzymes that catalyze the oxidation of monoamines in many tissues, including the brain. Increased MAO activity is involved in the etiology of depression, anxiety and neurodegenerative diseases. A clinical study showed that greater consumption of green tea led to a lower incidence of depressive symptoms among Japanese adults (Niu et al., 2009). Green tea polyphenols are known to inhibit MAO and thereby increase the level of monoamines in glial cells (Mazzio et al., 1998). This is one of the reasons why the polyphenols in green tea have an important role in depressive disorders. An in vitro assay with cyanidin and cyanidin-3-glucoside showed that they inhibit MAO A and MAO B (Dreiseitel et al., 2009).

Another possible mechanism in the improvement of depressive symptoms is supporting neurogenesis and restoring neuronal functional activity in the hippocampus and prefrontal cortex. Such an effect was found by Duman and Li, (2012) in depressed subjects. It is well known that BDNF regulates neuronal survival and differentiation, and also influences the formation of dendrites and dendritic spines (Licznerski & Duman, 2013). Decreased levels of neurotrophins can be increased by chronic treatment with antidepressant drugs (Castren et al., 2007). El-Marasy et al., (2014) reported similar effects of the natural flavanone glycoside - hesperidin, found mainly in citrus fruits, such as Citrus aurantium. Donato et al. (2014) found increased BDNF levels in the hippocampus in chronic administration of hesperidin. These authors conclude that the antidepressant effect of the flavanone glycoside is mediated by the inhibition of the L-arginine-NO-cGMP pathway and also by increasing BDNF levels in the hippocampus. The polyphenol-rich extract of Ginkgo biloba L. exhibits an antidepressant-like effect due to its ability to increase the levels of BDNF and its regulatory factor cAMP (CREB) (Hou et al., 2010). In a model of chronic unpredictable mild stress-induced depression, purified anthocyanin alleviated depression in mice by inhibiting MAO activity as well as by stimulating BDNF expression in the hippocampus, regulating the ERK/CREB/BDNF-signaling pathway (Fang et al., 2020).

Stress is one of the most important factors responsible for depressive disorders. Animal studies indicate that affecting antioxidant status is an important mechanism of action of antidepressants. In an experimental rodent model of stress-induced depression, antidepressant

drugs increased endogenous enzymatic antioxidant defenses and decreased lipid peroxidation (Zafir et al., 2009).

In this regard, one of the most pronounced and well-studied effects of polyphenolic compounds is the antioxidant. Due to its rich polyphenolic content, *Aronia Melanocarpa* fruit juice possesses significant antioxidant activity (Valcheva-Kuzmanova et al., 2012). It is known to be due to the redox properties of polyphenols, which allow them to act as donors of electrons or hydrogen atoms, scavengers of reactive oxygen species and metal chelators (Sandoval-Akunã et al., 2014). Polyphenols potentiate endogenous antioxidants (Valcheva-Kuzmanova et al., 2012) and also inhibit the expression of molecules, such as NF-kB and AP-1, sensitive to oxidative stress (Gupta et al., 2004). It is possible that the mechanism of the antidepressant-like action of polyphenols involves protection of the CNS from oxidative stress. Neuroprotective effects of *Aronia melanocarpa* extract have been demonstrated on cells from the hippocampus of mice *in vitro* conditions, where the production of ROS and glutamate-induced cell death were suppressed, accompanied by an increase in the activity of the antioxidant enzymes glutathione peroxidase and glutathione reductase (Lee et al. et al., 2017). Cyanidin-3-O-galactoside, a major compound in *Aronia melanocarpa* extract, ameliorates cognitive and behavioral decline in aging in mice by increasing antioxidant capacity and regulating ERK expression in the hippocampus of experimental animals (Tan et al., 2014). Thus, cyanidin-3-O-galactoside may be involved in the neuroprotective effect of *Aronia melanocarpa* extract. The potential of polyphenols to exert neuroprotective effects appears to be related to a number of mechanisms, including their ability to interact with intracellular neuronal and glial signaling, to affect peripheral and cerebrovascular blood flow and reduce neuronal damage and loss induced by neurotoxins and neuroinflammation (Smolensky et al., 2018). The role of pro-inflammatory cytokines in the pathogenesis of depression is known. IL-6, IL-1, TNF-α interact with mitochondria and increase the production of reactive oxygen species (ROS), which in turn increase cytokine expression (Sprague and Khalil, 2009). Polyphenols decrease the production of inflammatory cytokines in the CNS and promote the expression of anti-inflammatory markers in the microglia of experimental animals (Gomez-Pinilla and Nguyen, 2012). In the brain, the anti-inflammatory action of polyphenols leads to neuroprotective effects that may improve depressive symptoms.

Blueberry polyphenols were found to inhibit the production of NO·, IL-1β and TNF-α in activated microglial cells (Vauzour, 2012), and the flavonol quercetin (1-30 μM) (Chen et al.,

2005) and the catechin epigallocatechin gallate (1-50 μM) (Li et al., 2004), attenuate microglial and/or astrocyte-mediated neuronal inflammation (Si et al., 2016). Anthocyanins from Aronia melanocarpa reduce DNA damage in the brain of aging mice by suppressing the transcription of inflammatory cytokines (COX2, TGF-β1, and IL-1) involved in the DNA damage signaling pathway (Wei et al., 2007). Ferulic acid, a component of *Aronia melanocarpa* fruit juice, in a model of chronic unpredictable stress, ameliorates depressive symptomatology by influencing the activation of pro-inflammatory cytokines in the prefrontal cortex (Liu et al., 2017).

1.4. Effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on pain sensitivity in ovariectomized rats in the hot plate test after 79 days of treatment and discussion

The hot plate test is one of the main behavioral methods for assessing nociception in experimental animals. The results revealed that the latency time of OVX rats was shortened significantly ($p<0.05$) compared to the control group of animals. The latency time of OVX+AMFJ5 and OVX+AMFJ10 groups was prolonged significantly ($p<0.001$) compared to OVX group animals and was insignificantly different from that of the control group of rats (Figure 9). Latency time of OVX+CGA group was prolonged significantly ($p<0.05$) compared to OVX group of animals and non-significantly different from that of control group of rats (Figure 9). Treatment of OVX rats with AMFJ and CGA reduced nociceptive sensitivity in the hot plate test.

Pain is a complex experience that includes both sensory and affective dimensions. The decline in ovarian hormones during menopause can affect the body's somatosensory, cognitive, and affective response. Ovarian hormones alter behavior in response to pain.

Evidence suggests that estrogens play an important role in inducing antinociception in experimental animals (de Chaves et al., 2009). A possible function of ER in pain modulation is further supported by the fact that estrogen receptors are widely distributed in the CNS, particularly in several brain regions associated with the nociceptive neuronal system – the amygdala, thalamus, and frontal cingulate cortex (ACC) (Solum and Handa, 2002). Estrogens affect the sensory and pain systems (Chen et al., 2021). Ovariectomized rodents tend to exhibit mechanical and thermal hyperalgesia, while treatment with exogenous estrogen can prevent the observed symptoms (Chen et al., 2021). Pain-related signaling pathways are likely activated by ligands in a genetic or non-genetic manner. ERα and ERβ receptors interact with the nociceptive system and modulate pain through different cellular signaling pathways (MAPK/ERK; PKA; PKC, etc.) (Chen et al., 2021;

Kiss et al., 2012). Estrogen deficiency alters pain perception, yet the mechanisms by which ERs influence pain are still largely unknown. However, it has been found that the perception of pain depends on many factors (type of pain, its origin, level of estrogens, gender, etc.), which greatly complicates the overall description of the mechanisms underlying nociception in estrogen deficiency. The exact mechanism of this effect requires further research. Additionally, there may be interactions between different hormones that must also be taken into account.

The present study aimed to test the effects of *Aronia melanocarpa* fruit juice and chlorogenic acid on pain sensitivity in OVX-induced estrogen-deficient rats in a hot plate test. The hot plate test is the standard choice for evaluating analgesic activity of both newly synthesized and established drug compounds. The thermal stimulus used makes it possible to differentiate different mechanisms and levels of action of antinociception (Barrot, 2012). One way to assess nociception in experimental animals is by observing their behavior. The behavioral parameters observed in this test: paw licking, climbing attempts and vocalization are expressions of supraspinally integrated responses (Deuis et al., 2017).

In hot plate testing, OVX animals demonstrated thermal hyperalgesia, which is consistent with other studies (Li et al., 2014; Sanoja and Carvero, 2008). The results of the present study show that after 79 days of treatment, *Aronia melanocarpa* fruit juice at a dose of 5 ml/kg and 10 ml/kg, as well as chlorogenic acid at a dose of 20 mg/kg exert an antinociceptive effect, significantly prolonging the latent residence time compared to the OVX group and non-significantly different from that of the control (SO) group. *Aronia melanocarpa* fruit juice and chlorogenic acid effectively increase the sensitivity threshold of ovariectomized animals to heat-induced pain. There are mechanisms that could be proposed to explain these results. There is abundant evidence that some pro-inflammatory cytokines, such as interleukin 1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α), play a specific role in the process of nociceptive signaling (Zhang and An, 2007). Prostaglandins themselves do not induce pain, but enhance the pain-inducing effect of other algogenic substances such as kinins and 5-hydroxytryptamine (Basbaum et al., 2009).

On the other hand, there is a close relationship between cytokines and oxidative stress in the induction of pain. IL-1β and TNF-α can activate nicotinamide adenine dinucleotide phosphate (NADPH-) oxidase, leading to the production of superoxide anions. In turn, superoxide anion activates nuclear factor kappa B (NF-κB) and increases cytokine production (Verri et al., 2012).

Substances that suppress prostaglandin synthesis can have not only an anti-inflammatory, but also an analgesic effect.

From this point of view, quercetin, which is a component of AMFJ, demonstrates a good ability to reduce the expression of various interleukins (such as IL-6 and IL-2) and also the levels of iNOS, NF-κB, p38 MAPK and TNF-α (Caruso et al., 2017). In addition, quercetin has been shown to reduce heat hypersensitivity and mechanical allodynia, as well as oxidative imbalance and pro-nociceptive cytokine production in inflammatory pain. It was also found that in a model of hyperalgesia induced by Ehrlich carcinoma, quercetin exhibited good analgesic activity also dependent on endogenous opioid mechanisms (Caruso et al., 2017; Calixto-Campus et al., 2015).

Epigallocatechin-3-gallate (EGCG) has been reported to alleviate neuropathic pain symptoms in animal models, exhibiting anti-inflammatory and antioxidant activity (Bimonte et al., 2017).

A number of authors have reported the antioxidant and anti-inflammatory activity of *Aronia melanocarpa* fruit juice, which probably correlates with its antinociceptive effect (Jurikova et al., 2017; Banach et al., 2020). The anti-inflammatory actions of the juice may be due to its influence on inflammatory and anti-inflammatory cytokines. *Aronia melanocarpa* fruit juice has been shown to have an anti-inflammatory effect in models of histamine- and serotonin-induced inflammation of the rat hindpaw (Borissova et al., 1994). In a model of amiodarone-induced pulmonary toxicity, the juice decreased Il-6, increased Il-10 in serum, and decreased polymorphonuclear leukocytes in bronchoalveolar lavage fluid (Valcheva-Kuzmanova et al., 2012). In endotoxin-induced uveitis in rats, *Aronia melanocarpa* extract produced an anti-inflammatory effect resulting from reduced production of nitric oxide (NO) and prostaglandin E2 (PGE2) (Ohgami et al., 2005). The beneficial effects of *Aronia melanocarpa* fruit juice on the behavior of ovariectomized rats may be due, at least in part, to the phytoestrogenic properties of certain anthocyanidins (especially cyanidin) and quartzetin, both present in high concentration in *Aronia melanocarpa* fruits (Nanashima et al., 2015; Nanashima et al., 2018).

Chlorogenic acid reduces pain sensitivity in an inflammation model (Bagdas et al., 2020). The antinociceptive effects of chlorogenic acid in inflammatory and neuropathic pain are also associated with inhibitory effects on the release or synthesis of inflammatory mediators, such as TNF-α, NO and IL (Chauhan et al., 2012; dos Santos et al., 2006). Hyperpolarization of sensory ganglia may be an alternative explanation for the antinociceptive effects of chlorogenic acid in

animal models. Voltage-gated potassium channels (Kvs) are physiological regulators of membrane potential in sensory neurons. Because chlorogenic acid strongly increases the activity of voltage-gated potassium channels (Kv1.4), it leads to hyperpolarization and a gradual decrease in the excitability of neurons involved in neuropathic and inflammatory pain signaling (Zhang et al., 2014). Gallic acid, also a component of *Aronia melanocarpa* fruit juice, has been reported to exert antinociceptive effects in mice by acting as an antagonist of the transient receptor potential ankyrin 1 (TRPA1), thereby reducing TRPA1-mediated calcium influx (Trevisan et al., 2014). One of the specific functions of TRPA1 includes a role in the detection, integration and initiation of pain signals in the peripheral nervous system.

2. Effects of chlorogenic, ferulic and gallic acids on the behavior of rats with bilateral olfactory bulbectomy

2.1. Effects of chlorogenic, ferulic and gallic acids on anxiety in bulbectomized rats in the elevated plus maze test and discussion

The experiment was conducted on 30 male Wistar rats, divided into 5 groups, each consisting of 6 animals – Control (SO) sham operated, bulbectomized (OBX), OBX+CGA, OBX+FA and OBX+GA. Rats from OBX, OBX+CGA, OBX+FA and OBX+GA groups were bulbectomized bilaterally, and control animals were sham operated. After 15 days of recovery from surgery, for a period of 14 days, the animals were treated once (between 09:00 a.m. and 13:00 p.m.) by orogastric tube as follows: Control (SO) and OBX received saline at a dose of 10 ml/kg, and the groups OBX+CGA, OBX+FA and OBX+GA were treated respectively: with CGA, FA and GA in a dose of 20 mg/kg in the form of a solution in a volume of 10 ml/kg. The test was performed 60 min after the last treatment. During the 5-min test session, the following parameters were recorded for each animal: number of entries into the open and closed arms and the time spent in them in seconds, as well as the total number of arm entries and the ratio of entries into the open arms to the total number of entries. An increased number of entries into the open arms and time spent there is considered an indicator of an anxiolytic-like effect of the test substance (Pellow et al., 1986; Hritcu et al., 2012).

Bilateral bulbectomy induced a state of hyperactivity with a significant ($p<0.001$) increase in the total number of entries into the arms of the maze in OBX rats compared to controls (SO) animals. Changes in other indices demonstrate the development of a state of anxiety. Compared to

Control (SO), OBX animals have a significantly lower number of entries into the open arms ($p<0.05$) and time spent there ($p<0.01$), as well as a significantly higher number of entries into the closed arms ($p<0.001$) and time spent in them ($p<0.01$). In the OBX group, significantly lower ratios of the number of entries in the open arms to the total number of entries ($p<0.001$) and of the time spent in the open arms to the total time spent in the open and closed arms were found ($p <0.01$) compared to Control (SO) rats.

Treatment of OBX rats with chlorogenic, ferulic and gallic acids antagonized behavioral changes induced by bilateral bulbectomy. Ferulic and gallic acids led to a recovery of the measured indices to values that were significantly different from those of OBX rats and not different from those of Control (SO) animals. The effect caused by chlorogenic acid is even higher. Chlorogenic acid increased the number of open arm entries and the time spent there ($p<0.001$), as well as the ratios: number of open arm entries/total number of entries ($p<0.001$) and dwell time in open arms/total dwell time in open and closed arms to values that were significantly higher ($p < 0.001$) not only than those of OBX rats but also those of SO rats. Similarly, the time in the closed arms of OBX+CGA rats was shorter ($p<0.001$) than the corresponding time of both OBX and Control (SO) rats. Treatment of OBX rats with chlorogenic, ferulic, and gallic acids prevented the development of hyperactive and anxiety-like behaviors.

Due to their great social importance worldwide, anxiety-depressive disorders are the subject of many experimental developments. Experimental animal models are useful for studying the pathogenesis and treatment of these diseases.

Surgically induced bilateral olfactory bulbectomy (OB) has been used to screen antidepressant drugs for the past 40 years in both rodent groups – rats (Kelly et al., 1997, Mar et al., 2002) and mice (Han et al., 2009). OB induces significant neurochemical, neuroanatomical, physiological, endocrine, and behavioral changes that are similar to symptoms in patients with major depressive disorder (Song & Leonard, 2005).

Olfactory bulbs are bilateral extensions of the rostral telencephalon and constitute about 4% of the total brain mass in the adult rat (Cain, 1974). The presence of extensive efferent connections between the olfactory bulbs and the mesocortical and subcortical regions of the brain largely explain the effects on brain function observed when the olfactory bulbs are removed. The rat olfactory system is part of the limbic region, in which the amygdala and hippocampus contribute to the emotional and memory components of behavior (Song, 2005).

Bilateral removal of the olfactory bulbs disrupts the cortex-hippocampus-amygdala interconnection (Russo and Nestler, 2013), affecting food-seeking and avoidance behavior (Song and Leonard, 2005, Kelly et al., 1996). Bilateral destruction of the olfactory bulbs results in changes in serotonin and dopamine concentrations (Masini et al., 2004). Cognitive deficits, loss of libido (Larsson 1971), reduced social interaction and exploration of novel environments are associated with cortico-neuronal degeneration in OB rats (Wang et al., 2007). Impaired structural plasticity (dendritic reorganization, impaired cell growth) of the hippocampus is also associated with emotional deficits and changes in spatial memory of experimental OB animals (Morales-Medina et al., 2013; Morales-Medina et al, 2017). Bilateral olfactory bulbectomy subsequently reduced the volume of the cortex, hippocampus, and amygdala (Wrynn et al., 2000), as well as the expression of the NMDA (N-methyl-D-aspartate) receptor subunit NR1 (but not NR2A, B) in these regions, but it also reduced CREB phosphorylation in the prefrontal cortex and hippocampus (Song at el., 2011).

Neurotransmitter, structural and behavioral changes in OBX rats are also associated with neuroinflammatory events. Song et al. (2009) found increased expression of corticotropin-releasing factor in the hippocampus and increased secretion of corticosterone in OB rats compared with sham-operated controls. Rinva et al. (2013) demonstrated increased levels of inflammatory cytokines (TNF-α, IL-6, IL-1β) and caspase-3, together with a significant reduction of BDNF in the brain of OB rats. Song et al. (2009) studies in OB rats found significantly lower NGF mRNA expression in the hippocampus, while IL-1β and PgE2 levels were increased in serum and brain. The non-steroidal anti-inflammatory drug celecoxib significantly reduced the blood concentration of prostaglandin E2, IL-1β and corticosterone, and increased the expression of NGF, as well as normalized the behavior of OB rats.

Chronic antidepressant treatments can reverse the behavioral changes of OB rats to the normal phenotype (Song and Leonard, 2005). Human data on a direct relationship between olfactory sensory function and major depressive disorder are conflicting. Some clinical studies have shown reduced olfactory sensitivity in patients with an acute form of major depressive disorder (Atanasova et al., 2008) and the presence of a negative relationship between the volume of the olfactory bulbs and the severity of depressive symptoms (Negoias et al., 2010).

The elevated plus maze is the most commonly used experimental method to assess anxiety in rodents. The test is based on the animals' innate fear of open spaces. Most anxiolytic-acting

substances increase the exploration of the open arms of the maze. The increased number of entries into the open arms and the time spent in them are taken as an indicator of anxiolytic effect of the tested substance.

In this experiment, bilateral olfactory bulbectomy induced hyperactivity, demonstrated by an increase in the total number of entries into the open and closed arms of the maze. In other studies, similar hyperactive behavior of OB rats was associated with elevated glutamate levels in brain regions such as the *striatum* (Ho et al., 2000) and *nucleus accumbens* (Rudá-Kučerová et al., 2015). In the experiment conducted, chlorogenic, ferulic and gallic acids reduced the hyperactivity of rats with olfactory bulbs removed. Literature data indicate that in a cellular model, chlorogenic acid blocks glutamate-induced toxicity as well as prevents glutamate-induced death of primary cells isolated from mouse cortical neurons (Mikami and Yamazawa, 2015). In another study, chlorogenic acid and its metabolites counteracted glutamate-induced toxicity in primary cultures of rat cerebellar granule neurons (Taram et al., 2016).

Excessive concentration of glutamate can cause oxidative stress by increasing the production of RBC, which is closely related to the pathogenesis of anxiety disorders. Literature data show that the pathophysiology of anxiety and related affective disorders correlates with a wide range of epigenetic changes such as increased oxidative stress (Hassan et al., 2014), neuroinflammation (Ng et al., 2008), glutamatergic dysfunction (Pitsikas, 2014), dysregulation of synaptic plasticity through changes in the level of neurotrophins and inhibition of signaling pathways (Castrèn and Kojima, 2017).

In our study, chlorogenic, ferulic and gallic acids showed an anxiolytic effect. Most of the biological actions of phenolic acids on the brain are attributed to their anti-inflammatory and antioxidant properties (Mandrone et al., 2015). A study by Gul and colleagues (2016) revealed neuroprotective effects of chlorogenic acid. This polyphenol attenuated H2O2-induced increase in malondialdehyde and ROS levels in rat cortical slices (Gul et al., 2016). In another experiment with primary cultures of rat cerebellar granule neurons, chlorogenic acid was found to enhance protection against H2O2-induced proteasome inhibition and caspase-dependent apoptosis (Taram et al., 2016). Gallic acid owes its neuroprotective effects and ability to stabilize mood to antioxidant activity and ameliorate the loss of cell density in the hippocampus (Moghadas et al., 2016). Research by Lenzi et al. (2015) found that the effects of ferulic acid on the central nervous system also correlated with a demonstrated antioxidant capacity, evidenced by increased superoxide

dismutase and catalase activity in the hippocampus of treated mice, as well as by low levels of thiobarbiturate-reactive substances. acid. Ferulic acid has been shown to increase BDNF levels in the prefrontal cortex and hippocampus, and also inhibit microglial activation, proinflammatory cytokine expression, and NF-kB signaling (Liu et al., 2017). Reduction of pro-inflammatory cytokines could contribute to the anxiolytic-like effects of phenolic acids. Ferulic acid significantly inhibited the production of TNF-α, IL-6, IL-1β and NO, and reduced COX-2 and iNOS (Wu et al., 2015). Receptor activation may also be involved in the anxiolytic-like effects of phenolic acids. In a mouse model, chlorogenic acid demonstrated an anxiolytic effect examined in the light/dark box test and in the elevated plus maze test (Bouayed et al., 2007). In this study, the anxiolytic effect of chlorogenic acid was blocked by the benzodiazepine antagonist flumazenil, suggesting that chlorogenic acid probably acts as an agonist at benzodiazepine receptors (Ramboz et al., 1998). The manifested anxiolytic effect most likely has a complex mechanism and includes: stimulation of GABA-ergic neurotransmission, reduction of oxidative stress, stimulation of neuronal growth and differentiation, as well as maintenance of neuroplasticity (Bouayed et al., 2007; Saitou et al., 2018). Another possible mechanism of the anxiolytic action of the investigated phenolic acids is an influence on 5-HT1A receptor activation. 5-HT1A receptors are known to be involved in the modulation of exploratory and fear-related behaviors, and reduced 5-HT1A receptor density could increase anxiety (Bouayed et al., 2007). Gallic acid exhibited anxiolytic-like activity similar to the 5-HT1A receptor agonist buspirone in a rat elevated plus maze test (Mansouri et al., 2014). In conclusion, chlorogenic acid, gallic acid, and ferulic acid inhibited the development of hyperactive and anxiety-like behaviors in bilateral bulbectomized rats. The effect is most pronounced with chlorogenic acid.

2.2. Effects of chlorogenic, ferulic and gallic acids on memory and learning

2.2.1. Effects of chlorogenic, ferulic, and gallic acids on learning and memory in one-way passive avoidance test - step through

The experiment was conducted on 30 male Wistar rats, divided into 5 groups of 6 animals each designated as: Control (SO), sham operated, bulbectomized (OBX), OBX+CGA, OBX+FA and OBX+GA. Rats from OBX, OBX+CGA, OBX+FA and OBX+GA groups were bulbectomized bilaterally, and control animals were sham operated. After a 15-day recovery from surgery, for a period of 14 days, the animals were treated once (between 09:00 a.m. and 13:00 p.m.) by orogastric

tube as follows: Control and OBX received saline at a dose of 10 ml/ kg, and the groups OBX+CGA, OBX+FA and OBX+GA were treated respectively with CGA, FA and GA in a dose of 20 mg/kg in the form of a solution in a volume of 10 ml/kg. The last treatment of the animals was 60 min before the training session. Passive avoidance training with negative reinforcement consisted of a single training session conducted according to the method of Buresova and Bures (1983) using a step through apparatus. The memory test was conducted at the 3rd and 24th hour after training. The animals' stay in the illuminated chamber (latency time in sec) for a period of at least 180 sec is considered as training criteria

After 14 days of treatment, the latency time of the OBX group was 25.33±5.53 sec at the 3rd hour and 27.67±4.49 sec at the 24th hour, respectively. training session and was significantly ($p<0.001$) shortened compared to the Control (SO), which was 110.8±4.55 sec at the 3rd hour and 115.8±3.27 sec at the 24th hour. In the CGA-treated group, the latent time in the retention test at 3 and 24 hours was prolonged with significance $p<0.001$ compared to the OBX group and was not different from the Control (SO). In the OBX+FA group, the latency time in both retention tests was prolonged significantly ($p<0.001$) compared to the OBX group and did not differ from the Control group. Animals from the OBX+GA group also showed a significant prolongation of the latency time in the memory tests at 3 and 24 hours ($p<0.001$) compared to the OBX group and did not differ from the Control (SO) animals. These results show that after 14 days of treatment, CGA, FA, and GA significantly improve memory and learning processes in bilateral olfactory bulbectomy rats.

2.2.2. Effects of chlorogenic, ferulic and gallic acids on learning and memory in two-way active avoidance test – shuttle box

The experiment was conducted on 30 male Wistar rats, divided into 5 groups of 6 animals designated as: Control (SO) sham-operated, bulbectomized (OBX), OBX+CGA, OBX+FA and OBX+GA. Rats from OBX, OBX+CGA, OBX+FA and OBX+GA groups were bulbectomized bilaterally, and control animals were sham operated. After a 15-day post-operative recovery, for a period of 14 days the animals were treated once (between 09:00 a.m. and 13:00 p.m.) by orogastric tube as follows: Control (SO) and OBX received saline in a dose of 10 ml /kg, and the groups OBX+CGA, OBX+FA and OBX+GA were treated respectively: with CGA, FA and GA in a dose of 20 mg/kg in the form of a solution in a volume of 10 ml/kg. Experimental animals were treated 60 min before the start of the training sessions (on two consecutive days) and were accordingly not

treated before the memory test, which was 24 h after the second training session. As an indicator of learning and memorization, the number of conditioned reflex avoidances (avoidances) in the training sessions (on the 1st and 2nd day), as well as in the memory test, is counted.

The number of avoidance responses in OBX animals were statistically significantly reduced compared to the Control (SO) and were respectively 2.17±0.31 on the 1st training session day, 1.50±0.22 on the 2nd training day and 1.67±0.21 in the retention test. In animals treated with CGA, the number of avoidance-responses increased significantly on the second training day ($p<0.05$) compared to the control (FO) group, but was not significantly different from the number of avoidance-responses of the Control (SO) on the first training day and in the retention test. The CGA group significantly ($p<0.001$) increased the number of avoidance-responses on the first and second training days and in the retention test compared to the OBX group. In the FA-treated group, the number of avoidance-responses increased significantly on the first training day ($p<0.05$), on the second training day and in the retention test ($p<0.001$) compared to the OBX group and was not significantly different from the number of avoidance responses of the Control (SO). In animals treated with GA, the number of avoidance-responses in the retention test increased significantly ($p<0.05$) compared to the control group (SO), but did not differ significantly from the number of avoidance-responses of the Control (SO) on the first training session day and on the second training day. In the GA group, the number of avoidance reactions on the first training day, as well as on the second training day and in the retention test ($p<0.001$) increased significantly compared to the OBX group. These results indicate that CGA, FA, and GA significantly improve memory and learning processes in bilateral bulbectomized rats.

2.2.3 Discussion

As the aging of the human population continues to progress, scientific efforts are increasingly focused on improving the quality of life and delaying the onset of age-related diseases.

In the various types of dementia (eg, Alzheimer's disease, frontotemporal dementia, diffuse Lewy body disease, Parkinson's disease, Huntington's disease, vascular dementias, dementias associated with depression, etc.) there is a decline in multiple cognitive and behavioral functions, which is the reason for the deterioration of the quality of life and independence of the patients. By 2050, dementia is expected to reach 135.5 million people (Winblad et al., 2017). The

progressive loss of intelligence and autonomy affects not only the elderly population, but changes the quality of life of their loved ones. Neurodegenerative diseases are often accompanied by affective problems that worsen their course. They can worsen patients' social and functional status (Kessler et Greenberg, 2002). Zhao and colleagues (2016) reported that 40% of Alzheimer's disease cases have comorbid clinical depression. There is also a high percentage of patients diagnosed with Parkinson's disease who have comorbidity with anxiety, eating disorders (Kessler and Greenberg, 2002) and persistent/chronic pain symptoms (Lee et al, 2012).

Bilateral bulbectomy induces neurochemical, neuroanatomical, physiological, endocrine, and behavioral changes that are similar to symptoms in patients with major depressive disorder (Song & Leonard, 2005). The rat olfactory system is part of the limbic region, in which the amygdala and hippocampus contribute to the emotional and memory component of behavior (Song, 2005).

The present study aimed to test the effects of chlorogenic, ferulic and gallic acids on learning and memory in bulbectomized rats. Bilateral bullectomy was performed according to the method of Kelly et al. (Kelly et al., 1997). To test the memory of OB rats, two classical methods were used – passive avoidance training with negative reinforcement (step-through) and active two-way avoidance training with negative reinforcement (shuttle box).

The results of the study showed that the removal of the olfactory bulbs caused, 30 days later, a significant decrease in the latency time in the memory tests of the OB group, which is consistent with data from other researchers (Kelly et al., 1997; Tashev et al., 2010). After 14 days of treatment, chlorogenic, ferulic and gallic acids improve memory and learning processes in bilateral olfactory bulbectomy rats. Chlorogenic acid, ferulic acid and gallic acid have been reported to improve learning and memory in young healthy rats (Georgieva et al., 2015). A number of studies suggests that food-derived polyphenols have the potential to improve human memory and neurocognitive functions through their ability to protect vulnerable neurons, enhance existing neuronal function, and stimulate neuronal regeneration (Spenser, 2010; Vauzour D, 2012).

The mechanisms underlying the observed effects of chlorogenic, ferulic and gallic acids in the present study can be sought in their neuroprotective action, which is favored by the ability to pass through the BBB. It has been reported that chlorogenic acid or its metabolites can cross the BBB and exert neuroprotective effects on brain tissue (Ohnishi et al., 2006). Approximately 30 minutes after oral administration, ferulic acid was detected in rat brain (Chang et al., 1993).

Ferulic acid has been shown to stimulate the proliferation of neural progenitor cells in vitro and in vivo (Yabe et al., 2010). Ferulic acid and chlorogenic acid have demonstrated neuroprotective effects by improving cognition in models of Alzheimer's disease (Szwajgier et al., 2018). Gallic acid (100 mg/kg) has been reported to improve hippocampal neurodegeneration and cognitive changes (spatial memory and learning) in rats exposed to aluminum chloride (AlCl 3) (Ogunlade et.al., 2020). The central effects thus demonstrated confirm that chlorogenic acid, ferulic acid and gallic acid reach therapeutic concentrations in the CNS (Caruso et al, 2022).

Apoptosis is a form of programmed cell death that plays a crucial role in normal development and tissue homeostasis. However, inappropriate or excessive apoptosis is involved in the pathogenesis of a number of diseases, including some neuropsychiatric disorders (Kim et al., 2010). There is evidence that excessive neuronal apoptosis in the cortex and hippocampus contributes to memory dysfunction and impaired learning. Oxidative stress is thought to be capable of inducing neuronal death, probably by activating apoptosis (Kudryashov et al., 2002). In this regard, it would be expected that antioxidant compounds could suppress this cellular process. Phenolic acids have a proven antioxidant capacity. The anti-apoptotic effect of phenolic acids could contribute to the memory improvement of OB rats in the present study. In an experimental model of alcohol-induced cell injury, chlorogenic acid increased cell viability, promoted proliferation of damaged cells, increased mitochondrial transmembrane potential, and also inhibited apoptosis by increasing Bcl-2 expression and decreasing capsase-3 activation (Fang et al., 2016). Literature data suggest that gallic acid counteracts amyloid-induced neuronal programming death in rats by reduced release of glutamate and RBC (Ban et al., 2008). To some extent, the positive influence of phenolic acids on memory and learning processes is due to their antioxidant capacity. In a model of oxidative neuronal death in cell cultures, chlorogenic acid suppressed hydrogen peroxide-induced apoptotic condensation of the neuronal cell nucleus and down-regulation of the anti-apoptotic proteins Bcl and Bcl-XL. While blocking the pro-apoptotic factors caspara-3 and pro-poly (ADP-ribose) polymerase (Kim et al., 2012). In experiments with mice with induced diabetes, Stefanello and colleagues (2014) found that chlorogenic acid improved memory, exhibited an anxiolytic-like effect, and protected against the production of thiobarbituric acid-reactive substances. These effects are also believed to be due to a reduction in acetylcholinesterase activity (Stefanello et al., 2014). The central cholinergic system is essential for the regulation of cognitive functions, with acetylcholine playing an important role in memory processes, particularly in brain

areas important for retaining new memories such as the hippocampus, perirhinal and entorhinal cortex (Hasseimo and Chantal, 2006). The antioxidant properties of ferulic acid have been demonstrated in a model of hypoxia-induced neurotoxicity in cell cultures (Lin et al., 2015). Along with scavenging free radicals, ferulic acid increases cell viability and superoxide dismutase activity. It also reduced the increase in intracellular free Ca 2+, lipid peroxidation, apoptosis and PGE2 production in treated PC12 cells (Lin et al., 2015).

Activation of the transcription factor CREB regulates the expression of a number of important genes, including BDNF, thereby participating in the control of synaptic function and neuronal survival in the central nervous system (Carito et al., 2014; Pardon MC, 2010). These neurotrophins are required during memory acquisition and consolidation (Spencer, 2010). Agents that can induce pathways leading to activation of CREB/BDNF are believed to have the potential to improve both short-term and long-term memory (Spencer, 2009). In this regard, there is evidence that chlorogenic acid increases BDNF levels in the hippocampus of rats with diabetes-induced cognitive deficits (Xianchu et al., 2021). In an animal model, ferulic acid increases BDNF expression and promotes functional brain recovery from ischemia-induced injury (Baek et al, 2014).

Suppression of pro-inflammatory factors may be another possible mechanism of memory improvement by chlorogenic, ferulic and gallic acids in the present study. Chlorogenic acid exhibits neuroprotective and anti-inflammatory activity in herpes simplex virus-infected microglial cells. In infected cells chlorogenic acid increased the survival rate, decreased the release of TNF-α and interleukin IL-6, as well as the expression of NF-κB p65 (Guo et al., 2015), and the antioxidant properties of this acid reduced the levels of malonaldehyde (MDA) and reactive oxygen species in rat brain slices (Gul et al., 2016). A combination of antioxidant and anti-inflammatory activities was also demonstrated by ferulic acid, which inhibited TNF-α, IL-6, IL-1 and NO and reduced COX-2 and iNOS, in a model of LPS-induced microglial inflammation (Huang et al., 2011). Gallic acid exerts a neuroprotective effect in a model of traumatic brain injury, improving memory and long-term potentiation (LTP) by reducing lipid peroxidation and pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) in the brain of experimental animals (Caruso et al., 2022).

The potential effect of phenolic acids on the vessels is significant, as improved cerebrovascular function is known to facilitate neurogenesis in adults (Shohayeb et al., 2018). The

vascular effects of phenolic acids probably contribute to the improved memory and learning processes in the present experiment. Ferulic acid has been reported to enhance angiogenesis by influencing the activity of the main factors involved in it, namely vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and hypoxia-inducible factor 1 (HIF-1). In a test with human umbilical vein endothelial cells, Lin and colleagues (2010) found that ferulic acid enhanced the expression of VEGF and PDGF and increased the amount of hypoxia-inducible HIF-1, which generated hypoxia-responsive responses. A number of other in vivo and in vitro studies also demonstrate that ferulic acid improves angiogenesis and promotes new vessel formation (Yang et al., 2017; Lin et al., 2010). Chlorogenic acid has been shown to improve endothelial function by increasing NO bioavailability in rat arterial muscle, leading to vasodilation (Suzuki et al., 2006). In addition, in a mouse model, chlorogenic acid demonstrated an antiatherogenic effect by lowering plasma levels of total cholesterol, triglycerides, low-density lipoprotein, and inflammatory markers. In cell culture, chlorogenic acid significantly increased the transcription of PPARγ, LXRα, ABCA1/ABCG1, which are key regulators in cholesterol efflux (Wu et al., 2014).

According to the studied scientific data, chlorogenic, ferulic and gallic acids can improve memory and learning processes in bilateral bulbectomized rats, most likely through several mechanisms: antioxidant action, suppression of the release of pro-inflammatory factors, beneficial vascular effects, activation of signaling pathways and inhibition of acetylcholinesterase, as well as by increasing the levels of CREB-BDNF and suppressing apoptosis and stimulating the proliferation of nerve cells.

V. CONCLUSIONS

1. In an experimental model of bilateral ovariectomy in rats, the following behavioral changes were found:

1.1. Motor activity in an open field test

1.1.1. Bilateral ovariectomy non-significantly decreased the total locomotor activity recorded on days 30 and 75 from the start of treatment;

1.1.2. *Aronia melanocarpa* fruit juice in doses of 5 ml/kg and 10 ml/kg significantly reduced the locomotor activity of ovariectomized animals after 30- and 75-day administration, probably due to a sedative effect;

1.1.3. Chlorogenic acid at a dose of 20 mg/kg after 30- and 75-day administration suppressed locomotor activity in ovariectomized rats to the extent that it was significantly lower than that of sham operated rats, but not significantly different from that of ovariectomized control animals.

1.2. Anxiety in a social interaction test

1.2.1. Bilateral oophorectomy did not induce anxiety-like behavior on day 31 from the start of treatment;

1.2.2. Bilateral oophorectomy resulted in anxiety-like behavior on day 76 from the start of treatment, as measured by a reduction in social interaction time;

1.2.3. On day 31st, when no development of anxious behavior was reported in the ovariectomized animals, *Aronia melanocarpa* fruit juice in doses of 5 ml/kg and 10 ml/kg decreased the time of active social contacts. This effect may be due to the reduced total locomotor activity of these animals registered in the open field test;

1.2.4. *Aronia melanocarpa* fruit juice at a dose of 5 ml/kg prolonged the time of active social contacts after 76-day of administration, possibly due to an anxiolytic-like effect. The lack of a similar effect of the juice at a dose of 10 ml/kg may be the result of a more pronounced suppression of locomotor activity than this dose, registered in the same animals in the open field test;

1.2.5. Chlorogenic acid at a dose of 20 mg/kg after a 31-day administration did not significantly change the social interaction time of ovariectomized rats, in which no anxious behavior developed during this period;

1.2.6. Chlorogenic acid at a dose of 20 mg/kg after 76-day of administration prevented ovariectomy-induced anxiety by increasing the time of active social contacts to values not significantly different from those of sham-operated rats.

1.3. Depressive behavior in the forced swim test

1.3.1. Bilateral ovariectomy resulted in depressive-like behavior by prolonging the immobility time recorded on days 33 and 78;

1.3.2. *Aronia melanocarpa* fruit juice at a dose of 5 ml/kg had no significant effect on immobility time after 33- and 78-day administration;

1.3.3. *Aronia melanocarpa* fruit juice at a dose of 10 ml/kg after 33- and 78-day administration reduced depressive-like behavior by shortening immobility time. This effect is even more significant due to the fact that in the open field test the same animals have reduced locomotor activity;

1.3.4. Chlorogenic acid at a dose of 20 mg/kg did not reduce depressive symptoms after 33- and 78-day administration.

1.4. Pain sensitivity in the hot plate test

1.4.1. Bilateral ovariectomy reduced thermal pain threshold sensitivity registered at day 79;

1.4.2. *Aronia melanocarpa* fruit juice administered at doses of 5 ml/kg and 10 ml/kg increased the hit pain threshold sensitivity of ovariectomized animals after 79 days of administration;

1.4.3. Chlorogenic acid at a dose of 20 mg/kg increased the thermal pain threshold sensitivity of ovariectomized animals after 79 days of administration.

2. In an experimental model of bilateral olfactory bulbectomy, the following changes in behavior are found:

2.1. Hyperactivity in the elevated plus maze test

2.1.1. Olfactory bulbectomy induces hyperactivity, demonstrated by an increase in the total number of entries into the open and closed arms of the maze;

2.1.2. After 14 days of administration, chlorogenic acid, ferulic acid and gallic acid at a dose of 20 mg/kg prevented the development of hyperactive behavior.

2.2. Anxiety in the elevated plus-maze test

2.2.1. Olfactory bulbectomy resulted in the development of anxiety-like behavior, manifested by significantly lower number of entries into and time spent in the open arms of the maze, and greater number of entries and time spent in the closed arms. Low ratios of open arm entries to total entries and open arm dwell time to total open and closed arm dwell time ratios were found;

2.2.2. Chlorogenic acid, ferulic acid and gallic acid after 14 days of administration at a dose of 20 mg/kg showed an anxiolytic-like effect in bullectomized animals, increasing the number of entries into the open arms of the maze and the time spent there, as well as the ratio of the number of entries into the open arms to the total number of entries and the dwell time in the open arms to the total dwell time in the open and closed arms. The effect is most pronounced with chlorogenic acid.

2.3. Memory and learning in a one-way passive avoidance test

2.3.1. Olfactory bulbectomy causes deterioration of learning and memory processes, manifested by a shortening of latency in memory tests at 3^{th} and 24^{th} hours after training;

2.3.2. Chlorogenic acid, ferulic acid and gallic acid after 14-day administration at a dose of 20 mg/kg improve memory and learning processes in bulbectomized animals, manifested by prolongation of the latency time at the 3rd and 24th hours in the memory tests.

2.4. Memory and learning in a two-way active avoidance test

2.4.1. Olfactory bulbectomy caused a deterioration of learning and memory processes, manifested by a decrease in the number of conditioned reflex avoidances in the training sessions on days 1^{st} and 2^{nd}, as well as in the memory test;

2.4.2. Chlorogenic acid, ferulic acid, and gallic acid at a dose of 20 mg/kg after 14 days of administration improved memory and learning processes in bulbectomized animals, demonstrated by an increase in the number of conditioned reflex avoidances in training sessions on days 1^{st} and 2^{nd}, as well as in the test of memory.