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Effects of *Rhodiola rosea* and aspirin on behaviour and some biochemical parameters in old mice

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Abstract. This study investigates the effects of aqueous *Rhodiola rosea* extract and low-dose and high-dose aspirin on behaviour and biochemical parameters in aged C57BL/6J mice. Both substances are widely recognized for their therapeutic properties and potential geroprotective effects in simpler organisms such as yeast and *Drosophila*. Aspirin, or acetylsalicylic acid, is a medication used for the treatment of pain and fever and also possesses anti-inflammatory and antipyretic properties. It is found to prolong lifespan of model organisms if taken throughout life but not in the old age. *R. rosea* is considered a geroprotector with rejuvenating properties. It contains a variety of compounds that may act as anti-inflammatory or anti-cancer agents. Additionally, evidence suggests that *R. rosea* extracts improve quality of life in the elderly and extend lifespan.

Mice aged 17 months (comparable to approximately 80 human years) were administered *R. rosea* extract or aspirin (5 mg/kg and 25 mg/kg body mass daily) for two months. Behavioural assessment using the open field test revealed no significant differences between the groups, although trends suggested increased anxiety-like behaviour in mice receiving 25 mg aspirin. Biochemical analysis showed no statistically significant changes in lipid peroxidation in the brain or liver enzyme activities (alanine aminotransferase, ALT, and aspartate aminotransferase, AST), although a trend toward elevated ALT activity with aspirin supplementation was observed, indicating potential liver stress. These findings highlight the complexity of assessing geroprotective interventions in aged organisms and suggest that both *R. rosea extract* and aspirin, at the tested doses, may have limited efficacy in improving healthspan in older mice. Further studies with larger cohorts and additional biomarkers are needed to explore these preliminary findings.

Keywords: aspirin, ageing, healthspan, *Rhodiola rosea*, mice, food additive, behaviour.

1. INTRODUCTION

From an early history of humankind, herbs and mushrooms were used as medications in folk medicine. Over time, the healing properties of folk remedies have been and are being scientifically confirmed. These include plant adaptogens, which exhibit stimulating activity on physical or mental health (Todorova et al., 2021a). The term adaptogens was firstly honed by soviet scientists in 1947. Being relatively young word, it is well received in global community and by this moment there are nearly a thousand publications about different adaptogens in PubMed. These substances do not have a specific defined mechanism of action. In 1980 it was shown that adaptogens not only influence physical resistance but also resistance to biological and chemical factors. Some of adaptogens became prohibited at the sports events because of their obvious benefits to performance. Our adaptogen of choice in this study is extract from the rhizomes of *Rhodiola rosea*. Though *R. rosea* is a fairly old medication, as it was used by the Vikings or in ancient

China, studies of *R. rosea* adaptogenic properties are conducting since the middle of 20th century (Han et al., 2022). Complete properties of each of *R. rosea* compounds is yet to be studied and discovered. Some of most active compounds are phenylpropanoids, phenylethanol, flavonoids, monoterpenes and phenolic acids, with salidroside, tyrosol, rosavin, and triandrin. Athletes who want to enhance their durability during training sessions are already using *R. rosea* on a daily basis (Tinsley et al., 2024).

One of most commonly mentioned and studied compound of *R. rosea* is salidroside. Salidroside by itself shows wide range of effects including neuroprotective, geroprotective and many other(Calabrese et al., 2023). However, it is known that the effect of pure compounds can be weaker than in a complex herbal preparation, where a synergistic effect is exhibited (Bayliak and Lushchak, 2011). Another compound we focus in this study is acetylsalicylic acid, or aspirin, a derivative of salicylic phenol acid found in willow bark, another herb that is widely used for medicine. Before 1897, up until aspirin was discovered by Felix Hoffman, people were using willow bark and other salicylate-containing plants for pain relief. Knowledge of these plants was present during times of ancient Egypt and Greece (Miner & Hoffhines, 2007). Additionally, aspirin has the ability to inhibit platelet aggregation and is commonly used to prevent thrombosis and myocardial infarction. Aspirin is classified as a non-selective cyclooxygenase inhibitor and is available in various forms. Nowadays aspirin is widely used for its anti-inflammatory properties, pain relief, antiplatelet effect and many more. It was also shown to potentially reduce risk of cancer and improve lifespan but only when used from young age (Skriver et al., 2024). Both *Rhodiola rosea* and aspirin were found to have lifespan prolonging effects in model organism like yeast and Drosophila (Bayliak and Lushchak, 2011; Gospodaryov et al., 2013) and therefore are considered as geroprotectors. By definition, a good geroprotector should not show any difference from control or even restore some parameters to their level in young organisms. The main function of geroprotectors is to fight with dysfunctionalities which occur in the old body. Despite, there is a scientific debate to call aging a disease, (De Winter, 2015), but researchers try to find effective remedy to prevent or treat aging. In this study, we compare certain anti-aging effects of *R. rosea* aqueous extract and acetylsalicylic acid in old mice. We focused on the behavioural changes in mice and some biochemical parameters showing liver and cortex health.

2. Materials and Methods

2.1 *Design of experiment*

In our study we use C57BL/6J female mice. Mice were obtained from Bohomoletz Institute of Physiology (Kyiv, Ukraine) and bred in our animal facility. When mice reached 17 months of age we randomly divided mice in 4 groups. Approximately from 5 to 8 mice in the group. First group was control, which consists of mice which continued to be fed a standardized diet. Second group of mice was fed a standard diet with adding of aqueous *R. rosea* extract to drinking water. Third and fourth groups were supplemented by acetylsalicylic acid in concentrations of 5 and 25 mg/kg body mass/day. Concentrations were used those described in literature (Teramoto et al., 2021). Respective solutions of acetylsalicylic acid were added to drinking water. It was taken into account that one mouse consumes 5 ml of water per day. Mice were kept on the respective regimes for two months. Mice were housed under a 12-h light/dark cycle (6 a.m./6 p.m.) at 22 ± 2 ◦C with 50–60% humidity. The standard diet consisted of 21.8% protein, 4.8% fat, 69.1%

carbohydrates, and 3.9% fiber. All experimental protocols were approved by the Animal Experimental Committee of Vasyl Stefanyk Precarpathian National University (Ukraine) and were conducted in accordance with Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

2.2. Preparation of aqueous R. rosea extract

To prepare the extract, we used the dried rhizome of *R. rosea*, which was ground with a coffee grinder. Next, we mixed the powder with the boiled water in a ratio of 1:20 (1 g of crushed plant material: 20 ml of water), followed by boiling for 30 minutes in a water bath. After that, the filtration procedure was carried out using filter paper. The volume of the filtrate was made up to the original volume with water. The received extract was aliquoted and frozen and kept at -20C in the refrigerator. Before using, the aliquots were thawed at room temperature.

2.3. Behavioural parameters

The open field test was conducted in a polyvinyl chloride chamber measuring 40 × 40 × 40 cm, divided into 16 squares, each 10 × 10 cm in size. The experimental room maintained the same air temperature and lighting conditions as the mouse breeding rooms. Motor, exploratory, and anxiety-like behaviours were analyzed using ToxTrac software (version 2.98) developed by Magnus Andersson's team (https://sourceforge.net/projects/toxtrac/) (Rodriguez et al., 2018). Over a 10-minute observation period, motor activity was measured by the total distance travelled. Exploratory behaviour was evaluated based on the distance covered in the maze and the time spent in the outer squares. Anxiety-like behaviours were assessed by recording the time spent in the central squares (inner zone) and the latency time for each mouse (Seibenhener & Wooten, 2015).

2.4. Measurement of level of lipid peroxides

The frozen cortex was homogenized in 96% ethanol at a ratio of 1:10, centrifugated for 10 min at 13,200 rpm, 4°C, and supernatants were collected. For determination of lipid peroxides (LOOH), we used a method based on the ability of lipid peroxides to oxidize Fe^{2+} to Fe^{3+}. Then Fe^{3+} ions form a coloured complex with xylenol orange that absorbs light at 580 nm at low pH. The reaction mixture contained $FeSO_4$*7H2O (1 M), xylenol (4 mM), water and supernatant. As an inner control was used cumene hydroperoxide (1 mM) (Lushchak et al., 2008).

2.5. Measurement of activities of aspartate aminotransferase (ALT) and alanine aminotransferase (AST)

The next step in our study was to determine the activity of ALT and AST in blood plasma by the Reitman-Frenkel method. We used ready-made kits from the Reagent company (Dnipro, Ukraine). The determination of ALT activity is based on the the ability of pyruvate, a product of ALP reaction, to form a yellow compound at addition of 2,4-dinitrophenylhydrazine, namely pyruvic acid hydrazone, with an absorbance at 500-550 nm. To determine the activity of ALT, we used a substrate-buffer solution (pH 7.4), which contained 100 mM phosphate buffer, 200 mM alanine and 2 mM alpha-ketoglutarate, 1 mM pyruvate. Also, after incubation, we added 1 mM

2,4-dinitrophenyl-hydrazine in 1 M HCl. And then we added 0.4 M NaOH to stop reaction. We performed the measurements on a microplate using a HiPo MPP-96 reader.

The method for determination of AST activity is similar to the method for ALT activity assay. The only difference is that alpha-ketoglutarate is aminated with aspartate under the action of the enzyme aspartate aminotransferase to form glutamate and oxaloacetate, which is spontaneously decarboxylated to pyruvic acid. After the addition of 2,4-dinitrophenylhydrazine, pyruvic acid hydrazone is formed, with an absorbance optimum at 500-550 nm. We used a substrate-buffer solution (pH 7.4), which contained 100 mM phosphate buffer, 200 mM aspartate, 1 mM alpha-ketoglutarate, 1 mM pyruvate. After incubation, we added 1 mM 2,4-dinitrophenyl-hydrazine in 1 M HCl. Then we added 0.4 M NaOH. Measurements were carried out on a microplate using a HiPo MPP-96 microplate reader at a wavelength of 492 nm.

2.6. Statistical analysis of the results

Statistical analyses were conducted using Microsoft Office LTSC Professional Plus 2021 and GraphPad Prism 10.3.0(507) software. To identify statistical differences between groups, one-way ANOVA was applied, followed by Holm-Sidak test for multiple comparisons. A difference was considered statistically significant at $P \leq 0.05$.

3. RESULTS AND DISCUSSION

Fig. 1 shows the dynamics of body mouse mass over 15 weeks for 4 different groups: a control group, a group that received *Rosea aqueous extract*, a group that received 5 mg of aspirin (ASA 5mg), and a group that received 25 mg of aspirin (ASA 25mg).

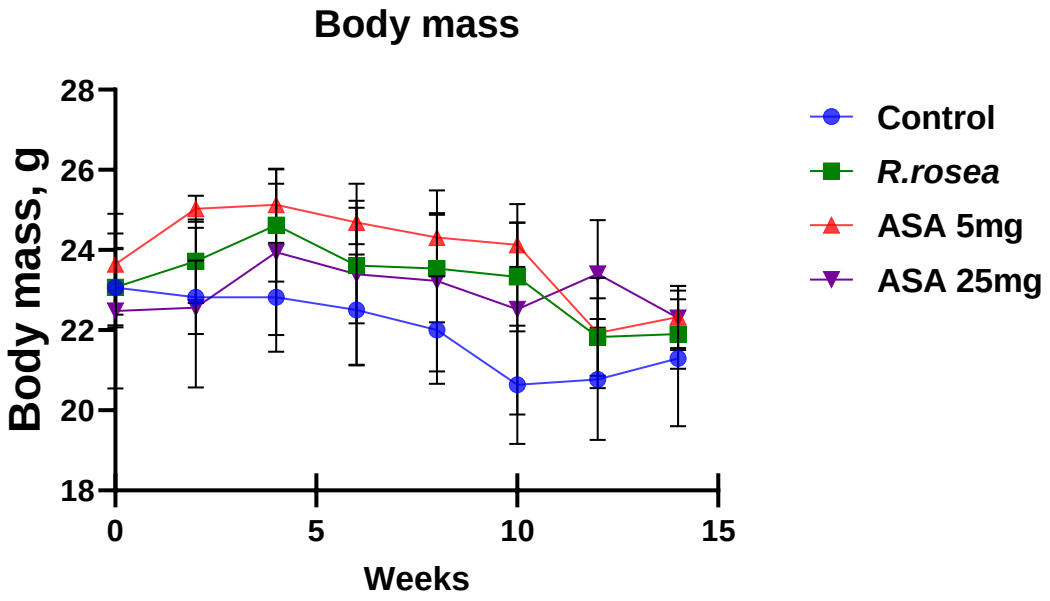

Figure 1. Changes in body mass of mice during the 15-week experiment. Mice fed the basic food (control group) or basic food supplemented with *R. rosea* (*R. rosea* group), 5 mg aspirin (ASA 5mg) and 25 mg aspirin (ASA 25mg) in drinking water

In the control group, the body mass relatively stable during first 8 weeks, but then body mass showed a tendency to decrease, that it can be connected with senescence of the body. In the *R. rosea* group, there was a gradual increase in body mass up to week 4, which may indicate a stimulating effect of this plant. Further, body mass of mice fed *R. rosea* extract decreased over time, but retained higher than in the control group. In the groups that received 5 mg aspirin, there was similar effects on body mass showed in *R. rosea* treated group. In the ASA 25mg group, there was a sharp increase in body mass from the beginning of the experiment, and then body mass decreased gradually. Overall, the graph demonstrates significant differences in the dynamics of body mass between the groups that received different experimental interventions, allowing us to suggest different effects of *R. rosea* and different doses of aspirin on body mass indicators.

Before euthanasia we conducted an open field behavioural test. We determined the anxiety and mobility of old mice fed aspirin and *R. rosea*. The recording lasted 10 minutes. As can be seen from Fig. 2(A), there was no significant difference in the average speed.

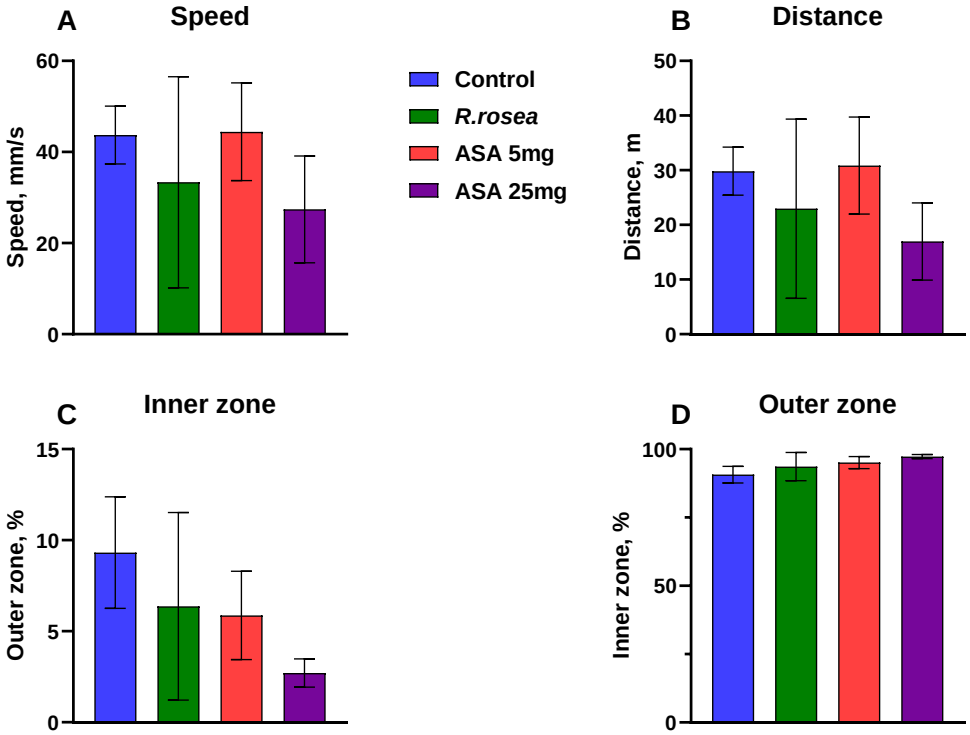

Figure 2. Behaviour in mice in open field test. Mice were fed the basic food (control group) or basic food supplemented with *R. rosea* (*R. rosea* group) or different dosed of aspirin (ASA 5mg and ASA 25mg) in drinking water for 15 weeks. Average speed (A), total distance (B) time spent in inner zone (C), time spent in outer zone (D). n = 3-6. Holm-Sidak test was used for multiple comparisons

However, there was a tendency to increase the speed among mice that consumed 5 mg of aspirin The next step was to determine the distance travelled by the mice. As can be seen from Fig. 2(B), there is no difference between the groups, although there was a tendency that the mice

that consumed 5 mg of aspirin travelled the biggest distance for 10 min among all mice. Next, we determined in which zone the open field mice spent the most time. As can be seen from Fig. 2(C), the mice that consumed 25 mg of aspirin spent the least time in the central quadrants, which may indicate the lowest level of stress, but there was no significant difference, only a trend. We also determined how much time the mice spent in the outer squares. As can be seen from Fig.2(D), the mice that consumed 25 mg of aspirin spent the most time in the outer quadrants, that corresponds data on time spent in inner quadrants.

Since we observed tendency to changes in behaviour of old mice fed with supplements, we decided to determine the presence of biochemical alterations in the brains of experimental mice. For this, we determined the levels of lipid peroxides (LOOH), a marker of the intensity of lipid peroxidation, in the mouse cortices. There was no significant difference observed.

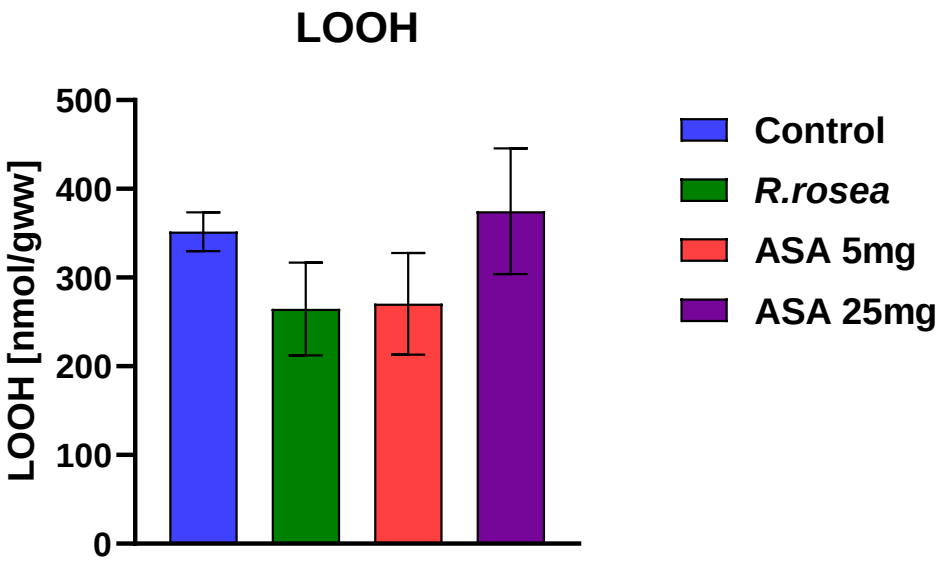

Figure 3. Lipid peroxide level in the cortexes of 20-month-old mice fed the basic food (control group) or basic food supplemented with *R. rosea* extract (*R. rosea* group), or different dosed of aspirin (ASA 5mg and ASA 25mg) in drinking water for 15 weeks. n = 3-6. Holm-Sidak test was used for multiple comparisons.

For next, we decided to check how the supplements affected some biochemical parameters, related to liver functioning, in mouse blood. To check this, we measured the activity of two liver enzymes, namely aspartate aminotransferase and alanine aminotransferase (Fig. 4). As we can see in Fig. 4A and B, there was no statistical difference in the enzyme activities between all groups. However, the de Ritis ratio, or AST/ALT ratio, showed a downward trend, but ALT activity demonstrated the opposite trend.

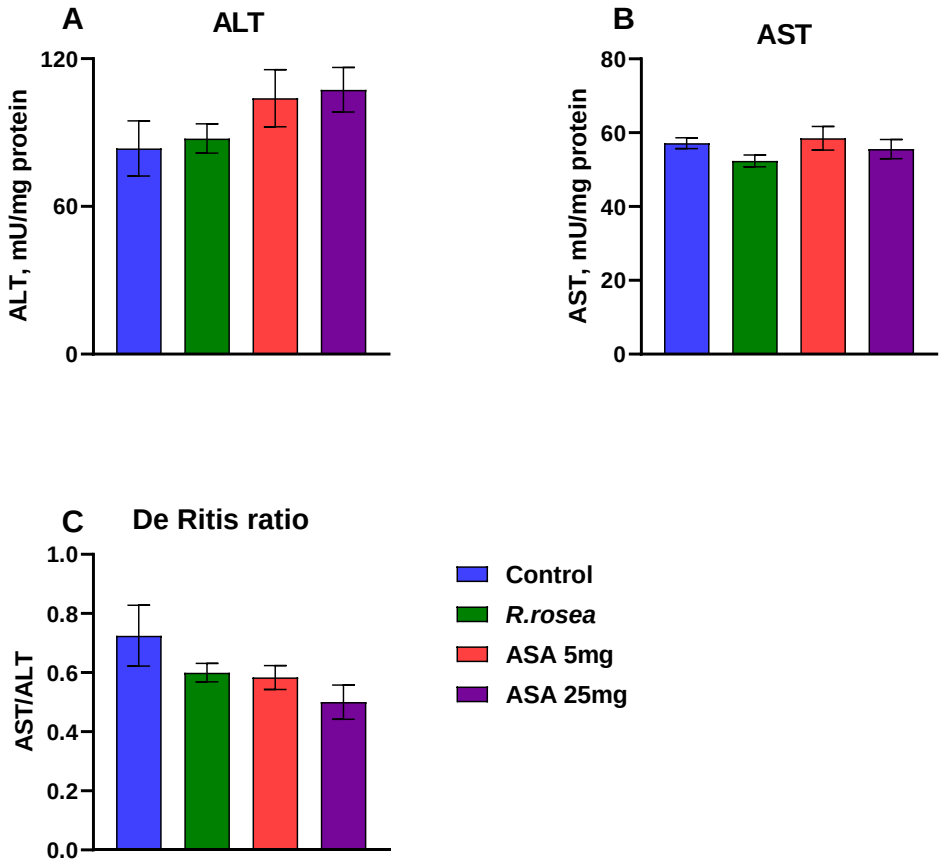

Figure 4. Enzyme activity in blood of 20-month-old mice fed the basic food (control group) or basic food supplanted with *R. rosea* (*R. rosea* group) or different dosed of aspirin (ASA 5mg and ASA 25mg) in drinking water. Alanine aminotransferase activity (A), aspartate aminotransferase activity (B), and De Ritis ratio (C). n = 3-6. Holm-Sidak test was used for multiple comparisons

By investigating the effects of substances which are known to prolong lifespan, we aimed to assess their impact on healthspan and examine whether *R. rosea* and aspirin could rejuvenate old mice. Across all measured parameters, no statistically significant differences were observed, but there were some important non-significant trends.

Firstly, aspirin in our experimental groups showed a non-significant change in serum ALT activity at both concentrations tested. The role of ALT activity in aging organisms remains unclear, as existing studies report conflicting results: some indicate a decrease in ALT activity with age, while others suggest an increase (Dong et al., 2012). In our study, the De Ritis ratio in old mice was less than 1. By contrast, a previous study reported on a De Ritis ratio of approximately 3.8 in younger mice of the same strain (Kawashita et al., 2019). Typically, a De Ritis ratio below 1 is associated with a healthy, recovering, or chronic state (Botros & Sikaris, 2013). This discrepancy suggests that ALT activity may increase in older mice. Our data indicate that aspirin might elevate ALT activity, although most studies report that aspirin reduces ALT activity, with an increase observed in only 3% of cases (Teramoto et al., 2021).

In summary, while the De Ritis ratio < 1 in our study aligns with a chronic or recovering state, the potential influence of aspirin on ALT activity in aged mice warrants further investigation. These findings highlight the complexity of ALT dynamics in aging and its modulation by pharmacological agents.

Secondly, our study did not reveal any significant differences in behavioral tests across the experimental groups. This outcome prompted us to compare our findings with those of previous studies investigating the effects of *Rhodiola*. Interestingly, several researchers reported that mice treated with *Rhodiola* exhibited increased time spent in the central zone during open field tests, a behavior commonly interpreted as reduced anxiety levels (Li et al., 2022). In addition, the behavioral effects of *Rhodiola* might be influenced by the strain, age, or sex of the mice, as these biological factors are known to modulate responses in behavioral tests. The mice used in our study may differ in their baseline anxiety or stress reactivity compared to those used in prior experiments. Additionally, environmental factors, such as housing conditions and test room settings, can significantly impact behavioral outcomes and might explain some variability between studies (Silvero-Isidre et al., 2018).

Finally, the effects of *Rhodiola* could depend on the specific behavioral paradigms used. While the open field test is a widely accepted measure of anxiety, it primarily assesses exploratory behavior in a novel environment (Perfumi & Mattioli, 2007). Other behavioral tests, such as the elevated plus maze or light/dark box, might provide complementary insights into anxiolytic potential of Rhodiola and could be worth exploring in future experiments (Kawashita et al., 2019).

Taken together, the lack of observable differences in our study underscores the complexity of evaluating natural adaptogens like *Rhodiola*. It highlights the importance of standardized methodologies and the need for further research to elucidate the underlying mechanisms driving its behavioral effects (Kawashita et al., 2019).

CONCLUSIONS

The study revealed that dietary supplements of *Rhodiola rosea* aqueous extract and aspirin, administered at concentrations of 5 and 25 mg/kg body mass per day, did not induce statistically significant changes in behavioural parameters such as average speed, distance travelled over 10 minutes, or the time spent in the inner and outer squares during the open field test. Additionally, these supplements did not significantly alter lipid peroxide levels in the brain or the activities of the enzymes AST and ALT in blood plasma of old mice. However, a trend toward increased ALT activity was observed in mice consuming aspirin, which may suggest potential liver damage. These findings indicate that while the tested doses of *R. rosea* extract and aspirin do not significantly impact behavioural or biochemical parameters in aged mice, caution may be warranted due to possible hepatic effects associated with aspirin supplementation. Obviously, the use of substances with a geroprotective effect at a very old age does not have the same protective effect as in earlier use.

Declaration of competing interest

The authors declare no competing interests.

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Віталій Деркачов, Владислав Березовський. Вплив *Родіоли рожевої* та аспірину на поведінку та деякі біохімічні показники у старих мишей. *Журнал Прикарпатського університету імені Василя Стефаника. Біологія*, Том 11 (2024), С.93-С.103.

Це дослідження вивчає вплив водного екстракту Rhodiola rosea та низьких і високих доз аспірину на поведінку і біохімічні показники у старих мишей лінії C57BL/6J. Обидві речовини широко відомі своїми терапевтичними властивостями та потенційними геропротекторними ефектами на простих організмах, таких як дріжджі та Drosophila. Аспірин, або ацетилсаліцилова кислота, є препаратом, що використовується для лікування болю та гарячки, а також має протизапальні та жарознижувальні властивості. Встановлено,

що аспірин подовжує тривалість життя модельних організмів, якщо його вживати протягом усього життя, але не в старшому віці. Rhodiola rosea вважається геропротектором із властивостями омолодження. Вона містить ряд сполук, які можуть діяти як протизапальні або протиракові агенти. Крім того, є свідчення, що екстракти R. rosea покращують якість життя у літніх людей і подовжують тривалість життя.
Мишам віком 17 місяців (що відповідає приблизно 80 людським рокам) протягом двох місяців вводили екстракт родіоли або аспірину (5 мг/кг і 25 мг/кг маси тіла щодня). Поведінкова оцінка за допомогою тесту "відкрите поле" не виявила значущих відмінностей між групами, хоча спостерігалися тенденції до підвищення тривожної поведінки у мишей, які отримували аспірин у дозі 25 мг. Біохімічний аналіз не виявив статистично значущих змін у рівні перекисного окислення ліпідів у мозку або активності ферментів печінки (аланінамінотрансфераза, АЛТ, і аспартатамінотрансфераза, АСТ), хоча було помічено тенденцію до підвищення активності ALT при вживанні аспірину, що може свідчити про потенційне навантаження на печінку. Ці результати підкреслюють складність оцінки геропротекторних втручань у старих організмах і свідчать про те, що екстракт R. rosea та аспірин у випробуваних дозах можуть мати обмежену ефективність у покращенні здоров'я в літньому віці. Для підтвердження цих попередніх висновків потрібні подальші дослідження з більшими групами та додатковими біомаркерами.

Ключові слова: аспірин, старіння, здоров'я, Роділа рожева, миші, харчова добавка, поведінка.