Journal of Experimental Biology and Agricultural Sciences, August - 2022; Volume – 10(4) page 852 – 860

Journal of Experimental Biology and Agricultural Sciences

http://www.jebas.org

ISSN No. 2320 – 8694

Antiradical and Oxidative Stress Release Properties of *Trifolium pratense* L. extract

Lyubov S. Dyshlyuk, Maria A. Osintseva, Oksana V. Kozlova, Natalya V. Fotina, Alexander Yu. Prosekov*

Kemerovo State University, 6 Krasnaya St., Kemerovo, Russia, 650000

Received – June 20, 2022; Revision – July 19, 2022; Accepted – August 04, 2022
Available Online – August 30, 2022

DOI: http://dx.doi.org/10.18006/2022.10(4).852.860

KEYWORDS

Caenorhabditis elegans

Trifolium pratense L.

Callus culture

Ononin

Chlorogenic acid

Genistein

Biochanin A

Antiradical activity

ABSTRACT

Low adaptive capacity and oxidative stress are the factors leading to cellular dysfunction, protein and lipid peroxidation, and the development of diseases. In recent decades, there has been a trend toward the active use of plant-based antioxidants. *Trifolium pratense L.* is a promising plant for the pharmaceutical and food industry and has anti-radical properties. This work is devoted to studying the antiradical and oxidative stress-released properties of *T. pratense* in *Caenorhabditis elegans* under oxidative and temperature stress. The objective of this research was to evaluate the anti-radical properties of the *T. pratense* extracts and individual BAS (chlorogenic acid, ononin, biochanin A, genistein) and analysis their influences on the oxidative stress of *Caenorhabditis elegans* in the presence of paraquat. Analysis of the antiradical properties revealed that chlorogenic acid has the maximum ability to neutralize the free radical (35.49µmol). A separate analysis of oxidative stress revealed high ononin activity at concentrations of 10, 50, and 100 µmol at 48 hours of cultivation. Biochanin A increases survival by 13.1% compared to the control. The use of the extract (500µmol) contributed to an increase in survival on day 1 of incubation. Under conditions of thermal stress, ononin (50 and 200 µmol) has a positive effect on the viability of *C. elegans*. The extract and BAS of *T. pratense* are characterized by high antiradical activity. In addition, the ability to influence the viability of *C. elegans* was revealed. Therefore, it is worthwhile to further study the biological properties of *T. pratense* for use in geroprotective therapy.

* Corresponding author

E-mail: a_piskaeva@mail.ru (Alexander Yu. Prosekov)

Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI] (http://www.horizonpublisherindia.in/).

1 Introduction

Aging is a process of degenerative changes in cells, tissues, and organs of the body that is accompanied by reduced adaptive activity to stressful environmental conditions and an increased risk of diseases and deaths (Hou et al. 2019). One key factor influencing the development and progression of many chronic diseases is oxidative stress. Oxidative stress is a state of the body resulting from excessive production and/or low ability to eliminate free radicals (reactive oxygen species, peroxides, etc.) (Leite et al. 2020; Monteiro-Alfredo et al. 2020). ROS plays an important role in maintaining cell homeostasis. However, their increased levels cause cellular dysfunction, protein and lipid peroxidation, and DNA damage, which eventually lead to irreversible damage and cell death with the gradual development of degenerative diseases like diabetes, arthritis, cardiovascular diseases, oncology, etc.) (van der Pol et al. 2019).

A key strategy for preventing the development of degenerative diseases and aging is the inclusion of antioxidants in the diet (Roxo et al. 2020). Synthetic antioxidants have several negative effects, including allergic reactions, liver damage, etc. on the human body. Plant raw materials rich in biologically active substances (BAS) of an adaptogenic orientation can be used as an alternative to synthetic antioxidants (Hou et al. 2019). Various studies have been conducted that show the influence of adaptogenic plants on the state of the body under stressful conditions (Chen et al. 2016; Jattujan et al. 2018; Tambara et al. 2018; Wang et al. 2018). According to Brekhman and Dardymov (1969), adaptogens are characterized by various properties, including an increase in the nonspecific resistance of the body, normalizing the state of the body regardless of the nature of the pathology, do not affect the normal functions of the body more than required, and be safe (Hou et al. 2019).

From a pharmacological point of view also, plant raw materials are a suitable source of chemical compounds for the treatment of various diseases, including those caused by oxidative stress. Further, the antioxidant activity of plants is related to their chemical composition, namely the presence of BAS (polyphenols, vitamins, organic acids, etc.). Thus, the search for promising sources of BAS is relevant to normalizing the reducing-oxidizing balance in the body.

Trifolium pratense is a representative of the genus Trifolium, a forage plant that is widely used in agriculture. The main biologically active compounds of the plant include flavonoids (Quercetin, Kaempferol, Apigenin, Hyperoside), isoflavonoids (Biochanin A, Formononetin, Daidzein, Genistein, Prunetin), phenolic acids (caffeic, rosemary, chlorogenic, salicylic, n-coumaric, ferulic), etc. (Akbaribazm et al. 2020). The rich composition of secondary metabolites gives the plant a wide range of pharmacological properties, including anti-inflammatory, antioxidant, antibacterial, antiviral, antitumor, antifungal, and neuroprotective (Luo et al. 2020).

To study the role of oxidative stress and phytochemical compounds in aging processes, a model system *Caenorhabditis elegans*, a soil free-living nematode are used in vivo (Wilson et al. 2006; Thabit et al. 2019; Roxo et al. 2020; González-Peña et al. 2021; Wang et al. 2021). The nonparasitic nematode *C. elegans* is characterized by changes in behavior and physiological health indicators (stress resistance, degeneration of the nervous system, changes in the structure of muscle tissue) which are similar to humans (Wang et al. 2020). Therefore, an assessment of the adaptogenic effect on model organism *C. elegans* can provide detail related to the positive effect of plant extracts on human life expectancy. The objective of this study was to examine the antioxidant activity and stress resistance of the *T. pratense L.* extract and its individual BAS in the *C. elegans* model system.

2 Materials and Methods

Callus culture of earlier stage *T. pratense* and standard BAS individual (Sigma-aldrich, USA) were used for the preparation of extract in this study (Dyshlyuk et al. 2021). For this, *T. pratense* callus was cultured on Gamborg nutrient medium supplemented with kinetin (2.00 mg/L), 6-BAP (0.10 mg/L), IUK (2.00 mg/L), and 2.4-D (2.00 g) from seedlings (Gamborg et al. 1968). The extract was prepared as described by Dyshlyuk et al. (2021).

The antioxidant activity of *T. pratense* callus extract and individual BAS were determined to the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical as per the method given by Re et al. (1999). To determine the antioxidant activity of the callus culture extract, it was dried with the help of Mini Spray Dryer B-290 (BUCHI, Switzerland) having parameters like the temperature of 105 °C, liquid feed rate of 6.2 mL/min, and main air flow rate of ~90 m^3/h (Langrish and Premarajah 2013).

Next, the effect of *T. pratense* callus cultures extracts and individual BAS was used to analyze the stress resistance in *C. elegans* against oxidative and temperature stress. For this *C. elegans* multiplication was carried out according to the standard method given by Rathor and Pandey (2018).

Individual BAS (chlorogenic acid, ononin, biochanin A, genistein) and their dry extract were prepared in DMSO of 10 mM concentration. Test solutions of BAS at various concentrations of 10, 50, 100, and 200 µmol and callus culture extract in three concentrations of 5, 50, and 500 µmol were prepared. The test solutions were stored at 4 °C until they were used.

Oxidative Stress release in *C. elegans* and effect of various concentrations of callus culture extract and standard extract

evaluated as per Rathor and Pandey (2018). For this, after adding 15 µl of the BAS or callus culture extract and 15 µl of 1M paraquat, the nematodes inoculated plates were left to incubate at a temperature of 20 °C, and after 24 and 48 hours of incubation, first and second counting of living and dead nematodes were carried out. The results of the effect of BAS on the oxidative stress resistance of nematodes were compared with the results of control nematodes that were incubated without adding the tested compounds.

For the estimation of temperature stress in *C. elegans* in the presence of BAS, the experiment was carried out as per the method described for oxidative stress release in *C. elegans* with some required changes. Further, the experiment was carried out without the addition of papaquat; throughout the experiment, the incubation temperature was 33 °C.

All analyses were repeated 3 times and statistical data was analyzed on Microsoft Office Excel 2007. Statistical analysis of the obtained data was carried out using a single-stage Student's paired criterion for each pair of interests. The differences were considered statistically significant at $p<0.05$.

3 Results and discussion

The results presented in Figure 1 revealed the antiradical activity of various concentrations of the *T. pratense* extract and BAS compared to standard Ascorbic acid in the neutralization of the ABTS. Results of the study suggested the highest ABTS radical scavenging activity from genistein (95.76%), followed by the chlorogenic acid (91.5%) and *T. pratense* callus culture extract (87.63%), and these were at par with the standard control ascorbic acid (94.09%). Among the various tested extracts lowest ability to absorb ABTS radicals (57.40%) was reported from Biochanin A.

Table 1 shows the EC_{50} values of *T. pratense* extracts, individual BAS, and ascorbic acid (control) in ABTS radical neutralization. The highest effective concentration was determined for ascorbic acid and

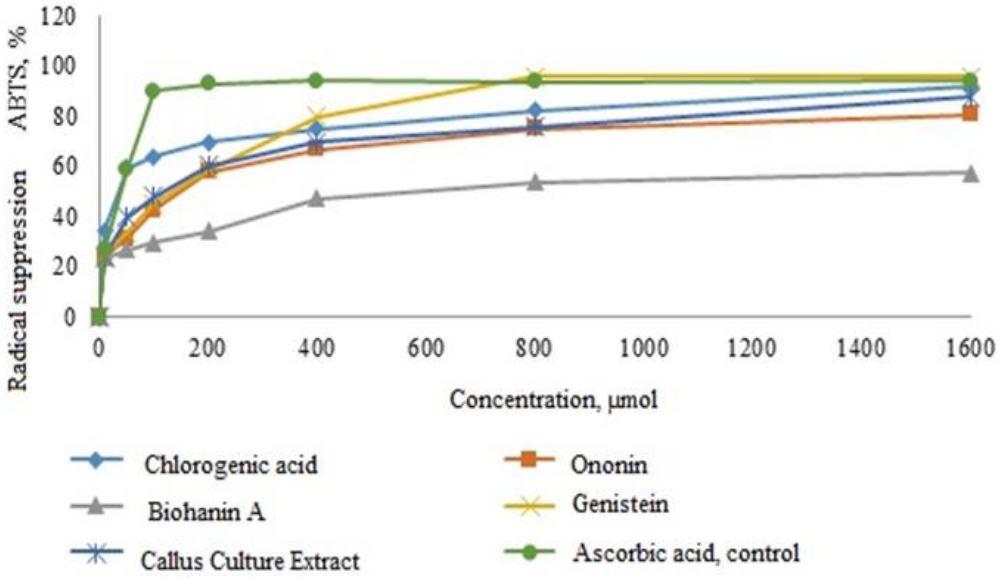

Figure 1 Antiradical activity of various concentrations of extract of callus cultures and individual BAS *T. pratense L.*

Table 1 Results of determining the effective concentration of ABTS radical suppression

No.	Name of sample	Effective concentration EC_{50}, µmol
1	Ascorbic acid (control)	38.24±1.89
2	Callus culture extract	117.60±3.62
3	Chlorogenic acid	35.49±1.59
4	Genistein	137.02±4.01
5	Biochanin A	582.20±6.64
6	Ononin	151.83±3.98

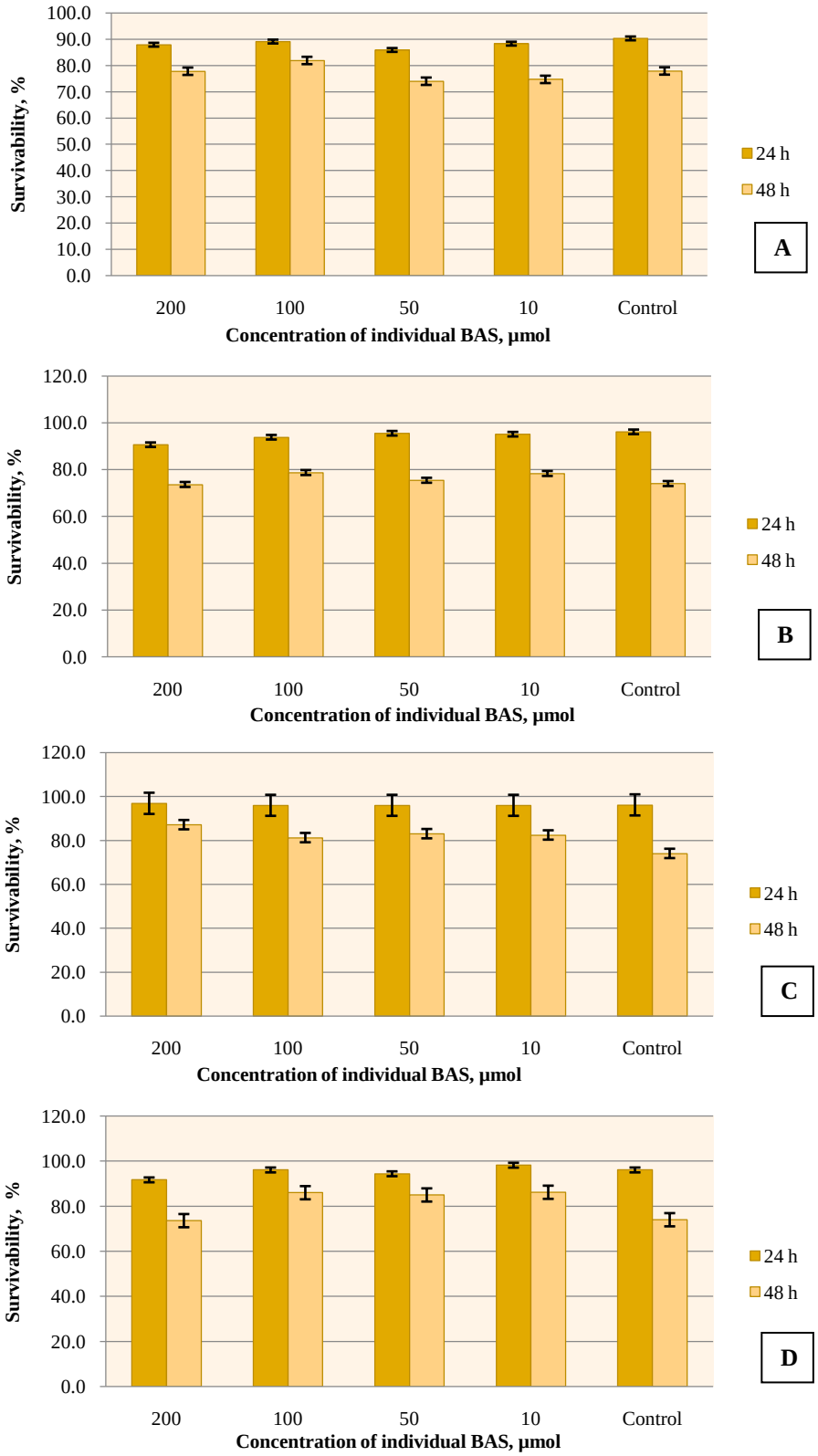

Figure 2 Effect of individual BAS on the resistance of *C. elegans* to oxidative stress (A) Effect of Chlorogenic, (B) Effect of Genistein, (C) Effect of Biochanin A, (D) Effect of Ononin

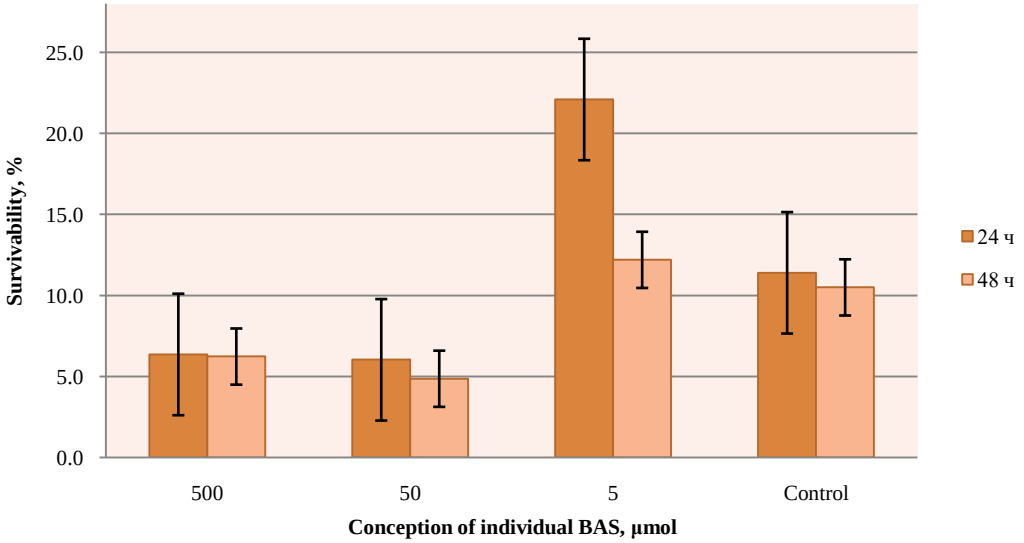

Figure 3 Effect of *T. pratense L.* callus culture extract on the resistance of *C. elegans* to oxidative stress

it is followed by chlorogenic acid (35.49 µmol), and these two are not significantly different. These results obtained are consistent with the literature data (Sun et al. 2014; Wang et al. 2021). The effective concentration of the callus culture extract was 117.60 µmol. From the studied literature, ABTS-radical absorption of methanol extract and various fractions of the plant has a value of 111.84 to more than 500 µg/ml (Esmaeili et al. 2015). Based on these indicators, it can be concluded that the extract has high antiradical activity.

To study the role of *T. pratense* callus culture extract and individual BAS (chlorogenic acid, ononin, biochanin A, genistein) in aging processes, a model system of the soil free-living *C. elegans* was used *in vivo*, since it is characterized by changes in behavior and physiological health indicators similar to humans (stress resistance, nervous system degeneration, changes in muscle tissue structure). Figure 2 presents a graphical representation of the effect of BAS tested at concentrations of 10, 50, 100, and 200 µmol on the oxidative stress resistance of *C. elegans*. The studied concentrations of individual BAS (10, 50, 100, 200 µmol) did not have any significant effect on the survival rate of *C. elegans* individuals under oxidative stress after 24 hours under the influence of 1M paraquat. After 48 hours of incubation ononin at concentrations of 10-100 microns had the greatest effect on the survival of nematodes. In addition to this BAS increased the survival rate of nematodes by 11.7% as compared to the control. The tendency to increase survival rate was also noted when using Biochanin A. The maximum survival rate was 87.1% compared to the control of 74.0%. The obtained data are in agreement with the findings of previous studies on the antioxidant properties of these BAS on other model systems (Wu et al. 2021; Dong et al. 2022). Chlorogenic acid and genistein did not affect the survival of nematodes under oxidative stress. It is important to note that there is no negative influence of chlorogenic acid and genistein on the survival of the model organism.

Figure 3 presents a graphical representation of the *T. pratense* callus culture extract for oxidative stress resistance with *C. elegans*. The results of the research revealed a positive effect of the extract in minimum concentration (500 µmol) on the survival of nematodes under oxidative stress. An increase in survival on the first day of cultivation by 93% compared to the control is associated with a wide range of compounds characterized by high antioxidant activity.

The results of the effect of individual BAS and extract on temperature stress are shown in Figures 4 and 5. At 48-hour incubation, the overall dynamics are characterized by an increase in the viability of *C. elegans* at ononin concentrations of 50 µmol and 200 µmol (by 9.4% and 8.6%, respectively). For the remaining BAS at 48 hours of incubation, there is a tendency to increase the survival rate of nematodes with an increase in the concentration of BAS. At 200 µmol of BAS, the average survival rate was increased by 11.8% as compared to the control samples. Use of the extract in a concentration of 500 µmol led to 100% death of *C. elegans* under both 24 and 48 hrs of incubation. However, when using concentrations of 5 µmol and 50 µmol, no changes in survival were reported as compared to the control. The data obtained are characterized by relatively low activity compared to data from the studied literature (Carranza et al. 2020; Cai et al. 2022). This is probably due to the deviation of the experimental conditions from the standard methodology.

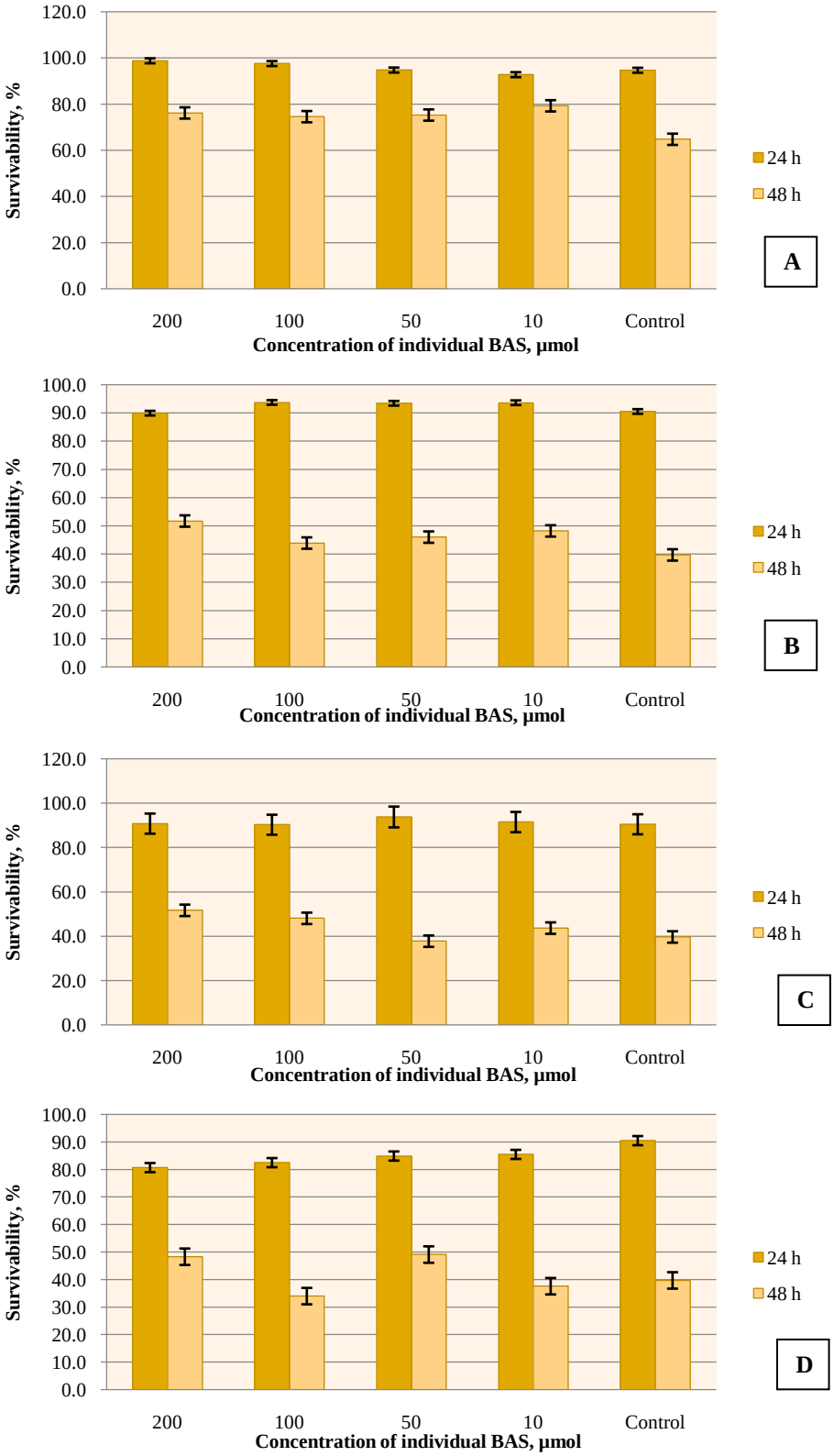

Figure 4 Effect of individual BAS of *T. pratense L.* callus culture on the resistance of *C. elegans* to temperature stress
(A) Effect of Chlorogenic, (B) Effect of Genistein, (C) Effect of Biochanin A, (D) Effect of Ononin

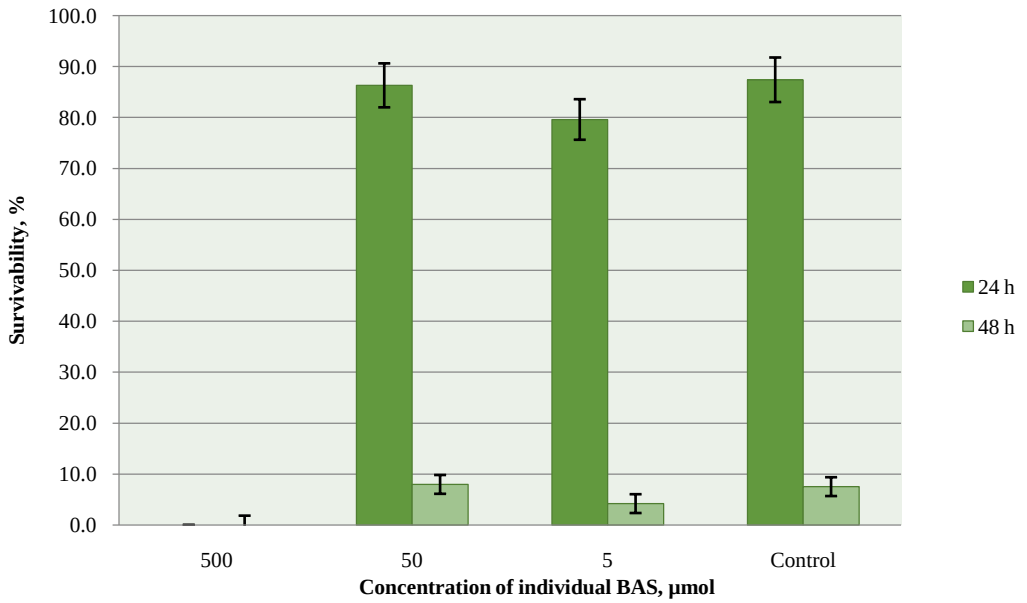

Figure 5 Effect of *T. pratense L.* callus culture extract. on the resistance of *C. elegans* to temperature stress

Conclusion

The antioxidant properties and the effect *T. pratense* callus culture extract and individual BAS (chlorogenic acid, ononin, biochanin A, genistein) on stress resistance were studied to determine the possibility of using this plant in geroprotective nutrition to increase the healthy life expectancy of the population. The data obtained showed a high antiradical activity of chlorogenic acid, followed by genistein, ononin, and callus culture extract. When studying the nematode survival under oxidative stress, callus culture extracts (5 µmol) and ononin (50 µmol) showed high indicators. When studying temperature stress, high rates are also characteristic of ononin and callus culture extract. Therefore, the results of the study suggested that *T. pratense* extract and BAS have a significant effect on the increase of the body's resistance to stressful conditions. In different cases, high rates of both antiradical activity and the ability to increase or decrease in vivo survival of the *C. elegans* were noted and leading to the further broadening of the study of the composition and biological properties of the *T. pratense* callus culture. This is relevant and promising for further use of geroprotective therapy.

Criterion of authorship

All authors are equally responsible for the research results and the manuscript.

Acknowledgments

The work was carried out with the financial support of the Ministry of Science and Higher Education of the Russian Federation (Project FZSR-2020-0006 – "Screening of biologically active substances of plant origin with geroprotective properties and development of technology for obtaining nutraceuticals that slow down aging".

Conflict of interest

The authors declare no conflict of interest.

References

Akbaribazm M., Khazaei M. R., & Khazaei. M. (2020). *Trifolium pratense* L. (red clover) extract and doxorubicin synergistically inhibits proliferation of 4T1 breast cancer in tumor-bearing BALB/c mice through modulation of apoptosis and increase antioxidant and anti-inflammatory related pathways. *Food Science & Nutrition*, *8*(8), 4276–4290. doi: 10.1002/fsn3.1724.

Brekhman I. I., & Dardymov I. V. (1969). New substances of plant origin which increase nonspecific resistance. *Annual Review of Pharmacology*, *9*, 419–430 doi: 10.1146/annurev.pa.09. 040169.002223.

Cai S. Q., Tang Z. M., Xiong C., Wu F. F., et al. (2022). The anti-inflammatory effects of apigenin and genistein on the rat intestinal epithelial (IEC-6) cells with TNF-α stimulation in response to heat treatment. *Current Research in Food Science*, *5*, 918–926. https://doi.org/10.1016/j.crfs.2022.05.011.

Carranza A. del V., Saragusti A., Chiabrando G. A., Carrari F., & Asis R. (2020). Effects of chlorogenic acid on thermal stress tolerance in *C. elegans* via HIF-1, HSF-1 and autophagy.

Phytomedicine, *66*, 153132. https://doi.org/10.1016/j.phymed.2019.153132.

Chen C., Song J., Chen M., Li Z., Tong X., et al. (2016). Rhodiola rosea extends lifespan and improves stress tolerance in silkworm, Bombyx mori. *Biogerontology*, *17*(2), 373–381 doi: 10.1007/s10522-015-9622-8.

Dong L., Yu L., Liu A., Alahmadi T. A., Almoallim H.S., & Durairaj K. (2022). Ononin mitigates streptozotocin-induced diabetic nephropathy in rats via alleviating oxidative stress and inflammatory markers. *Journal of King Saud University – Science*, *34*(6), 102029. https://doi.org/10.1016/j.jksus.2022.102029.

Dyshlyuk L. S., Fedorova A. M., Loseva A. I., & Eremeeva N. I. (2021). Callus cultures of *Thymus vulgaris* and *Trifolium pratense* as a source of geroprotectors. *Food processing: techniques and technology*, *51*(2), 423–432. doi: 10.21603/2074-9414-2021-2-423-432.

Esmaeili K. A., Taha M. R., Mohajer S., & Banisalam B. (2015). Antioxidant activity and total phenolic and flavonoid content of various solvent extracts from in vivo and in vitro grown *Trifolium pratense* L. (Red Clover). *BioMed Research International*, *643285* doi: 10.1155/2015/643285.

Gamborg O. L., Miller R. A., & Ojima. O. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, *50*, 151–158.

González-Peña M. A., Lozada-Ramírez J. D., & Ortega-Regules A.E. (2021) Carotenoids from mamey (*Pouteria sapota*) and carrot (*Daucus carota*) increase the oxidative stress resistance of Caenorhabditis elegans. *Biochemistry and Biophysics Reports*, *26*, 100989. doi: 10.1016/j.bbrep.2021.100989.

Hou L., Jiang M., Guo Q., & Shi W. (2019). Zymolytic Grain Extract (ZGE) Significantly Extends the Lifespan and Enhances the Environmental Stress Resistance of *Caenorhabditis elegans*. *International Journal of Molecular Sciences*, *20*(14), 3489. doi: 10.3390/ijms20143489.

Jattujan P., Chalorak P., Siangcham T., Sangpairoj K., & Nobsathian S. (2018). *Holothuria scabra* extracts possess anti-oxidant activity and promote stress resistance and lifespan extension in *Caenorhabditis elegans*. *Experimental Gerontology*, *110*, 158–171. doi: 10.1016/j.exger.2018.06.006.

Langrish T. A. G., & Premarajah R. (2013). Antioxidant capacity of spray-dried plant extracts: Experiments and simulations. *Advanced Powder Technology*, *24*(4), 771–779. https://doi.org/10.1016/j.apt.2013.03.020)

Leite N. R., de Araújo L., Dos Santos da Rocha P., Agarrayua D. A., et al. (2020). Baru Pulp (*Dipteryx alata* Vogel): Fruit from the Brazilian Savanna Protects against Oxidative Stress and Increases the Life Expectancy of Caenorhabditis elegans via SOD-3 and DAF-16. *Biomolecules*, 10(8), 1106. doi: 10.3390/biom10081106.

Luo L., Gao W., Zhang Y., Wang G., Wu H., & Gao W. (2020). Integrated Phytochemical Analysis Based on UPLC-MS and Network Pharmacology Approaches to Explore the Quality Control Markers for the Quality Assessment of *Trifolium pratense* L. *Molecules*, *25*(17), 3787. doi: 10.3390/molecules25173787.

Monteiro-Alfredo T., Matafome P., Iacia B. P., Antunes K. Á., et al. (2020). *Acrocomia aculeata* (Jacq.) Lodd. ex Mart. Leaves Increase SIRT1 Levels and Improve Stress Resistance. *Oxidative medicine and cellular longevity*, *5238650*. doi: 10.1155/2020/5238650.

Rathor L., & Pandey R. (2018). Age-induced diminution of free radicals by Boeravinone B in Caenorhabditis elegans. *Experimental Gerontology*, *111*(1), 94–106. DOI: 10.1016/j.exger.2018.07.005.

Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., & Rice-Evans C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, *26*(9–10), 1231–1237.

Roxo M., Peixoto H., Wetterauer P., Lima E., & Wink M. (2020). *Piquiá Shells* (Caryocar villosum): A Fruit by-Product with Antioxidant and Antiaging Properties in *Caenorhabditis elegans*. *Oxidative medicine and cellular longevity*, *7590707*. doi: 10.1155/2020/7590707.

Sun Z. X., Liu S., Zhao Z. Q., & Su R. Q. (2014). Protective effect of chlorogenic acid against carbon tetrachloride-induced acute liver damage in rats. *Chinese Herbal Medicines*, *6*(1), 36–41. doi: 10.1016/S1674-6384(14)60004-6.

Tambara A. L., de Los Santos Moraes L., Dal Forno A. H., Boldori J. R., et al. (2018). Purple pitanga fruit (*Eugenia uniflora* L.) protects against oxidative stress and increase the lifespan in *Caenorhabditis elegans* via the DAF-16/FOXO pathway. *Food and Chemical Toxicology*, *120*, 639–650. doi: 10.1016/j.fct.2018.07.057.

Thabit S., Handoussa H., Roxo M., Cestari de Azevedo B., El Sayed N., & Wink M. (2019). *Styphnolobium japonicum* (L.) Schott Fruits Increase Stress Resistance and Exert Antioxidant Properties in *Caenorhabditis elegans* and Mouse Models. *Molecules*, *24*(14), 2633. doi: 10.3390/molecules24142633.

van der Pol A., van Gilst W. H., Voors A. A., & van der Meer P. (2019). Treating oxidative stress in heart failure: past, present and

future. *European Journal of Heart Failure*, *21*(4), 425–435. doi: 10.1002/ejhf.1320.

Wang H., Liu J., Li T., & Liu R. H. (2018). Blueberry extract promotes longevity and stress tolerance via DAF-16 in *Caenorhabditis elegans*. *Food & Function*, *9*(10), 5273–5282. doi: 10.1039/c8fo01680a.

Wang J., Deng N., Wang H., Li T., Chen L., Zheng B., & Liu R. H. (2020). Effects of Orange Extracts on Longevity, Healthspan, and Stress Resistance in *Caenorhabditis elegans*. *Molecules*, *25*(2), 351. doi: 10.3390/molecules25020351.

Wang S., Li Y., Meng X., Chen S., Huang D., Xia Y., Zhu S. (2021). Antioxidant activities of chlorogenic acid derivatives with different acyl donor chain lengths and their stabilities during in vitro simulated gastrointestinal digestion. *Food Chemistry*, *357*, 129904. doi: 10.1016/j.foodchem.2021.129904.

Wilson M. A., Shukitt-Hale B., Kalt W., Ingram D. K., Joseph J. A., & Wolkow C. A. (2006). Blueberry polyphenols increase lifespan and thermotolerance in *Caenorhabditis elegans*. *Aging Cell*, *5*(1), 59-68. doi: 10.1111/j.1474-9726.2006.00192.x.

Wu Q., Shen T., Liu F., & Zhang W. (2021). Biochanin A protects SH-SY5Y cells against isoflurane-induced neurotoxicity by suppressing oxidative stress and apoptosis. *NeuroToxicology*, *86*, 10–18. https://doi.org/10.1016/j.neuro.2021.06.007.