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Geroprotective potential of *Thymus vulgaris* L. callus culture and its metabolites

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Abstract:
Healthy aging and active longevity need an integrated approach to a healthy lifestyle. Proper nutrition can effectively prevent the development of chronic diseases that lead to premature aging. Therefore, food scientists are searching for new bioactive substances with geroprotective potential to be introduced into functional food products and dietary supplements. We aimed to study the bioactivity of the *Thymus vulgaris* L. callus culture extract and its metabolites as potential geroprotectors.

We studied a hydroalcoholic extract of the *T. vulgaris* callus culture (70% ethanol) and its individual bioactive substances (thymol, oleanolic and ursolic acids), with *Caenorhabditis elegans* used as a model organism. IR spectroscopy and high-performance liquid chromatography were employed to analyze the effect of the extract and its metabolites on the growth of *C. elegans* larvae, as well as on the nematodes' lifespan and resistance to oxidative and thermal stress.

The extract of the *T. vulgaris* callus culture increased the length and surface area of the nematode body, producing an anabolic effect. In a 100-fold dilution, the extract enhanced the survival and stress resistance of the nematodes. Thymol, oleanolic and ursolic acids were obtained from the extract at 95% purification. Thymol completely inhibited the growth of nematode larvae, with no offspring produced from the eggs. At 10 μM, thymol increased the survival of the nematodes by a factor of 1.8 and also improved their stress resistance. In the presence of oleanolic acid (10 and 50 μM), some nematodes laid eggs; oleanolic acid increased the nematode lifespan be a factor of 1.9. Oleanolic acid (200 and 100 μM) and ursolic acid (50 and 100 μM) increased the survival of the nematodes exposed to thermal stress compared to the control.

The extract of the *T. vulgaris* callus culture and its metabolites (thymol, oleanolic and ursolic acids) have geroprotective potential for use in food supplementation. However, there is a need for further research.

Keywords: Geroprotectors, callus culture extract, thymol, oleanolic acid, ursolic acid, *Caenorhabditis elegans*

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INTRODUCTION

According to the World Health Organization, 2022 saw the global population aged 65 and over accounting for 9.4% of the total population. Across the countries, the proportion of older people reached 16.85% in Russia (similarly to Australia, Iceland, Belarus, and Montenegro), 14.5% in China, and about 19% in the USA and the UK [1]. The number of older people is growing every year worldwide. A country or a region is considered to be aging when its population aged 60 years and over reaches 10%. Whether aging is an inevitable destructive biological process remains controversial. Aging, however, leads to an increase in age-related diseases, including metabolic disorders, cancer, and neurodegeneration [2].

Aging is a complex process influenced by genetic, environmental, and lifestyle factors [3]. In 2023, a group of scientists led by K. Lopez-Otin added three new hallmarks of aging to the original 9 hallmarks, providing a more comprehensive framework for anti-aging research. They include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion,

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altered intercellular communication, chronic inflammation, and dysbiosis [4].

Currently, geroprotectors are actively used to combat or prevent premature aging by counteracting the hallmarks mentioned above [4, 5]. One of them is metroformin, which is primarily known as an antidiabetic agent [6]. Nicotinamide mononucleotide is another anti-aging agent that plays a vital role in various biological processes, including cell death, aging, gene expression, neuroinflammation, and DNA repair, as reported by Nadeeshani *et al*. [7]. Quercetin is a flavonoid that exhibits antioxidant activity *in vitro*. It has a positive effect on the lifespan of *Caenorhabditis elegans* and its survival under oxidative stress, on the expression of the *SOD-3* gene, and on the accumulation of lipid inclusions *in vivo* [8].

Thus, targeting the hallmarks of aging can slow down or reverse the aging process.

Some of the major challenges of the modern world are how to prevent aging and prolong an active life. They call for a complex approach integrating a healthy lifestyle, proper nutrition, physical activity, good mental health, and preventive healthcare [9]. Proper nutrition is one of the most effective measures to prevent the development of chronic diseases that lead to premature aging [10].

Therefore, there is a need to search for new bioactive substances (bioactive geroprotectors) to be included in the human diet as part of functional foods or dietary supplements.

Today, plant materials rich in bioactive geroprotectors are used to produce effective and safe drugs to prevent cardiovascular, oncological, and skin diseases, as well as diseases associated with premature wear, fragility, and other aging processes in the body [11–14]. Among various bioactive geroprotectors, polyphenols are increasingly attracting scientists due to their rejuvenating effect. According to many studies, polyphenols exhibit anti-inflammatory and antioxidant effects by stimulating cell regeneration [15, 16]. In addition, they can regulate the immune function and inflammatory response, as well as increase resistance to diseases such as lupus [17], rheumatoid arthritis [18], and multiple sclerosis [19]. Finally, polyphenols isolated from plants benefit human health by slowing down the aging of various organs and tissues, such as the brain, muscles, skin, heart, liver, and gut [20].

Thymus vulgaris L. is one of many plants containing a wide range of polyphenols. Growing in the Mediterranean region, this plant has been shown to protect the brain from neurodegeneration and neuroinflammation risk factors. Shimada *et al*. [21] described the inhibitory effect of three pentacyclic terpenes (ursolic, betulinic, and oleanolic acid) isolated from *T. vulgaris* on glutaminase activity. Glutaminase inhibitors are a potential therapeutic agent for the prevention and treatment of neurodegenerative diseases. Other researchers identified the anti-inflammatory activity of linalool and geraniol contained in *T. vulgaris* essential oil against lipopolysaccharide-induced neuroinflammation in BV-2 microglial cells [22].

In addition, thymol and para-cymene, both individually and in combination, contributed to the attenuation of cholinergic brain damage that leads to neurodegenerative diseases [23]. Warman *et al*. [24] established the effect of *T. vulgaris* essential oil on the attenuation of chronic low-grade inflammation of the brain caused by aging and the protection of blood telomeres in aged C57BL/6J mice. The oil was also found to exhibit spasmolytic and antimicrobial effects [25]. Many studies confirm the positive effect of *T. vulgaris* extracts on cardiovascular, oncological, and inflammatory diseases [26]. Furthermore, these extracts are also used to treat respiratory problems [27], relieve gastrointestinal spasms, and improve digestion [28]. Thus, *T. vulgaris* has been shown to have functional potential for preventing the development of chronic diseases that lead to premature aging.

T. vulgaris grows in the central and southern regions of the European part of Russia, in the Caucasus, and in the south of Eastern and Western Siberia [29]. However, its natural resources are insufficient to meet the needs of the whole population. Therefore, biotechnological cultivation of callus cultures [30] should be applied to preserve biodiversity and produce a sufficient amount of plant materials for obtaining bioactive substances. Plant cell cultures are increasingly being used as alternative sources of valuable secondary metabolites [31, 32]. Callus culture is one of the models of microclonal propagation of plants. It is a system of plant tissues consisting of thin-walled parenchymatous cells grown on artificial nutrient media [33].

The bioactivity of the *T. vulgaris* callus culture extract and its metabolites can be assessed using various models including nematodes, fruit flies, mice, rats, or primates [34]. Today, *C. elegans* nematodes are promising model organisms for fundamental research. Their aging is manifested through decreased mobility, tissue degeneration, and cessation of reproduction. This makes them effective models for preclinical trials of bioactive substances [35]. In addition, their anatomical and physiological features allow for short-term studies of their lifespan. According to Zhang *et al*. [36], bioactive substances extracted from *Portulaca oleracea* with ethyl acetate increased the average lifespan of nematodes by 5.3, 12.7, and 16.5% at concentrations of 250, 500, and 1,000 μg/mL, respectively. Also, the higher concentrations increased the worms' motility.

Many studies of *C. elegans* have shown the influence of metabolic pathways on aging. These nematodes have complex conservative pathways and gene expression programs. Their longevity and response to stress are controlled by such pathways as stress reactions (reactive oxygen species, heat shock). Oxidative stress is the main factor of aging in vertebrates, with reactive oxygen species accelerating the aging process. Song *et al*. [37] found that the nematodes incubated with raspberry extract had reduced levels of free radicals for five days. The SKN-1 protein is a transcription factor involved in the modulation of oxidative stress and, therefore, the

lifespan of the worms. During stress, the protein is phosphorylated and its accumulation in the nucleus activates the genes such as *gcs-1, gst-4,* and *gst-7.* These genes regulate metabolism, aging, and oxidative stress [38].

Thus, the nematodes *C. elegans* are used to study the effects of polyphenols on their viability and longevity. According to studies, bioactive substances at different concentrations have varying effects on the metabolism and physiological processes of the worms, increasing their life expectancy, reducing thermal and oxidative stress, etc.

In this study, we aimed to investigate the biological activity of the *T. vulgaris* callus culture extract and its individual bioactive substances (potential geroprotectors) for preventative functional food supplementation. In particular, we analyzed the geroprotective potential of the extract and three of its bioactive substances (thymol, oleanolic and ursolic acids) *in vivo.*

STUDY OBJECTS AND METHODS

Our study objects included: 50% alcohol solutions of the hydroalcoholic extract of *Thymus vulgaris* L. callus culture (70% ethanol), which were diluted with sterile distilled water at ratios of 1:10,000, 1:1,000, and 1:100; individual bioactive substances (thymol, oleanolic and ursolic acids) as solutions in dimethyl sulfoxide (DMSO) at concentrations of 200, 100, 50, and 10 μM.

The cultivation of the *T. vulgaris* callus culture, the hydroalcoholic extraction of its biomass, and the chemical analysis of the extract were performed at the early stages of our study at the Laboratory for Biotesting of Natural Nutraceuticals, Kemerovo State University, as described in [39].

The bioactive substances were isolated from the hydroalcoholic extract as follows. The extract was evaporated on an IKA RV 8 rotary vacuum evaporator (IKA-Werke GmbH & Co. KG, Staufen, Germany) at under 40°C. Deionized water, which had been sparged with an inert gas, was added to the residue evaporated to 1/4 of the initial volume and the vacuum evaporation was continued until a dry residue was obtained. The residue was then treated three times with dichloromethane (EKOS-1, Russia) for 5 min, each time with vigorous stirring. The resulting extracts were combined, and anhydrous sodium sulfate (Organic Mir, Russia) was added at 20.0 g per liter of the dichloromethane extract. The mixture was left to settle for 3 h and filtered through a degreased paper filter with a pore size of 8–12 μm (State Standard 12026-76; Ekokhim, Russia) followed by a membrane filter with a pore size of 0.45 μm (Membrane Solutions, LLC, USA).

The residue was used to isolate the flavonoid fraction, while the supernatant (filtrate) was used to isolate thymol.

IR spectra were recorded on an SF-2000 device (SPEKTR, Russia) for those bioactive substances of the *T. vulgaris* callus culture extract which were at least 95% pure.

Thymol was purified further by vacuum evaporation. For this, the supernatant was evaporated in a vacuum evaporator until the initial volume was reduced tenfold. The resulting solution of thymol in dichloromethane was subjected to column chromatography using aluminum oxide (activity II according to Brockmann) as a sorbent [40]. For this, aluminum oxide was dried in a drying cabinet at 150°C for 12 h, cooled in a desiccator, and then deactivated with 3% water by weight. The solution was eluted with dichloromethane using a BioLogic low-pressure chromatograph (BioRad, Hercules, CA, USA) on a 30×150 mm chromatographic column with a fraction collector, resulting in 1 mL of the eluate.

The purification of individual bioactive substances from the residue of the flavonoid fraction (flavonoids and their glycosides, phenolic carboxylic acids, triterpenoids) included the following stages. First, the residue was dissolved in 96% ethanol (RFK, Russia). The ethanol fraction was passed through an AN-1 anion exchanger (State Standard 20301-74). Then, it was washed with water-ethanol eluents (up to 50% ethanol), desorbed using 0.1 M HCl, and fractionated using a Sephadex LH-20 sorbent (GE Healthcare, Chicago, Illinois, USA) in an ethanol gradient of 10–80%. As a result, we obtained two bioactive substances – oleanolic and ursolic acids.

Figure 1 shows the stages of isolation and purification of metabolites from the extract of the *T. vulgaris* callus culture.

The isolated individual bioactive substances were identified using IR spectroscopy and HPLC (degree of oxidation on a glassy carbon electrode):

1. IR spectroscopy was performed on an FSM-1202 spectrometer (Infraspek, St. Petersburg, Russia). IR spectra were recorded in potassium bromide disks (Fluka, Germany). The conditions included a transmission mode of 4000–400 cm^{-1}, a resolution of 4 cm^{-1}, and 30 scans. Air was used as a reference sample and it was recorded before the analysis of each test sample. FSpec (4.0.0.2) and Aspec (1.1) softwares were used to control the process and analyze the spectral data.
2. The oxidation of the bioactive substances on a glassy carbon electrode was measured on a Tsvet Yauza-04 HPLC chromatograph, with an UV/Vis detector and a photodiode array at wavelengths of 255, 280, and 370 nm. The process was controlled and the data were analyzed using MultiChrom 3.1.1550 (Ampersend, Russia), with a Kromasil C18 110A column (5 μm, 250×4.6 mm; MERCK, USA) and a KJO-4282 analytical guard pre-column.

The bioactivity of the *T. vulgaris* callus culture extract and its individual bioactive substances was tested on nematodes *Caenorhabditis elegans*. The *C. elegans* N2 Bristol strain was provided by the Laboratory of Innovative Drug Development and Agrobiotechnology, Moscow Institute of Physics and Technology (National Research University, Dolgoprudny, Russia).

The nematodes were cultivated on solid agar according to the method described in [41]. They were fed on *Escherichia coli* OP50 strain provided by the Engelhardt Institute of Molecular Biology, Russian Academy of Sciences (Russia). To obtain a starter culture, one *E. coli*

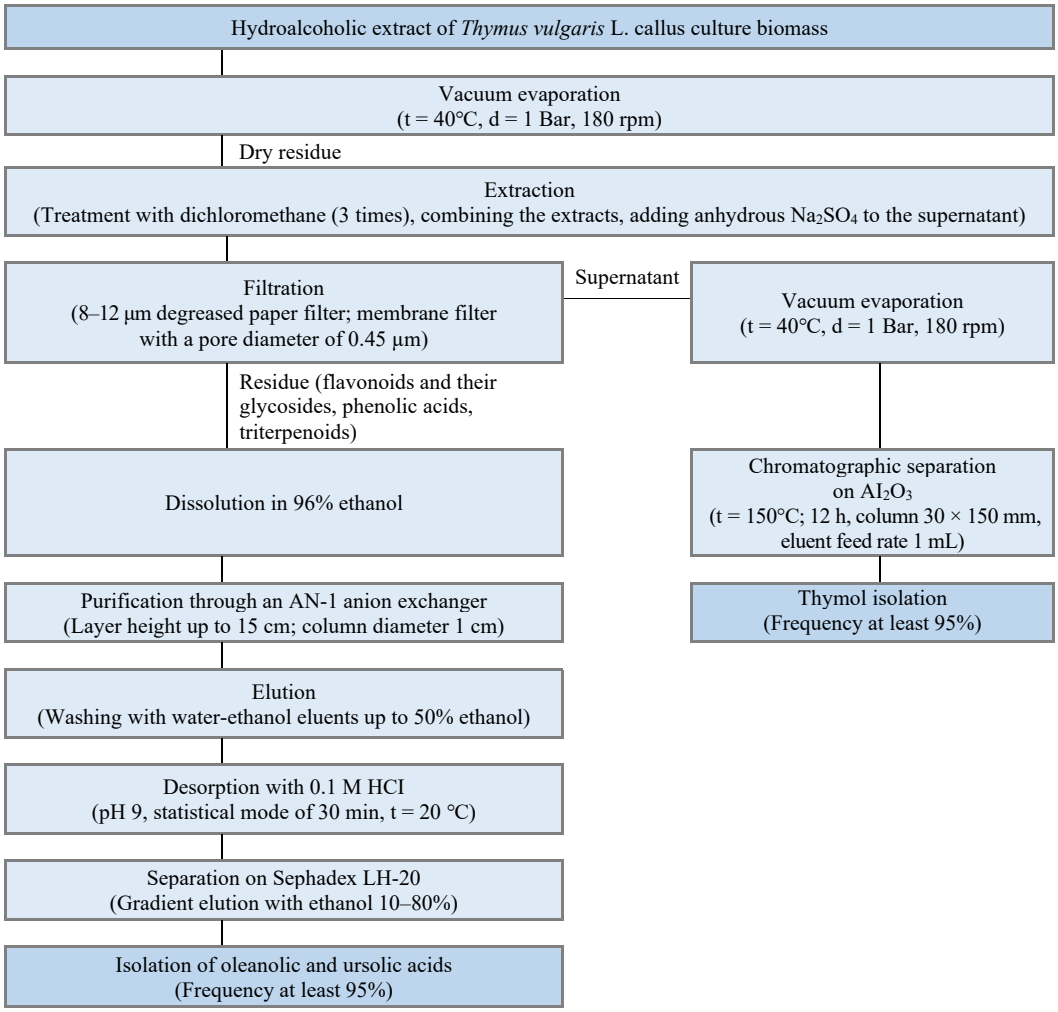


Figure 1 Isolation and purification of bioactive substances from the extract of the *Thymus vulgaris* L. callus culture

colony was grown in 5 cm^3 of LB medium (AppliChem GmbH, Germany) for 12 ± 0.5 h at 37 ± 0.5°C with vigorous stirring (120 rpm). At least 100 *C. elegans* worms were used in the experiments to test the *T. vulgaris* callus culture extract, as well as each individual bioactive substance.

To synchronize the nematodes, 5–10 mL of sterile water was added to an agar Petri dish with the nematodes and mixed several times until all the nematodes and their eggs were adhesively attached to the agar. The liquid was poured into a 50-mL tube and centrifuged for 2 min at 1,200 rpm. The supernatant was removed and the sediment was washed with 10 mL of distilled water. The supernatant was removed again and then 5 mL of a freshly prepared mixture of 1 mL of 10 N NaOH, 2.5 mL of sodium hypochlorite, and 6.5 mL of water was added to the sediment. The sediment was vigorously mixed for 5 min with a pause every 2 min to hydrolyze the nematodes. Upon completion, 5 mL of M9 medium (1,000 mL ddH_2O, 3 g KH_2PO_4, 5 g NaCl, 6 g Na_2HPO_4, 1 mL 1 M $MgSO_4$) was added to neutralize the reaction. The mixture was centrifuged for 2 min at 2500 rpm. The supernatant was removed and the sediment was resuspended in 10 mL of fresh sterile water. The neutralization and centrifugation were repeated 3 times. The sediment was washed for the fourth time with 10 mL of S medium (1,000 mL S base, 10 mL 1 M potassium citrate pH 6, 10 mL trace metal solution, 3 mL 1 M $CaCl_2$, 3 mL 1 M $MgSO_4$). The supernatant was removed, 10 mL of epy S medium was added, and the test tube with nematode eggs was left on a slow shaker for 24 h at room temperature until the nematodes entered the L1 development stage.

The *T. vulgaris* callus culture extract and its individual bioactive substances were tested in a liquid S medium to evaluate the growth rate of *C. elegans* larvae. For this, the substances at 100 μmol/mL (final) and the extract at a 100-fold dilution were added to the L1 nematodes simultaneously with the addition of *E. coli* OP50 bacteria. This time was considered the starting point (0 h) for assessing the effect of the tested compounds on the time it took *C. elegans* larvae to reach the sexually mature L4 development stage and lay eggs (the end point).

The growth parameters of the tested *C. elegans* larvae, including the nematodes' body surface area, length and width, were compared with those of the control nematodes. The effect of the extract and its bioactive substances on each of the mentioned parameters

of nematode ontogenesis was validated at three points of postembryonic development, including 72, 96, and 108 h. The experiments were carried out in 48-well plates (Eppendorf, USA) in 300 µL of a liquid S medium for culturing nematodes at 20 ± 0.5°C in a Binder climatic chamber (Germany). The plates were covered with a protective film to prevent evaporation of the culture liquid medium (SSI, USA). The experiments were carried out under sterile conditions in a FastFlow laminar flow hood (Italy).

An overnight culture of *E. coli* OP50, which had been washed from LB broth and resuspended in the S medium to a final bacterial concentration of 0.5 mg/mL, was added to the synchronized L1 nematodes in the S medium. Each well of the plate was filled with 270 µL of the nematode suspension with *E. coli* and then 30 µL of the test extract and its bioactive substances was added to the wells according to the dropping scheme. The plate was sealed with film and left to incubate for 72 h at 20 ± 0.5°C. Upon incubation, morphological changes in the nematodes were analyzed using an Axio Observer Z1 microscope (Karl Zeiss, Germany), with a series of images taken at a fixed magnification.

The plate was placed in a climate chamber and left to incubate for another 24 h at 20 ± 0.5°C. Then, the nematodes were tested for changes in the microscope at the same magnification. After 108 h of incubation, the procedure was repeated for the last time. The growth rates of the nematode larvae incubated in the presence of the tested extract and its individual bioactive substances were compared with those for the control nematodes (without the tested compounds).

The freeware CellProfiler 4.2.1 was used for image analysis [42]. First, the images were pre-processed to compensate for uneven illumination and remove undesirable artifacts such as well edges. Next, the nematodes and their clusters were separated by foreground/background segmentation. Then, 60 nematodes were manually selected for machine learning to identify them and separate their clusters. The resulting model was used to segment the nematodes in all the images using the UntangleWorms module [43]. The StraightenWorms and MeasureObjectSizeShape modules were used to measure the area, length, and width of the nematodes.

The nematode images were made to meet the requirements of the CellProfiler software. For this, the images were pre-processed with the ImageJ freeware to compensate for uneven illumination of the wells and to remove artifacts such as well edges, *E. coli* OP50 residues, eggs or L1 nematodes in the wells. The artifacts were "painted over" with the Pen tool (dark gray color) using the codes #a9a9a9, rgb (169, 169, 169), and cmyk (0, 0, 0, 34) to align the image background. The contrast parameter (Brightness/Contrast) was adjusted in the low-contrast images, where it was difficult to distinguish the nematodes from the background.

The digital data obtained from the nematode images during machine learning (StraightenWorms and MeasureObjectSizeShape modules) was filtered by leaving only those values that differed from the average by no more than a standard deviation. As a result of filtering, the number of measured nematode parameters (area, length, width) significantly reduced.

The digital images of the nematodes were converted to the metric scale of measurement. Using the ZenPro software of the Axio Observer Z1 microscope (Karl Zeiss, Germany), we determined the coefficient of converting pixels into millimeters (2.05×10^{-3}). So, the length and width of the nematodes in pixels were multiplied by this coefficient. To convert the area to the metric scale, the pixel values were multiplied by the square of this coefficient.

The effect of the *T. vulgaris* callus culture extract and its individual bioactive substances (0, 10, 50, 100, and 200 µM) on the lifespan of *C. elegans* was assessed in 10-, 100-, and 1000-fold dilutions based on the number of surviving worms. The experiment was conducted in 96-well plates in the liquid S medium for culturing nematodes. The number of live and dead nematodes was counted every 4–7 days during 59 days. The experiment was considered complete when there were no live nematodes left in the control group [41].

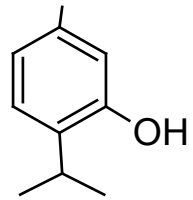
To determine the effect of the *T. vulgaris* callus culture extract and its individual bioactive substances on the resistance of *C. elegans* to oxidative and thermal stress, we modified the experiment for cultivating *C. elegans* in the liquid medium. After adding 15 µL of the bioactive substances, the plate was left for incubation at 20 ± 0.5°C for 5 days. Then, live and dead worms were counted in each well of the plate. To study the worms' resistance to oxidative stress, 15 µL of 1 M hydrogen peroxide (working concentration 100 mM) was added to each well and the incubation continued at 20 ± 0.5°C. To study the resistance of *C. elegans* to thermal stress, the plate was left for incubation at 20 ± 0.2°C. The first and second counts of live and dead nematodes were made after 24 and 48 h of incubation, respectively. The worms' resistance to oxidative and thermal stress in the presence of bioactive substances was compared with the results for the control nematodes, which were incubated without the tested compounds [44, 45].

All the experiments were conducted in triplicate. The obtained data were expressed as an average of three measurements with standard deviation. The data were analyzed using the Microsoft Office Excel 2007. The statistical analysis was performed using the ANOVA with the Scheffe's criterion.

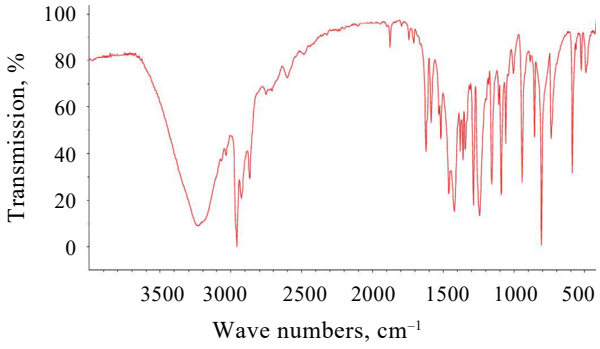
RESULTS AND DISCUSSION

Isolation and purification of metabolites from the hydroalcoholic extract of the *Thymus vulgaris* L. callus culture. Thymol, oleanolic acid, and ursolic acid were isolated and purified from the extract of *T. vulgaris* L. callus culture using chromatography, with at least 95% of purification.

IR spectroscopy of the metabolites. The IR spectroscopy results for the bioactive substance thymol obtained from the extract of the *T. vulgaris* callus culture are presented in Fig. 2 and Table 1.



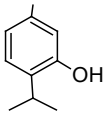
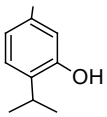
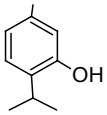
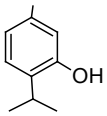
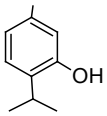
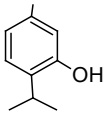
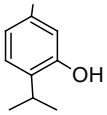
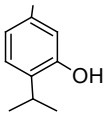
a



b

Figure 2 Structure (a) and IR spectrum (b) of thymol isolated from the extract of *Thymus vulgaris* L. callus culture

Table 1 Characteristic vibrational frequencies and their correlation with the main structural fragments of thymol isolated from the *Thymus vulgaris* L. callus culture extract

Structure	Wave numbers, cm^{-1}	Type of vibrations/bonds in structural fragments
	2960	Asymmetric stretching vibrations of C-H in methyl groups
	2865	Symmetric stretching vibrations of methyl groups
	2926	Asymmetric stretching vibrations of the methylene group
	1583, 1460, 1420	Stretching vibrations of C=C bonds in a ring
	1422	Asymmetric C-C-O vibrations
	1380, 1361, 1344	Symmetric stretching C-C-O vibrations
	856, 806, 738	Out-of-plane deformation vibrations of C-H bonds in an aromatic ring
	588	Out-of-plane deformation vibrations of the O-H group

As can be seen, the IR spectrum of thymol (2-isopropyl-5-methylphenol) has the following distinctive features. The absorption band at 2960 cm^{-1} is due to asymmetric stretching vibrations of C-H in methyl groups, while the band at 2865 cm^{-1} results from symmetric stretching vibrations of the molecule's methyl groups. The band at 2926 cm^{-1} is caused by asymmetric stretching vibrations of the methylene group. The first and second overtones (1877, 1744, and 1709 cm^{-1}) are due to the presence of substituents in the para- and meta- positions. Stretching vibrations of the C-C ring are at 1583, 1460, and 1420 cm^{-1}. The triplet bands at 1380, 1361, and 1344 cm^{-1} characterize an isopropyl fragment. The band around 1422 cm^{-1} is due to in-plane deformation vibrations of the O-H group. Out-of-plane deformation vibrations of C-H bonds in the aromatic ring are at 856, 806, and 738 cm^{-1}. Out-of-plane deformation C-C vibrations in the aromatic ring are at 745 cm^{-1}. A broad band of out-of-plane deformation vibrations of the O-H group is found around 588 cm^{-1}.

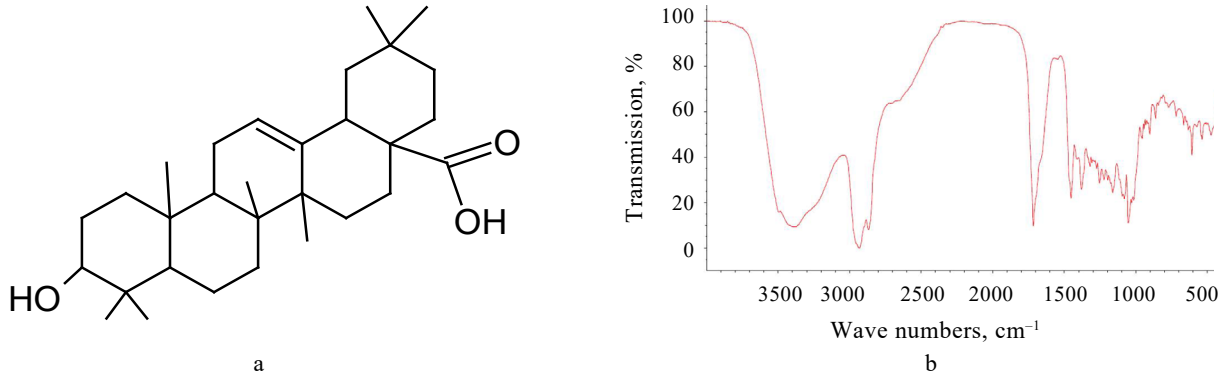
The IR spectroscopy results for oleanolic acid obtained from the extract of the *T. vulgaris* callus culture are presented in Fig. 3 and Table 2.

As can be seen, the IR spectrum of oleanolic acid isolated from the extract of *T. vulgaris* callus culture has the following distinctive features. The band with the absorption maximum at 3386 cm^{-1} is specific for the associated O-groups. The band at 2931 cm^{-1} is due to symmetric stretching vibrations of C-H in methyl fragments (groups). The band at 2867 cm^{-1} also corresponds to symmetric stretching vibrations of C-H in methyl fragments (groups). The strong intensity band at 1716 cm^{-1} is due to stretching vibrations of C=O in the carboxyl group.

The band at 1451 cm^{-1} is due to scissor vibrations of C-H in methylene groups, which is specific for cyclohexane structures. The band at 1377 cm^{-1} corresponds to deformation vibrations of C-H in geminal methyl groups. The bands at 1252 and 1162 cm^{-1} are due to stretching vibrations of the C-O-H bond. The broad band at 1050 cm^{-1} is caused by planar deformation vibrations of the C-O bond. The band at 960 cm^{-1} results from methylene vibrations of C-H. Finally, the bands at 861 and 771 cm^{-1} are formed by pendulum vibrations of methylene groups.

The IR spectroscopy results for ursolic acid obtained from the extract of the *T. vulgaris* callus culture are presented in Fig. 4 and Table 3.

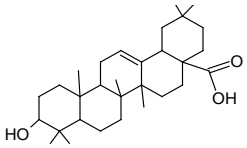
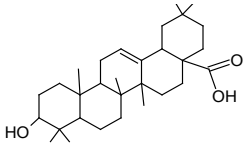
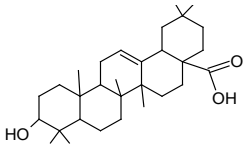
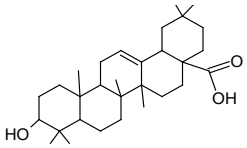
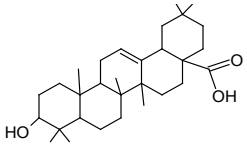
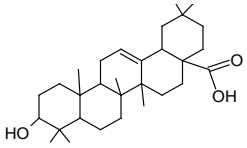
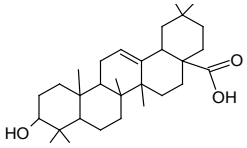
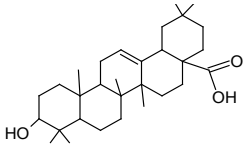
As can be seen, the IR spectrum of ursolic acid (3β-hydroxyurs-12-ene-28-oic acid) isolated from the extract of *T. vulgaris* callus culture has the following distinctive features.

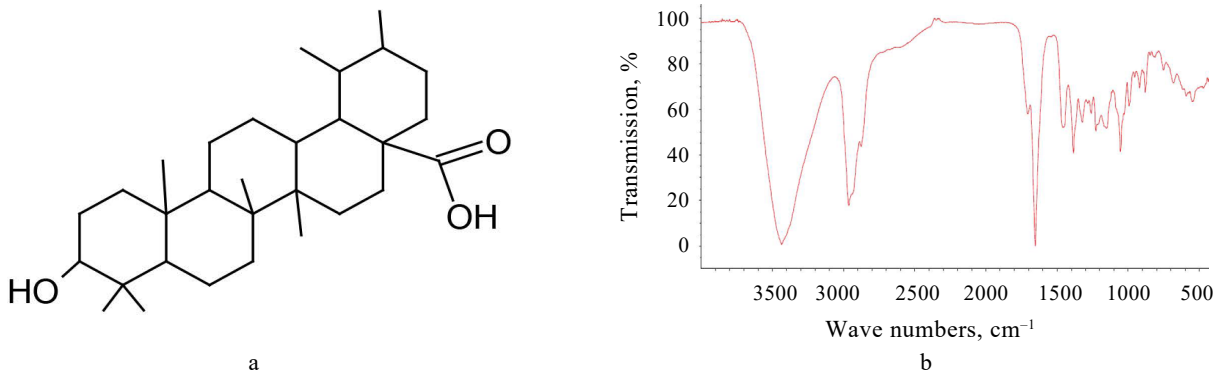


a b

Figure 3 Structure (a) and IR spectrum (b) of oleanolic acid isolated from the extract of *Thymus vulgaris* L. callus culture

Table 2 Characteristic vibrational frequencies and their correlation with the main structural fragments of oleanolic acid isolated from the *Thymus vulgaris* L. callus culture extract

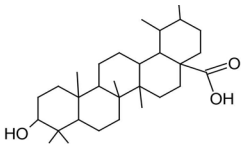
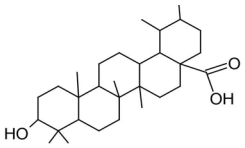
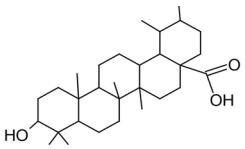
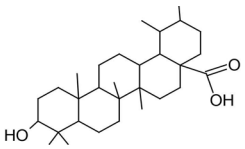
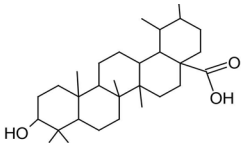
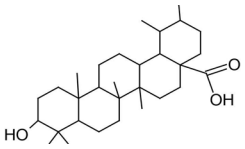
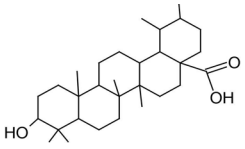
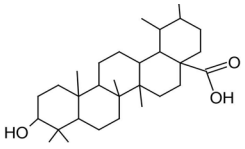
Structure	Wave numbers, cm^{-1}	Type of vibrations/bonds in structural fragments
	3433	Associated OH groups
	2962	Stretching symmetric C-H vibrations in methyl groups
	1652	Stretching vibrations of C=O in a carboxyl group
	1451	Scissor vibrations of C-H in methylene groups
	1377	Deformation vibrations of C-H in methylene structures
	1320	Planar deformation vibrations of C-O
	1252, 1162	Stretching vibrations of the C-O bond
	960	Planar deformation vibrations of the hydroxyl (alcohol) group in ring A
	861, 771	Pendulum vibrations of methylene groups



a b

Figure 4 Structure (a) and IR spectrum (b) of ursolic acid isolated from the extract of *Thymus vulgaris* L. callus culture

Table 3 Characteristic vibrational frequencies and their correlation with the main structural fragments of ursolic acid isolated from the *Thymus vulgaris* L. callus culture extract

Structure	Wave numbers, cm^{-1}	Type of vibrations/bonds of structural fragments
	3433	Associated OH groups
	2962	Stretching symmetrical vibrations of C-H in methyl fragments
	1652	Stretching vibrations of C=O in a carboxyl group
	1453	Scissor vibrations of C-H in methylene groups
	1384	Deformation vibrations of C-H in methyl structures
	1320	Planar deformation vibrations of C-O
	1261, 1226	Stretching vibrations of the C-O bond
	993	Planar deformation vibrations of the hydroxyl (alcohol) group in ring A
	880, 730	Pendulum vibrations of methylene groups

The band with the absorption maximum at 3433 cm^{-1} is specific for the associated OH groups. The band at 2962 cm^{-1} is due to symmetric stretching vibrations of C-H in methyl fragments (groups). The band at 2877 cm^{-1} also corresponds to symmetric stretching vibrations of C-H in methyl fragments (groups). The strong intensity band at 1652 cm^{-1} is caused by stretching vibrations of C=O in the carboxyl group.

The band at 1453 cm^{-1} is caused by scissor vibrations of C-H in the methylene groups, which is specific for cyclohexane structures. The band at 1384 cm^{-1} is due to deformation vibrations of C-H in geminal methyl structures. The band at 1320 cm^{-1} results from planar deformation vibrations of O-H. The bands at 1261 and 1226 cm^{-1} are caused by stretching vibrations of the C-O-H bond. The broad band at 1053 cm^{-1} is formed by planar deformation vibrations of the O-C-C, C-O bond. The band at 993 cm^{-1} is due to methylene vibrations of C-H. Finally, the bands at 881 and 749 cm^{-1} are caused by pendulum vibrations of the methylene fragments of ursolic acid.

According to the results, the spectral activity of the individual bioactive substances corresponds to their structural features. Their characteristic features, including those in the fingerprint zone of the IR spectrum, are also consistent with their cultivation, extraction, and preparation.

HPLC identification of the metabolites. The HPLC results for the bioactive substances isolated from the extract of *T. vulgaris* callus culture are presented in Fig. 5.

The biological activity of the extract of *T. vulgaris* L. callus culture and its metabolites. ***The effect of the extract and its bioactive substances on the growth of C. elegans larvae.*** The effect of the *T. vulgaris* callus culture extract and its individual bioactive substances

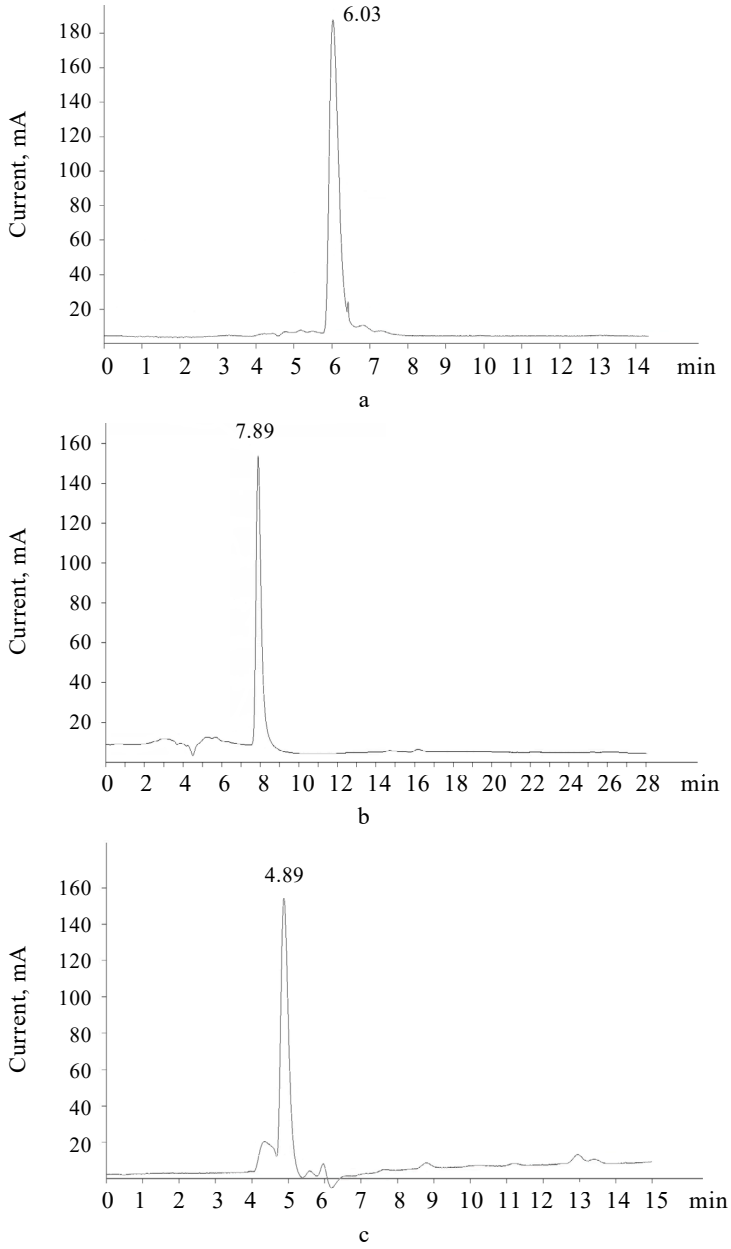


Figure 5 HPLC chromatogram: (a) oleanolic acid; (b) ursolic acid; and (c) thymol

(thymol, oleanolic and ursolic acids) at 100 μM on the growth of *C. elegans* larvae was determined as a duration of larval development from stage L1 (larvae) to stage L4 (sexually mature nematodes). The control nematodes were incubated without adding the tested compounds. The results are presented in Table 4.

When cultivated on solid agar, it usually takes a *C. elegans* larva (stage L1) three days to reach a sexually mature stage of development (L4), when it lays eggs formed in the intestinal cavity. In a liquid medium, however, neither the control nor the test nematodes laid eggs after 72 h. The control worms had a slight shift in ontogenesis and laid eggs after 96 h of incubation. None of the tested bioactive substances accelerated the development of the L1 larva to stage L4 compared to the control. However, the compounds had a different effect on the growth rate of nematode larvae, namely:

1. In the presence of ursolic acid (sample 4), the entire population of nematodes reached stage L4 after 96 h, laying eggs with a new generation of L1 larvae.
2. In the presence of oleanolic acid (sample 3), only part of the nematode population began laying eggs after 96 h (some even produced an insignificant number of new L1 larvae), but some nematodes did not reach stage L4 even after 108 h.
3. In the presence of thymol (sample 2), larval growth was fully inhibited and none of the nematodes reached stage L4 even after 108 h, with no new larvae produced.

To further study the effect of the *T. vulgaris* extract and its metabolites on the growth rate of nematode larvae, we also determined their body surface area, length, and width.

To determine the body surface area of the L4 nematode larvae, we performed image processing with machine learning, using the programs described in the methods section above. Initially, the images were preprocessed in the ImageJ program to compensate for uneven lighting and to remove undesirable artifacts for subsequent machine learning.

Then, the nematodes and their clusters were separated from the image background using foreground/background segmentation. The next step was machine learning of 60 nematode images using the UntangleWorms, MeasureObjectSizeShape, and StraightenWorms modules of CellProfiler 4.2.1. Its aim was to write an algorithm for identifying and segmenting the nematodes from the entire pool.

Applying the machine learning algorithm, we obtained a number of nematode parameters, including their area, length, and width. After filtering, these data in pixels were converted to metric units (mm). The larva body surface area was recorded until the nematodes reached stage L4.

The effects of the *T. vulgaris* callus culture extract and its metabolites (thymol, oleanolic and ursolic acids) on the nematodes' surface area, length, and width are presented in Figs. 6–8.

As can be seen in Fig. 6, the extract of the *T. vulgaris* callus culture increased the surface area of the nematodes throughout the entire experiment, with the maximum area after 108 h of incubation. The delay in the larval growth from L1 to L4 correlated with the increased surface area over time. The area of the control nematodes remained unchanged during 108 h of obser-

Table 4 Development from larva (L1) to adult nematodes (L4)

Sample	Name of sample	72 h	96 h	108 h
1	*Thymus vulgaris* L. callus culture extract	–	+	n.a.
2	Thymol	–	–	–
3	Oleanolic acid	–	–/+	+/–
4	Ursolic acid	–	+	n.a.
Control		–/+	+	n.a.

"+" – L1 larvae are present; "–/+" – small numbers of L1 in the population; "+/–" – not all the nematodes have laid eggs or produced a new L1 generation; n.a. – not available

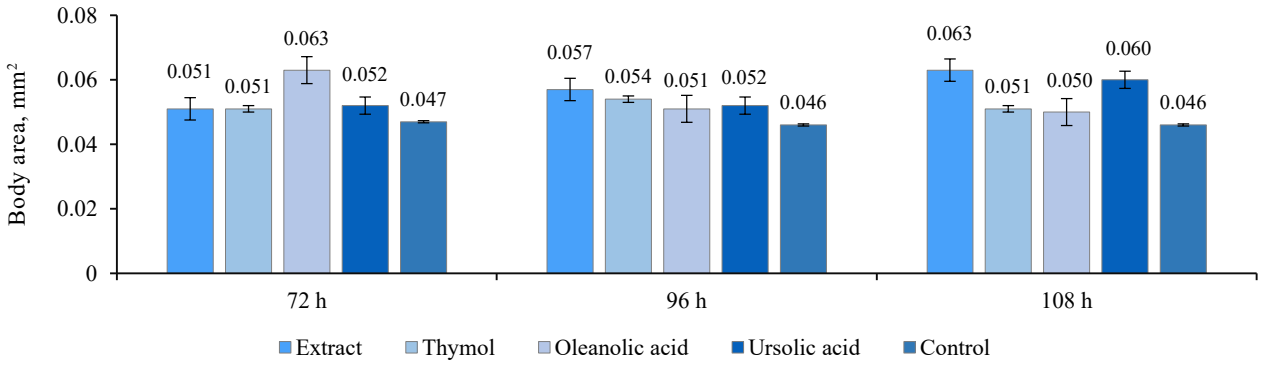


Figure 6 Effect of the *Thymus vulgaris* L. callus culture extract and its bioactive substances on the *Caenorhabditis elegans* body surface area

vation. Regardless of the effect that the extract and its bioactive substances had on the growth rate of the larvae, their surface area exceeded the area of the control larvae throughout the experiment.

The fastest larva development was observed in the presence of oleanolic acid, with all L4 nematodes producing a new generation of L1 larvae after 72 h. In the presence of ursolic acid, the fastest developing larvae to stage L4 in 96 h had a decreased surface area. Thymol had an insignificant effect on the surface area of the larvae, with only a 1.17-fold increase over 96 h of incubation, compared to the control nematodes.

As can be seen in Fig. 7, the body length of the tested nematodes significantly exceeded the length of the control nematodes after 72, 96, and 108 h of incubation.

The *T. vulgaris* extract increased the length of the nematodes during 108 h of incubation. Also, it might have had an anabolic effect expressed in a larger surface area. In the presence of ursolic acid, the maximum length of the larvae was observed after 72 h. It was higher than the length of the control nematodes by an average of 23.3%. The longest length was registered in the presence of oleanolic acid after 108 h of incubation. Furthermore, the effect of oleanolic acid was greater than that of thymol and ursolic acid by factors of 1.16 and 1.24, respectively.

According to Fig. 8, the width of the control larvae remained unchanged throughout the experiment (108 h). No significant differences against the control were found in the presence of the *T. vulgaris* extract and thymol. Oleanolic and ursolic acids had an insignificant effect on the body width of the nematode larvae, increasing it only 1.01 times compared to the extract, thymol, and the control group.

The effect of T. vulgaris callus culture extract and its individual bioactive substances on the lifespan of C. elegans. The effect of the *T. vulgaris* extract and its metabolites on the lifespan of *C. elegans* was determined as the number of surviving worms.

The extract was tested in 10-, 100-, and 1000-fold dilutions (Fig. 9).

We found that the extract of *T. vulgaris* callus culture in a 10-fold dilution was toxic to the nematodes: they died after 3 weeks of incubation in its presence. In a 100-fold dilution, the extract had little effect on the lifespan of the nematodes. Their survival began to decline on day 36 of incubation, and on day 40, they all died. In a 1,000-fold dilution, the extract had no effect on the lifespan of the nematodes in comparison with the control group.

The effects of the bioactive substances isolated from the *T. vulgaris* extract at concentrations of 0, 10, 50, 100, and 200 μM on the lifespan of *C. elegans* are presented in Figs. 10–12.

As shown in Fig. 10, thymol at 10 μM increased the survival of the nematodes on day 8 by a factor of 1.8 compared to the control group. Overall, however, thymol at various concentrations had no positive effect on the

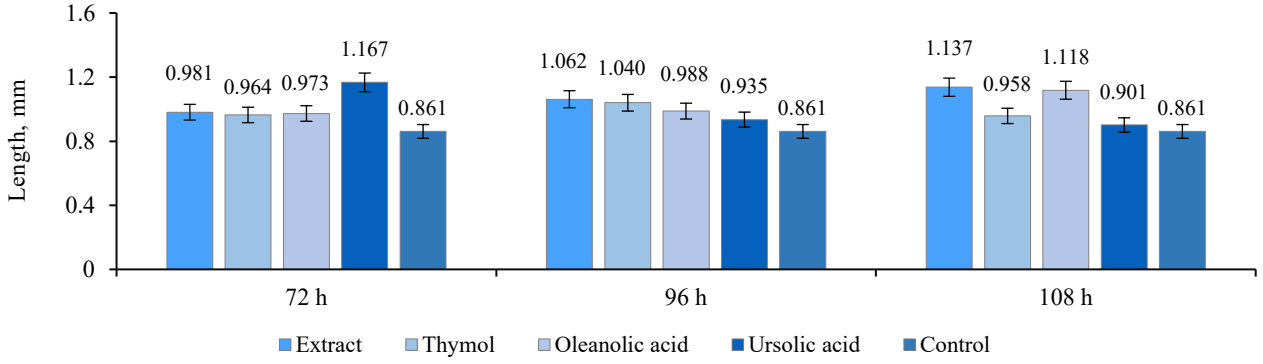


Figure 7 Effect of the *Thymus vulgaris* L. callus culture extract and its bioactive substances on the *Caenorhabditis elegans* length

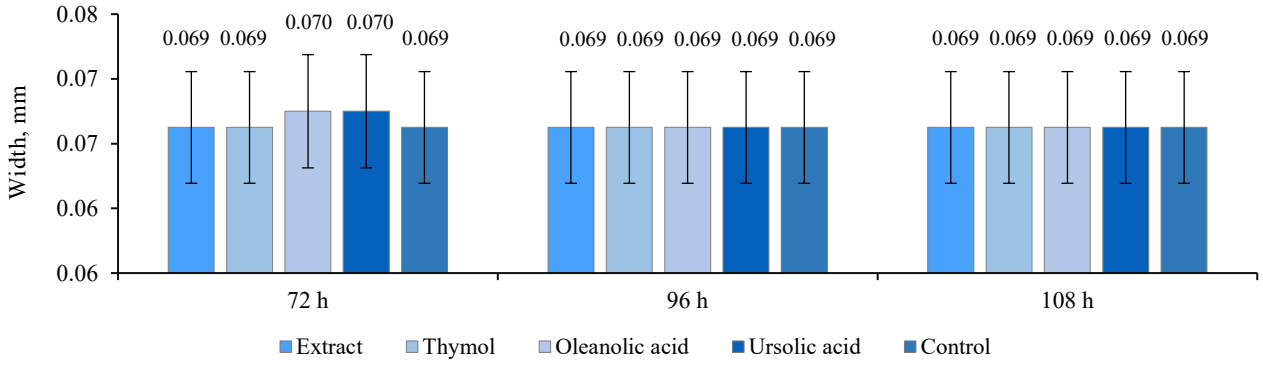


Figure 8 Effect of the *Thymus vulgaris* L. callus culture extract and its bioactive substances on the *Caenorhabditis elegans* width

lifespan of *C. elegans*. Moreover, it turned out to be toxic since the nematodes began to die on day 13 of incubation.

According to Fig. 11, oleanolic acid at 10 and 50 μM increased the lifespan of the nematodes by a factor of 1.9. On day 61 of the experiment, 16% of the nematode population remained alive and active. Higher concentrations of oleanolic acid (100 and 200 μM) had a negative effect on the lifespan of *C. elegans*.

As shown in Fig. 12, ursolic acid had no significant effect on the lifespan of the nematodes. At concentrations of 50 and 100 μM, it had a positive effect from day 3 to day 30 of nematode incubation. After day 30, the lifespan of the worms became comparable with that of the control group.

We found no publications on the effect of the *T. vulgaris*. callus culture extract on the lifespan of *C. elegans*.

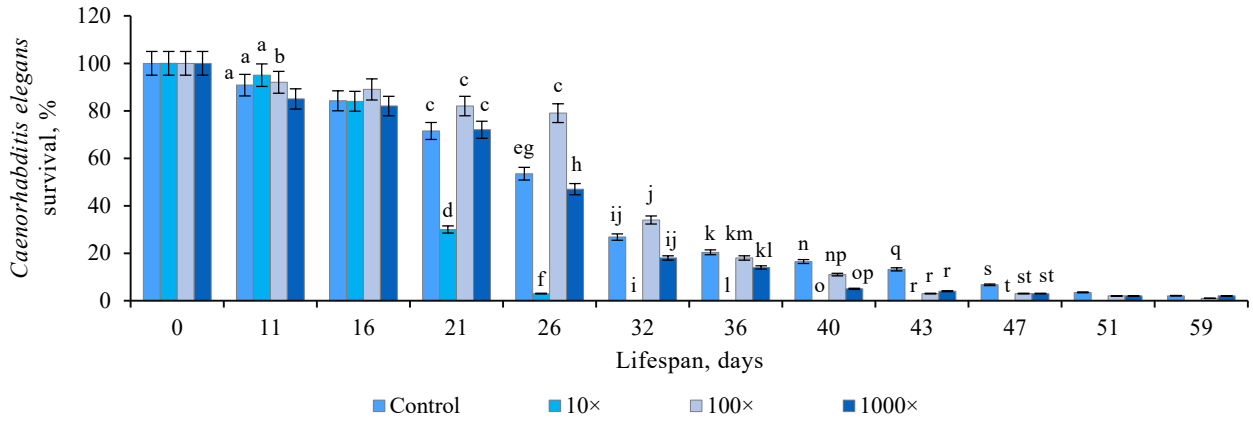
Our data confirmed that thymol, oleanolic acid, and ursolic acid can influence and increase the lifespan of the nematodes [46–48].

The effect of T. vulgaris callus culture extract and its individual bioactive substances on stress (oxidative and thermal) resistance of C. elegans. The effect of the *T. vulgaris* callus culture extract and its metabolites on the resistance of *C. elegans* to oxidative stress was assessed by counting the nematodes that died within 24 and 48 h at 20°C after the addition of 1 M hydrogen peroxide (working concentration 100 mM) under oxidative stress conditions.

Figure 13 shows the results for the *T. vulgaris* callus culture extract in 10-, 100-, and 10.00-fold dilutions.

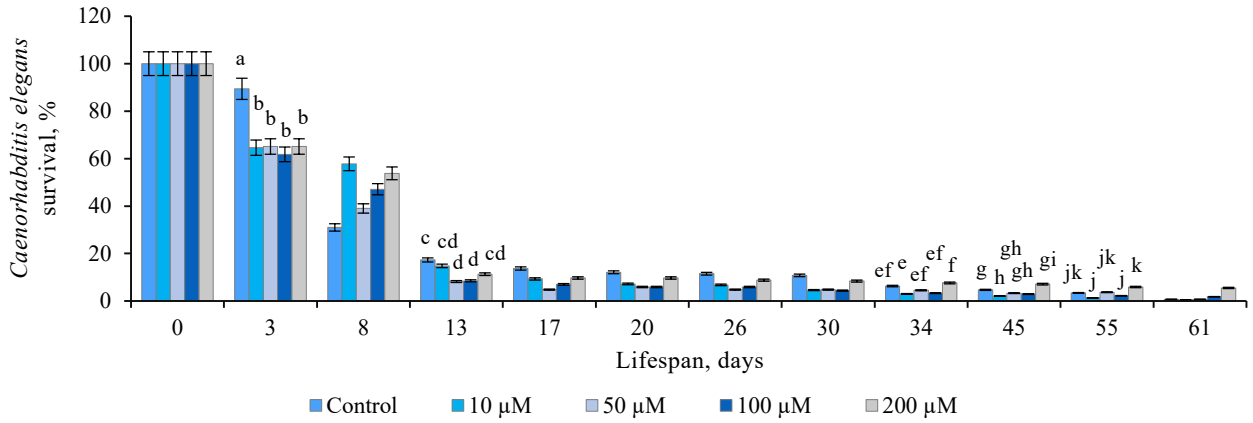
As can be seen in Fig. 13, the 10.00-fold dilution of the *T. vulgaris* extract increased the resistance of *C. elegans* to oxidative stress. Their survival rate was 1.5 times higher compared to the control. However, after 48 h of incubation, the extract lost its effect on the resistance of the nematodes to oxidative stress. Furthermore, the *T. vulgaris* extract did not show any stress-resistant effect on *C. elegans* under heat stress during 24 and 48 h.

Figure 14 shows the effect of the individual bioactive substances at concentrations of 0, 10, 50, 100, and 200 μM on the resistance of *C. elegans* to oxidative stress during 24 and 48 h of exposure.



Different letter indices denote statistically significant differences between the samples (within a parameter) according to ANOVA with Scheffe's post hoc test. Identical letter indices denote the absence of significant differences

Figure 9 Effect of *Thymus vulgaris* L. callus culture extract on the lifespan of *Caenorhabditis elegans* nematodes

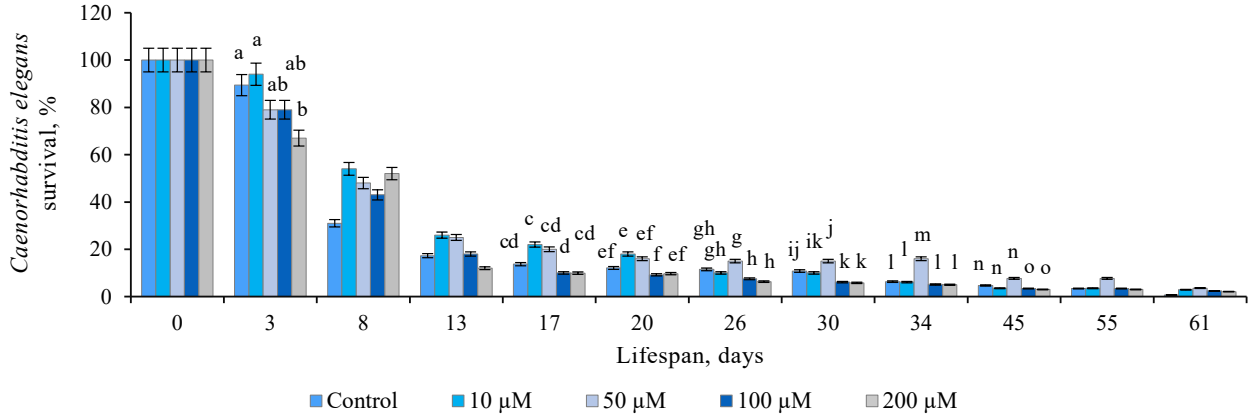


Different letter indices denote statistically significant differences between the samples (within a parameter) according to ANOVA with Scheffe's post hoc test. Identical letter indices denote the absence of significant differences

Figure 10 Effect of thymol isolated from *Thymus vulgaris* L. callus culture extract on the lifespan of *Caenorhabditis elegans* nematodes

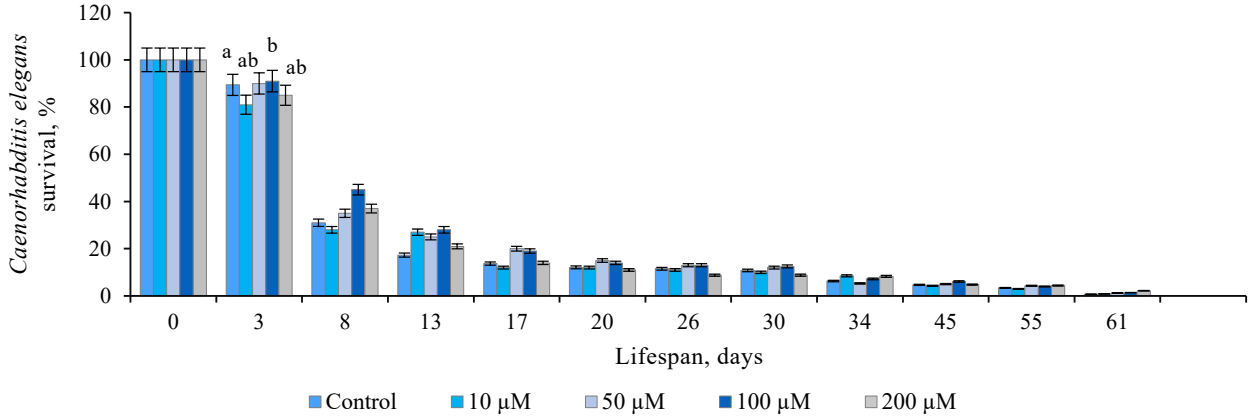
As can be seen, oleanolic and ursolic acids at all tested concentrations (10–200 μM) affected the survival of *C. elegans,* compared to the control. Thymol showed an insignificant effect on the resistance of the nematodes to oxidative stress. At 50 μM, this substance increased the survival of the nematodes 1.01 times compared to the control, but at the other concentrations, it had no effect on *C. elegans* stress resistance.

The analysis of *C. elegans* resistance to oxidative stress during 48 h of exposure revealed that thymol and



Different letter indices denote statistically significant differences between the samples (within a parameter) according to ANOVA with Scheffe's post hoc test. Identical letter indices denote the absence of significant differences

Figure 11 Effect of oleanolic acid isolated from *Thymus vulgaris* L. callus culture extract on the lifespan of *Caenorhabditis elegans* nematodes



Different letter indices denote statistically significant differences between the samples (within a parameter) according to ANOVA with Scheffe's post hoc test. Identical letter indices denote the absence of significant differences

Figure 12 Effect of ursolic acid isolated from *Thymus vulgaris* L. callus culture extract on the lifespan of *Caenorhabditis elegans* nematodes

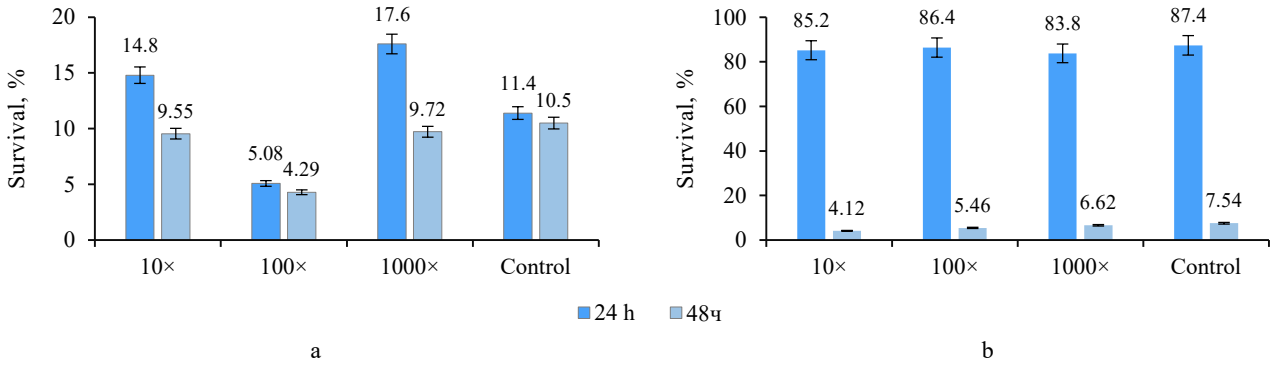


Figure 13 Effect of *Thymus vulgaris* L. callus culture extract on the stress resistance of *Caenorhabditis elegans*: (a) oxidative stress; (b) thermal stress

oleanolic acid at 10–200 μM increased the survival rate of the nematodes 1.1 times compared to the control. Ursolic acid showed the same result but only at 10–100 μM. At a concentration of 200 μM, ursolic acid decreased the survival of the nematodes compared to the control.

Figure 15 shows the effect of the individual bioactive substances at concentrations of 0, 10, 50, 100, and 200 μM on the resistance of *C. elegans* to thermal stress during 24 and 48 h of exposure.

During 24 h of *C. elegans* exposure to thermal stress, thymol at 10 μM increased the survival rate of the nematodes by a factor of 1.01 compared to the control. However, at higher concentrations of 50 to 200 μM, thymol led to a decrease in the survival rate. Oleanolic

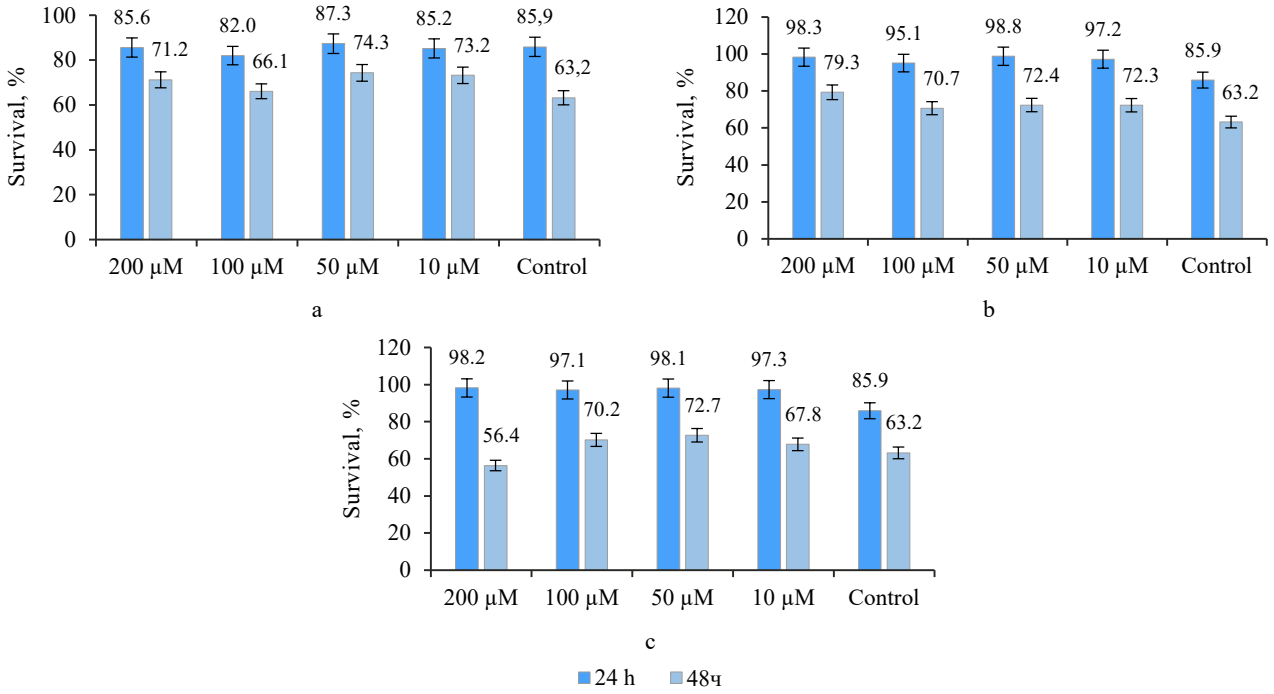


Figure 14 Effect of individual bioactive substances on *Caenorhabditis elegans* resistance to oxidative stress: (a) thymol; (b) oleanolic acid; and (c) ursolic acid

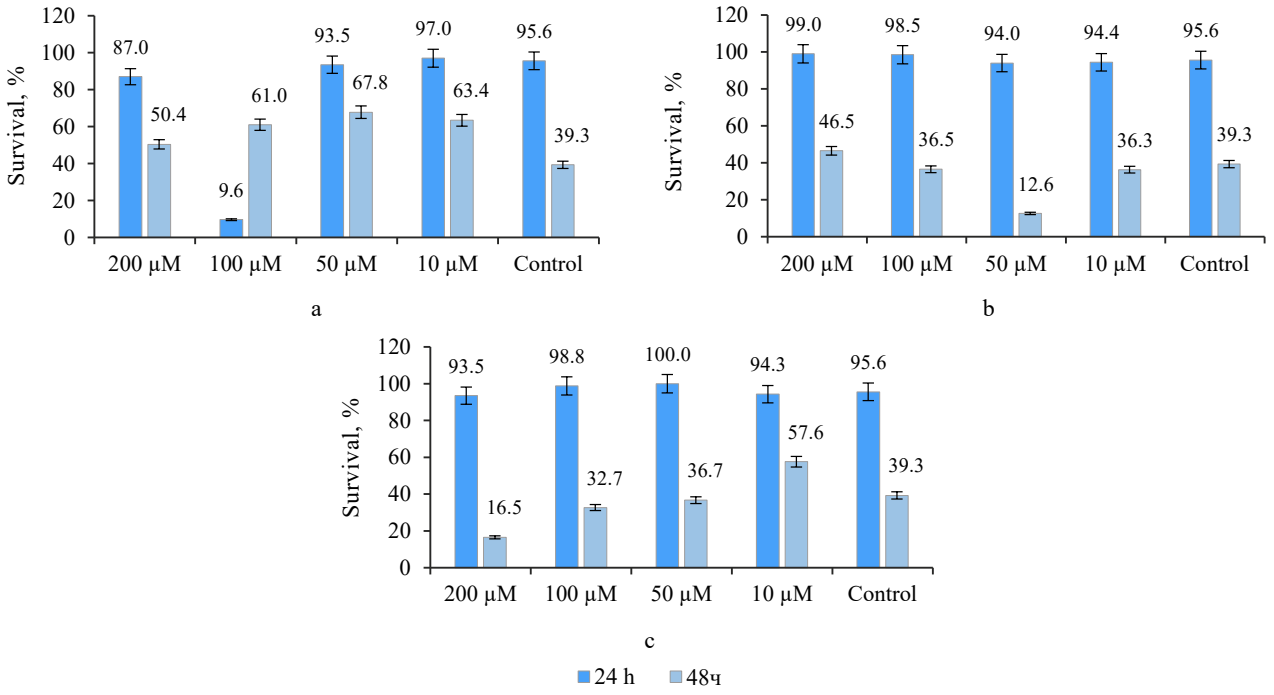


Figure 15 Effect of individual bioactive substances on *Caenorhabditis elegans* resistance to thermal stress: (a) thymol; (b) oleanolic acid; and (c) ursolic acid

acid increased the survival rate only at high concentrations of 200 and 100 μM, compared to the control. Ursolic acid at 100 and 50 μM showed a significant increase in the survival of the nematodes, compared to the control.

During 48 h of *C. elegans* exposure to thermal stress, thymol at concentrations of 10–200 μM led to a high survival rate of the nematodes. Oleanolic acid at 10 μM increased their survival rate by a factor of 1.18, compared to the control, but significantly decreased it at its other concentrations. Ursolic acid at 10 μM insignificantly increased the survival rate of the nematodes exposed to thermal stress, compared to the control. Yet, its higher concentrations of 50 to 200 μM decreased the number of surviving nematodes.

We found no publications on the effect of the *T. vulgaris* callus culture extract on the resistance of *C. elegans* to oxidative and thermal stress.

Our findings on *C. elegans* stress resistance under the influence of thymol, oleanolic acid, and ursolic acid are consistent with those reported in scientific literature [46]. For example, Zhang *et al*. [47] demonstrated that oleanolic acid can increase stress resistance and reduce intracellular reactive oxygen species in *C. elegans*. A study by Yue *et al*. [48] shows that ursolic acid can reduce the level of reactive oxygen species during oxidative stress in the nematodes. Naß *et al*. [49] also reported that ursolic acid increased resistance to oxidative and thermal stress.

Our data differed from the data reported by other authors since we used different concentrations of the metabolites and a different source of their isolation.

To sum up, our results suggest that a 70% hydroalcoholic extract of the *T. vulgaris* callus culture and its individual bioactive substances (thymol, oleanolic and ursolic acids) have geroprotective potential since they can affect the growth rate of *C. elegans* larvae, as well as the lifespan and resistance of the nematodes to oxidative and thermal stress. This makes them good candidates for use in functional foods and preventative dietary supplements.

CONCLUSION

Our study found that the *Thymus vulgaris* L. callus culture is a promising source of metabolites such as thymol, oleanolic acid, and ursolic acid. These substances have a number of bioactive properties, including a positive effect on the growth of *Caenorhabditis elegans* larvae, as well as their lifespan and survival under oxidative and thermal stress.

Effects on the growth rate of *C. elegans* larvae:

– the *T. vulgaris* callus culture extract increased the length of the nematodes during 108 h of incubation and, possibly, exhibited an anabolic effect by increasing their body surface area;
– in the presence of ursolic acid, the entire population of the nematodes developed from larvae to sexually matured nematodes (stage L4) after 96 h, laying eggs with a new generation of L1 larvae;
– in the presence of oleanolic acid, some nematodes began laying eggs after 96 h and even produced an insignificant number of new larvae, but all the nematodes did not reach stage L4 even after 108 h;
– thymol fully inhibited the growth of nematode larvae, with no larvae reaching the reproductive L4 stage even after 108 h. The eggs formed in that period failed to hatch into larvae;
– in the presence of ursolic acid, the fastest development of larvae to stage L4 in 96 h was accompanied by a decrease in the nematodes' body area; and
– under the action of ursolic acid, the nematodes reached their maximum length after 72 h, exceeding the length of the control nematodes by an average of 23.3%.

Effects on the lifespan of *C. elegans*:

– in a 100-fold dilution, the *T. vulgaris* callus culture extract increased the lifespan of the nematodes;
– in the presence of thymol at 10 μM, the survival of the nematodes on day 8 increased by a factor of 1.8 compared to the control group; and
– oleanolic acid at 10 and 50 μM increased the lifespan of the nematodes by a factor of 1.9. On day 61 of the experiment, 16% of the nematodes remained alive and active.

Effects on the resistance of *C. elegans* to oxidative and thermal stress:

– in a 1000-fold dilution, the *T. vulgaris* callus culture extract increased the survival of the nematodes under oxidative stress by a factor of 1.5 compared to the control;
– at all concentrations (10–200 μM), thymol, oleanolic acid, and ursolic acid affected the survival of the nematodes under oxidative stress, compared to the control;
– during 24 h of incubation under thermal stress, thymol at 10 μM increased the percentage of surviving nematodes by a factor of 1.01; oleanolic acid increased nematode survival only at 200 and 100 μM; and ursolic acid significantly increased nematode survival at 100 and 50 μM, compared to the control; and
– during 48 h of incubation under thermal stress, thymol at 10–200 μM showed a high percentage of nematode survival; oleanolic acid at 10 μM increased nematode survival by a factor of 1.8; and ursolic acid at 10 μM insignificantly increased nematode survival, compared to the control.

CONTRIBUTION

A.M. Fedorova and A.A. Shevel designed the research concept; the formal analysis was performed by E.S. Miller; S.V. Kovalenko, A.I. Loseva, A.V. Zaushintsena, O.G. Altshuler, and V.P. Yustratov developed the methodology; A.M. Fedorova wrote, reviewed, and proofread the manuscript. All the authors have read and approved the manuscript.

CONFLICT OF INTEREST

The authors declared no potential conflict of interest regarding the research, authorship, and/or publication of this article.

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