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RESEARCH ARTICLE

# Inability of 7,8 – Dihydroxy-4-Methylcoumarin Antioxidant Activity, to Prolong Longevity and to Protect Against Stress in *Caenorhabditis Elegans* Worms

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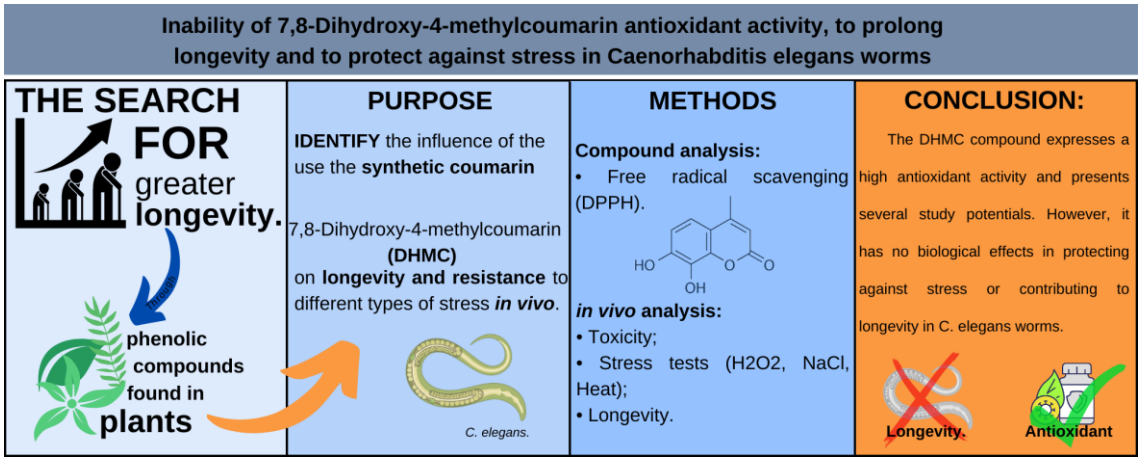
## Abstract:

Coumarins are secondary metabolites found in several plants, such as guaco, and are part of the polyphenol family capable of producing up to 1300 derivatives, with potential effects against metabolic stress, in addition to being used in the cosmetics industry with the objective of improving fragrance of products. Therefore, it seems necessary to seek to elucidate molecular mechanisms that can regulate this aging process, which can be associated with the gradual loss of physiological functions of cells and tissues, increasing the number of cells in senescence that may be related to increased oxidative stress. PURPOSE: The aim of this study was to identify the influence of the use of the coumarin synthetic compound 7,8-Dihydroxy-4-methylcoumarin (DHMC) on longevity and resistance to different types of stress in vivo. METHODS: Free radical scavenging (DPPH) analyzes were performed for the compound, longevity, and stress tests (H2O2, NaCl, and Heat) for C. elegans worms. RESULTS: In the first analysis, the compound showed 92% of antioxidant activity already in small concentrations (25ug/ml) through DPPH analysis. In the following tests DHMC did not show antibacterial responses against Escherichia coli, and Caenorhabditis elegans worms did not show stress reduction or significant improvement in longevity with the use of the compound. CONCLUSION: Therefore, the DHMC compound expresses a high antioxidant activity and presents several study potentials. However, it has no biological effects in protecting against stress or contributing to longevity in C. elegans worms.

Keywords: Caenorhabditis elegans, Antioxidant, Longevity, Coumarin

## Graphical Abstract




## Introduction

Cellular processes in eukaryotes depend on refined adjustments, triggered through signaling cascades, which depend on interactions between internal and external signals to the cytoplasmic environment (1). These processes lead to physiological responses, modifications and adaptations of organisms. It is known that extracellular signals can even alter the ability to divide; activate senescence pathways and promote DNA damage that can alter the natural cell aging process (2, 3).

The aging process is characterized by a natural and unavoidable process, marked by an increase in cellular oxidative stress, which can be caused by an increase in free radicals. A persistent inflammatory picture can also be observed, in addition to a failure in mitochondrial function, which can lead to loss of cellular energy capacity. Specifically, oxidative stress is related to greater synthesis of reactive oxygen species (ROS) and reactive nitrogen species (RNS). It is known that the increase of these toxic molecules to the body can trigger diseases such as cancer, diabetes, and neurological diseases (4, 5). Together, these aspects modulated by ROS and RNS can decrease cell, tissue and body longevity (6).

In these aspects, the pharmaceutical industry has highlighted substances from plants as a controlling factor and aid in the regulation of cellular processes. Coumarin is a natural compound found in plants such as Mikania glomerata and Arnica montana, it is part of the polyphenol family and can be synthesized using chemical methodologies, such as the Perkin reaction, enabling a wide variety of compounds with beneficial physiological effects, such as: antiviral, anticancer, cytotoxic, anti-acne and antioxidant (7). One of these derivatives is 7,8-Dihydroxy-4-methylcoumarin, however, studies investigating its physiological effects and longevity in C. elegans were not yet known. Longevity studies in another models take years and there can be a high maintenance value until the end of the experiment.

In the search for experimental models that could bring quick answers in studies capable of elucidating molecular mechanisms, the nematode Caenorhabditis elegans (C. elegans) presents itself as an alternative, as it has a short shelf life, is easy to handle, and has high similarity with humans (8). These aspects became clearer after 1998, when C. elegans had its DNA sequenced (9). Studies dating from the 1960s show that initially the worm was used to understand aspects involving the nervous system, later it was adopted to assess many other cellular processes in other tissues (10)

Therefore, this experimental model seemed interesting to us to verify the influence of the use of the Coumarin synthetic compound DHMC on resistance to different types of stress in vivo, these aspects may open new ways to elucidate important mechanisms related to longevity.

## Methodology

Experimental design:

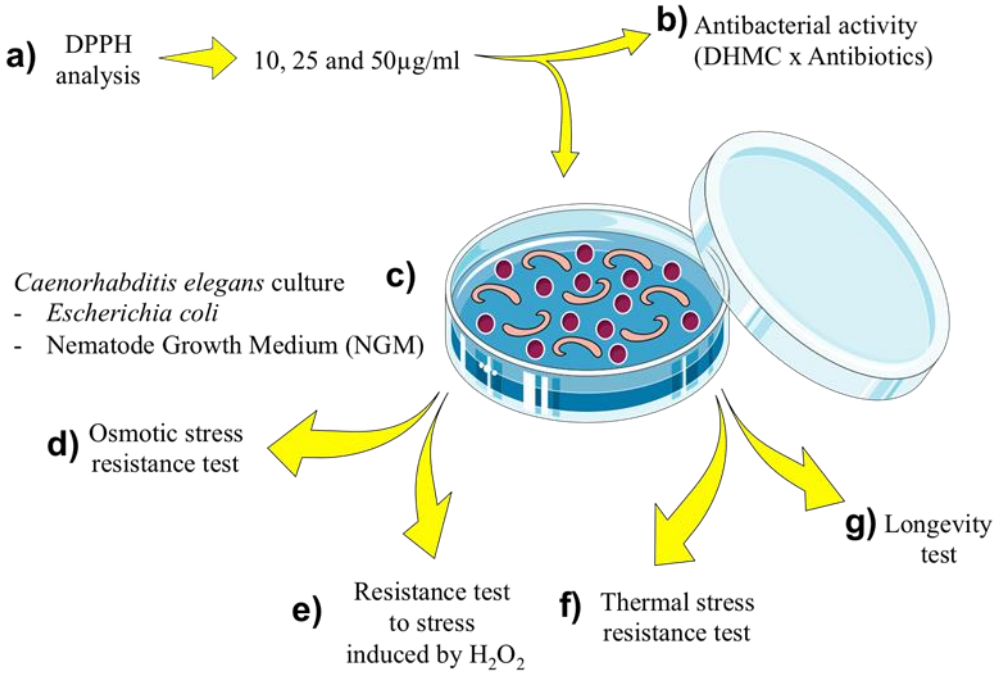



Figure 1: Experimental design. Coumarin treatment concentrations were determined by DPPH method (a). Then an antibacterial assay (b) was performed. At different concentrations, DHMC was used to treat Caenorhabditis elegans worms (c) that were evaluated for osmolality change by NaCl (d), H2O2 (e), in addition to temperature survival (f) and longevity (g) tests.

Strains: Wild strains Bistrol N2 and GLP-4; SEK-1 mutants (capable of becoming sterile at 25ºC) were used, both fed with Escherichia coli (OP50). Each experimental group consisted of 75 worms of each mutation, divided into triplicate (11). For maintenance and care, the worms were kept at a temperature of 20ºC in an incubation oven. In experimental periods, the worms were kept at 25ºC, to accelerate the development and inhibit the progeny in GLP-4; SEK-1 strains (12-14). In all tests, synchronizations of the worms were performed following the

protocols described by Porta-de-la-Riva, Fontrodona (15), Sugawara and Sakamoto (16).

Analysis of the antioxidant activity of coumarin by free radical stabilization (DPPH): To carry out the experiment, test tubes were used with a volume of 0.03ml in the respective concentrations of the compound 25, 50, 100, 150, 200, 250 and 500 µg/ml and completed with 2.7 of an ethanolic solution of DPPH. To improve the results, a positive control group made with Butylated Hydroxytoluene (BHT), a synthetic antioxidant, and a negative control made with ethanolic solution were used. All analyzes were performed in triplicate. Data were analyzed following the protocols of Sirivibulkovit, Nouanthavong (17), Garcia, Oldoni (18).

Compound: Initially, 10 mg of the DHMC compound was diluted in 500µl of Dimethylsulfoxide (DMSO) and later completed up to the volume of 1ml with distilled water and subsequently diluted to concentrations 10-25-50µg/ml. All concentrations were below 1% DMSO avoiding interference with worm survival according to Katiki, Ferreira (19).

Coumarin DHMC toxicity test: The worms were synchronized and caught at the L1 stage and transported to 24-well plates containing E. coli (OP50) and different concentrations of compound. The survival rate of the worms was analyzed every day until the beginning of their progeny, in order to verify if there will be changes in their behavior (20).

Antibiogram and antibacterial analysis of coumarin against E. coli OP50: For the antibacterial test, the disk diffusion protocol through antimicrobial sensitivity was used. The test was performed in triplicate following the procedures proposed by Photolo, Mavumengwana (21), Hoelzer, Cummings (22).

Essay: All tests were performed in triplicate with 25 worms per well, being divided into a control group (treated with E. coli + DMSO vehicle solution at a concentration of 50µl/ml) and a treated group (being treated with E. coli + the compound in the concentration of 10, 25 or 50µl/ml).

Osmotic Stress Resistance Test by Sodium Chloride (NaCl): Divided into groups, synchronized Lineage N2-worms were treated from the first day of life with the compound and control solution. For the osmotic stress assay, 2ml (500 mM of NaCl) was added to 24-well plates containing only Nematode Growth Medium (NGM). Thus, the worms were kept at 20ºC and observed after 24 hours, the worms were stimulated by a platinum wire to verify the survival rate (14, 23).

Resistance test to stress induced by H2O2: Synchronous and compound-treated worms. In early adulthood, they were transferred to a 24-well plate with NGM containing 2ml of 0.3% Hydrogen Peroxide (H2O2) and kept at 20ºC. Survival rate after transfer was defined as 100% and assessed every hour after an initial 2-hour stress period. The test was performed in triplicate (16).

Thermal stress resistance test: On the fourth day of adult life, the worms were transferred to plates containing only agar medium without food and compost and then submitted to an incubator at a temperature of 35ºC and observed every 2 hours to the stimulation of a platinum wire in order to check survival rate, the process was adapted following the baseline study (16).

Longevity test: To have the greatest control of the worms, GLP-4 mutant worms exposed to 25ºC were used to control the progeny. In sets of 25 GLP-4 worms made in triplicate, the groups were divided into: negative control group fed with a concentration of 50µl/ml of DMSO + E. coli and treated group with concentrations of 10, 25, 50 µl/ml of Coumarin + E. coli. Throughout the experiment, every day the number of dead worms was counted and removed from the plate, every 4 days the plate was changed by means of transfer with a platinum wire.

Statistic: The statistical software Graph Prism 8 was used for T-test analysis for comparison between two groups, Two-way ANOVA with independent samples with post Sidak multiple comparison tests. For the survival rate analyses, the kaplan meier curve was used. In all analyses, the pre-established level of significance was $p<0.05$.

## Results

*DHMC antioxidant capacity test by DPPH method*

It is described in the literature that one of the effects that can negatively modulate longevity is free radical. Free radicals are molecules that are toxic to cells naturally released by the body. As they are released by metabolism, other molecules can quickly eliminate them. In this sense, we sought to assess whether our compound had any antioxidant activity. One of the most effective evaluations of this process is through the use of the DPPH. Our data show that the use of 25mg had a high capacity for scavenging free radicals (Fig. 2), in addition the 240 and 500mg tools also provided significantly better values when compared to the test control (BHT).

From the results obtained in the DPPH assay, we infer three procedures for further testing using the coumarin compound DHMC: a concentration lower than the first observed in the assay (10µg/ml), the first concentration with high sequestering capacity (25µg/ml), and a concentration above the first observed in the graph (50µg/ml).

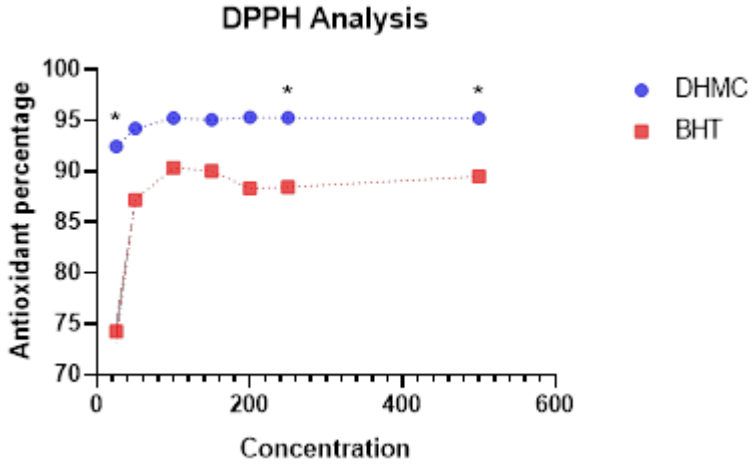



Figure 2: Effect of DHMC compound on free radical scavenging. The samples were separated into different concentrations: 25, 50, 100, 150, 200, 250 and 500 µg/ml. As test control we used Butylated Hydroxytoluene (BHT). All analyzes were performed in triplicate. Statistically the concentrations of 25, 240 and 500 µg/ml were significantly different when compared to BHT.

*Possible bactericidal effect of synthetic coumarin on E. coli bacteria*

It is known that the worm Caenorhabditis elegans can feed on Escherichia coli bacteria. In this case, it seemed interesting to us to investigate whether the study compound could have any bactericidal effect, and once this ability was proven, it could be possible to modulate the worm longevity in feeding on live or dead bacteria. For this assay we used three bactericides as controls: Ampicillin, Norfloxacin and Nitrofurantoin; in addition, the three concentrations of Coumarin: 10, 25 and 50µg/ml (Fig. 3a-b). Our results show that commercial antibiotics were effective in the formation of halo, we saw that Ampicillin, Norfloxacin and Nitrofurantoin presented results of 26, 30 and 38mm of halo, respectively, which confirms the sensitivity of E. coli to drugs. However, our compound did not show any change in the halo at different concentrations, showing that the bacteria are resistant to synthetic Coumarin (Table 1).

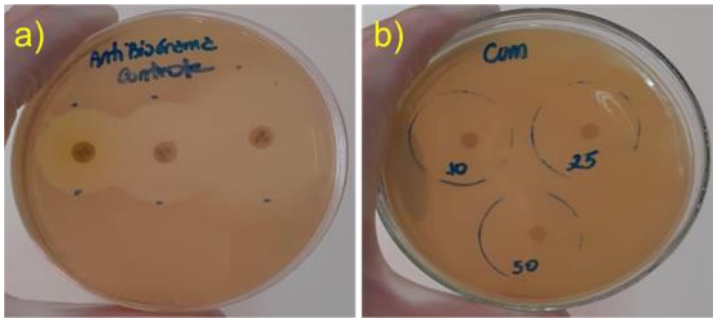



Figure 3: Antibacterial test in E. coli bacteria treated with DHMC. Escherichia coli bacteria were seeded in Agar Cled. culture medium, to assess resistance or sensitivity, they were treated with commercial drugs (Ampicillin, Norfloxacin and Nitrofurantoin) considered controls in the experiment (Fig. 3a), furthermore treated with DHMC at concentrations of 10, 25 and 50µg/ml (Fig. 3b). At the end of the experiment, resistance or sensitivity was determined based on the bacterial growth inhibition zones.

Our results show that commercial antibiotics were effective in the formation of halo, we saw that Ampicillin, Norfloxacin and Nitrofurantoin presented results of 26, 30 and 38mm of halo, respectively, which confirms the sensitivity of E. coli to drugs. However, our compound did not show any change in the halo at different concentrations, showing that the bacteria are resistant to synthetic Coumarin (Table 1).

Table 1: Method of determination of resistance or resistivity of bacterial growth using a growth halo.

| | Resistant | Intermediate | Sensitive | Results | Classification |
|---|---|---|---|---|---|
| **Ampicillin (10µg)** | ≤13 | 14-16 | ≥17 | 26mm | Sensitive |
| **Norfloxacin (10µg)** | ≤12 | 13-16 | ≥17 | 30mm | Sensitive |
| **Nitrofurantoin (300µg)** | ≤14 | 15-16 | ≥17 | 38mm | Sensitive |
| **7,8-Dihydroxy-4-methylcoumarin** | ** | ** | ** | 0mm | Resistant |

### *Survival overview of Caenorhabditis elegans worms treated with Coumarina compound*

Living organisms can be influenced by the concentration of leaving the external environment, a fact that favors water loss through osmotic processes. We have so far observed that our synthetic compound Coumarin (DHMC) is highly capable of scavenging free radicals at a concentration of 25µg/ml (≅95% antioxidant capacity), in addition we have seen that DHMC has no bacterial effect. Now, we decided to directly investigate the effect of our compound on Caenorhabditis elegans species. Initially the worms were kept in two solutions (M9 buffer solution and Sodium Chloride) and divided in two groups: Control (DMSO) and treated with DHMC (10, 25 and 50µg/ml), our results show that when the worms remained in buffer solution there was no significant difference between the groups when evaluating the total number of dead worms, but when the animals were subjected to a solution with Sodium Chloride (NaCl), there was a significant increase in death in the DHMC-50µg group (14 deaths and $p<0.001$), furthermore, there is a trend towards a lower death rate in the DHMC-25µg group when compared to the other experimental groups (Fig. 4a).

Another important mechanism that makes it possible to assess the mortality rate of organisms is through tests that assess oxidative stress. The stressful situation is due to imbalances between molecules/compounds with oxidizing activity and other compounds/molecules with antioxidant activity. This process leads to an increase in free radicals in an exacerbated way that the molecules responsible for fighting them do not increase proportionally, this mechanism can result in loss of biological functions and cell death, in addition to homeostatic imbalance. To assess the ability of our compound to act as an anti-stress molecule, we treated the worms with Hydrogen Peroxide (H2O2) and with DHMC, in addition to DMSO which was used as a control. Our data show that at a concentration of 50µg/ml, the compound coumarin promoted greater cell death ($p<0.0001$) when compared to the other groups (Fig. 4b).

Next step was to evaluate the survival capacity of DHMC-treated worms when induced to thermal resistance, kept at 35ºC. After 10 hours of experiment, death rate was measured every 2 hours, we observed the death of all animals, including the control group (DMSO). Only in the DHMC-10ug/ml group was found a significant difference ($p<0.0001$) compared to DMSO 50µg/ml (Fig. 4c), leading to a higher resistance in the mortality rate to heat stress.

It also investigated the survival rate of animals over 25 days. Every 4 days the worms were transferred from plates using platinum wire and the number of deaths counted. At the end of 20 days, all animals died and no statistical difference was found between the experimental groups (Fig. 4d).

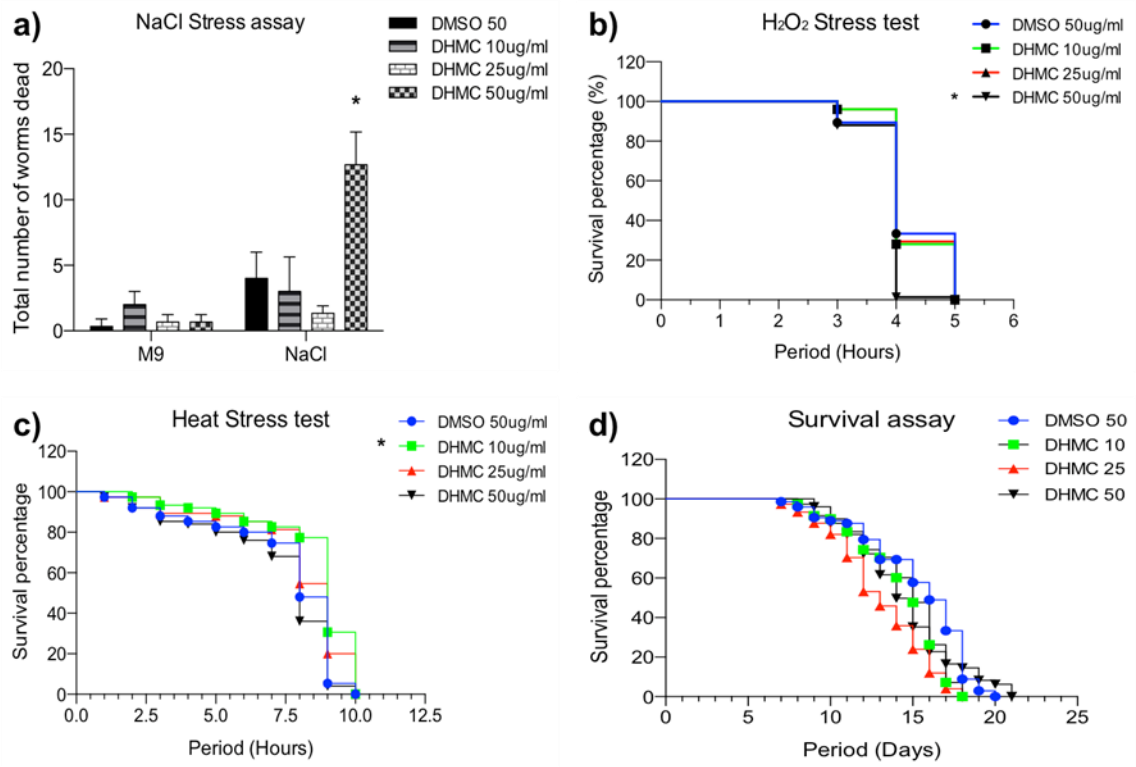



Figure 4: DHMC alters osmotic resistance and survival of Caenorhabditis elegans worms. To investigate the osmolality change profile of the animals, Sodium Chloride assays (a) with the use of M9 buffer as a control, Hydrogen Peroxide (b) were used. Furthermore, survivability was measured by increasing temperature (c) and death rate over days (d). Data represent independent triplicates.

Finally, in Table 2 we can see the quality of life of the animals over time, considering the treatment doses, experimental control and mean standard aviation, there is no statistically significant difference.

Table 2: Mean and standard deviation of lifetime

| Strain | Treatment | Life mean (± SD) |
|---|---|---|
| **GLP-4;SEK-1** | DMSO 50ug/ml | 13,76 ± 2,98 |
| **GLP-4;SEK-1** | DHMC 10ug/ml | 12,84 ± 2,75 |
| **GLP-4;SEK-1** | DHMC 25ug/ml | 12,12 ± 2,06 |
| **GLP-4;SEK-1** | DHMC 50ug/ml | 13,37 ± 2,49 |

## Discussion

The present study aimed to identify the influence of the use of synthetic compound coumarin (DHMC) on longevity and resistance to different types of stress. Caenorhabditis elegans are an important methodological tool for pharmacology, their similarity to intervention responses is compatible with more evolved mammals (20). Making the model an excellent choice for initial compound testing. In our findings, the 4-methyl class coumarin compound corroborates and reinforces the study conducted by Sharma, Rajor (24), demonstrating in our analysis an antioxidant action above the half-maximal inhibitory concentration (IC50) from small concentrations.

In recent decades, there have been constant relationships between a better quality of life or body maintenance and consumption of foods with high antioxidant potential (25). It is known that the high production of reactive oxygen species (ROS) can lead to mitochondrial damage, increasing cell wear with a potential decrease in lifespan, in addition to accelerating (26-28). However, there is a hypothesis in the literature that high levels of antioxidants may have a negative influence. Pisoschi and Pop (29) describe that the ingestion of foods or supplements that contain excess antioxidant activity can negatively affect the action of endogenous ROS, causing a condition known as "antioxidative stress" by inhibiting hormesis stress. In response to food restriction, hypoxia, and controlled oxidative stress, the body tends to activate autophagic pathways controlled by AMPK and ATGs that can lead to better cell maintenance (30, 31).

Our second hypothesis is guided by an elegant study conducted by Goel, Prasad (32) in lung cells with adenocarcinoma, it was identified that these cells treated with DHMC, when exposed to the stress factor TNF-α, underwent apoptosis through the BCL-2 pathway. It is known that there are two apoptotic pathways known as intrinsic and extrinsic, in the extrinsic pathway the pro-apoptotic family of BCL-2 is regulated by death factors such as TNF-a through Fas receptors, leading to cytochrome C extravasation and consequently apoptosis (33). On the other hand, the intrinsic pathway is regulated by Bcl-2 homologus antagonist killer (Bak) and Bcl-2 associated X protein (Bax) and is activated by ROS (33). That is, apoptosis can occur regardless of a good antioxidant system. Sumorek-Wiadro, Zając (34) identified similar results to the use of 4-hydroxycoumarin coumarin, being used for alternative treatment of gliomas, leading to an antiangiogenesis and higher apoptotic levels through the inhibition of the anti-apoptotic expression of BCL-2. Our results show that the DHMC compound has high antioxidation efficiency at a concentration of 25µg/ml, detected by DPPH.

As already mentioned, hormesis stress is beneficial to the body, in that small amounts of ROS can play an important role in regulating the defense of pathogens and systemic signaling. Most cells release ROS and use it as a form of chemical signaling (35-37). Thus, compounds such as DHMC could have performed an antioxidant action above the ideal, causing a negative effect. However, this hypothesis needs more data and studies to make such an assumption about DHMC. The above hypothesis could also justify the results of the heat stress test, in which a significant difference was found in the 10µg/ml group with greater resistance to worm death. Groups with higher concentrations could completely inhibit hormesis stress, affecting the body's defense system and preventing the accumulation of misfolded proteins (38).

In the longevity test, we identified results similar to another study using the same strain. With an approximate average of 14 days of life in SEK-1 mutant

strains (39). In the study by Henderson, Bonafè (40) it is described that food excess can lead to an activation of the insulin/IGF-like signaling pathway (IIS) and rapamycin target (mTOR) regulated by DAF-16 and consequently a decrease in nematode life through super expression of insulin pathways. Zhang, Sowers (30) in their review, analyze the cascades of reactions through the insulin-related pathway, showing that an overexposure to nutrients can lead to a decrease in beneficial autophagy. Henderson, Bonafè (40), show that hungry animals showed a higher expression of the antioxidant enzyme SOD:3. Thus, food scarcity can activate DAF-16-dependent pathways increasing longevity (41), while autophagy-related pathways are inhibited through excess food (30).

## Conclusion

Therefore, our results show that coumarin 7,8-Dihydroxy-4-methylcoumarin (DHMC) has a high antioxidant activity, in addition to protecting against effects caused by increased osmolarity at low concentrations. In addition, it can protect from thermal effects, but studies with new tools need to be carried out to investigate the potential in longevity processes. Our results open new avenues to investigate the potential of DHMC in in vitro or in vivo studies showing new molecular mechanisms caused by stress and ROS.

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