Effect of whole plant extract of *Evolvulus alsinoides* on thermal stress resistance and longevity in *Caenorhabditis elegans*

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Abstract

The popular Ayurvedic plant *Evolvulus alsinoides* is known to have adaptogenic properties. Adaptogens reduce stress and anxiety and thereby promote the overall well-being of the individual. Since chronic stress is associated with lower than normal lifespan expectance, any herbs known to reduce the stress should have the reverse impact. Therefore, this research aimed to study the anti-ageing activity of Evolvulus alsinoides in the well-established aging model *Caenorhabditis elegans*. The longevity-enhancing impact was assessed under the optimum growth and survival conditions for the *C. elegans*. Oxidative stress was induced by the use of Paraquat in N2 wild-type *C. elegans*, and the thermal stress was induced in transgenic *C. elegans* TJ 356, which expressed Green fluorescence Protein (GFP) under the control of heat shock protein promoters for the visualization of induction of anti-stress genes. The impact of the stress was analyzed by the lifespan analysis, and data were analyzed by the Kaplein Meyer statistical analysis. The results indicated that *E. alsinoides* extracts dose-dependently increased the mean lifespan of *C. elegans* by 18.0% and 26.2% at the concentrations of 0.1 mg/mL^{-1} and 1 mg/mL^{-1} under optimum growth and survival conditions, respectively. The survival rates of *E. alsinoides* extract-fed *C. elegans* have been greater than those of untreated *C. elegans* against thermal-induced stress. For Oxidative stress, the *E. alsinoides* treatment was non-significant. It was found that *Evolvulus alsinoides* extract promotes longevity in *Caenorhabditis elegans* by promoting stress tolerance and by tinkering with the insulin/IGF signaling pathway.

Keywords: genus *Caenorhabditis*, genus *Evolvulus*, longevity studies, thermal resistance.

Efeito do extrato da planta inteira de *Evolvulus alsinoides* na resistência ao estresse térmico e na longevidade em *Caenorhabditis elegans*

Resumo

A popular planta ayurvédica *Evolvulus alsinoides* é conhecida por suas propriedades adaptogênicas. Adaptógenos reduzem o estresse e a ansiedade, promovendo, assim, o bem-estar geral do indivíduo. Como o estresse crônico está associado a uma expectativa de vida menor, qualquer erva conhecida por reduzir o estresse pode ter o impacto oposto. Portanto, esta pesquisa teve como objetivo estudar a atividade antienvelhecimento de Evolvulus alsinoides no modelo de envelhecimento amplamente estabelecido *Caenorhabditis elegans*. O impacto na longevidade foi avaliado em condições ideais de crescimento e sobrevivência para C. elegans. O estresse oxidativo foi induzido com o uso de Paraquat em *C. elegans* do tipo selvagem N2, e o estresse térmico foi induzido em *C. elegans* transgênico TJ 356, que expressa a proteína fluorescente verde (GFP) sob o controle de promotores de proteínas de choque térmico, para visualizar a indução de genes antiestrésse. O impacto do estresse foi analisado por meio de análise de tempo de vida, e os dados foram tratados utilizando a análise estatística Kaplan-Meier. Os resultados indicaram que os extratos de *E. alsinoides* aumentaram a média de vida de *C. elegans* de forma dependente da dose em 18,0% e 26,2%, nas concentrações de 0,1 mg/mL^{-1} e 1 mg/mL^{-1}, respectivamente, sob condições ideais de crescimento e sobrevivência. As taxas de sobrevivência de *C. elegans* alimentados com extratos de *E. alsinoides* foram maiores do que as de *C. elegans* não tratados, sob estresse térmico induzido. Para o estresse oxidativo, o tratamento com *E. alsinoides* não apresentou efeito significativo.

Concluiu-se que o extrato de *Evolvulus alsinoides* promove a longevidade em *Caenorhabditis elegans* ao aumentar a tolerância ao estresse e ao modular a via de sinalização da insulina/IGF.

Palavras-chave: gênero *Caenorhabditis*, gênero *Evolvulus*, estudos de longevidade, resistência térmica.

1. Introduction

The increasing segment of the older population in our society is considered one of the most significant transformations the twenty-first century is witnessing. This transformation has important implications for all societies, including the economy and social welfare. As populations become increasingly aged, maintaining a healthy lifespan has become a critical challenge. Along with modern medicines, functional foods, herbal extracts, and dietary supplements are known to contain special metabolites that provide numerous health benefits, especially in aging-related disorders (Dhanjal et al., 2020).

Several herbs associated with healthy aging are also adaptogens and, when evaluated for their mode of action, were found to be working via their ability to modulate stress, enhance physical endurance, provide antioxidants, and at times also due to their nootropic abilities (Esmaealzadeh et al., 2022). Herbal extracts such as *Rhodiola rosea*, *Ginkgo biloba*, and *Withania somnifera*, the known sources of the adaptogens, are proven to increase the lifespan of *Caenorhabditis elegans* by increasing their ability to resist physical, biological, and chemical stress (Kumar et al., 2013; Shen et al., 2021; Wiegant et al., 2009).

Evolvulus alsinoides is an ingredient in the popular formulations of Ayurveda known as Medhya Rasayana. It is a brain tonic and has been used to treat various ailments, such as neurodegenerative disorders, amnesia, and asthma, for centuries (Yadav et al., 2019a). A study conducted on Male Sprague–Dawley rats to evaluate the adaptogenic ability of *E. alsinoides* on acute and chronic stress proved that the plant reduces the peripheral stress marker and the effect was equivalent to the known adaptogen *Panax quinquefolium* (Siripurapu et al., 2005).

Because of this, we have evaluated the crude hydroethanolic extract of *E. alsinoides* on *C. elegans* to evaluate its stress-modulatory, antioxidant, and longevity-promoting activity. *C. elegans* is a useful *in vivo* model to validate longevity-promoting agents because of its intricate cellular structure, conservation of numerous metabolic pathways with complex higher organisms, ease of cultivation, and low cost (Liao et al., 2018). Since a publication by Klass in 1977, which established the method to measure *C. elegans* lifespan consistently and the utilization of 5-Fluoro-2'-deoxyuridine (FUDR) to sustain synchronous cultures of aged *C. elegans*, it has become the well-known model for aging research (Tissenbaum et al., 2015).

C. elegans as a model has shown the anti-ageing potential of various phytomolecules like curcumin, reservatol, Ursolic acid, aspalathin, speciose, and carotenes (Asthana et al., 2015; Gruber et al., 2015). These plant-derived molecules have therapeutic properties and minimal off-target effects compared to their chemical substitutes, making them suitable alternatives to synthetic drugs (Wiegant et al., 2009). Furthermore, studies on *C. elegans* have provided insight into the anti-ageing properties of crude extracts, which are frequently attributed to the antioxidants present in those extracts. Therefore, *C. elegans* has been utilized in this study as a model system to assess the adaptogenic and anti-ageing properties of *E. alsinoides* (Van Raamsdonk et al., 2010). Our study may promote the development of *Evolvulus alsinoides* as an adaptogen and a potential nutritional supplement to promote a healthy lifespan.

2. Materials and Methods

2.1 Preparation of plant extracts

Saplings of *E. alsinoides* were attained from the Anand Agricultural University and cultivated at the LCRD ("Loyola Center for Research and Development") (Doshi; Braganza, 2018). To prepare plant extracts, the entire *E. alsinoides* plantlet was collected, carefully cleaned under running tap water, and then shade-dried. The dried material was ground into a powder, and extracts were made using a cold maceration at 20 ºC for 72 h with a 70:30 ratio of ethanol to water as the solvent (Doshi; Braganza, 2019).

2.2 Quantification of phenolics and flavonoids

The plant extract's total phenolics and flavonoids were estimated as per "the Folin–Ciocalteu's (F-C) and $AlCl_3$ methods, correspondingly, with the slight modification adapted (Herald et al., 2012; Doshi; Braganza, 2018; Doshi; Braganza, 2019). Briefly, for total phenolics, in a 96-well plate, 5 µL of standard or sample was diluted

with 75 µL of distilled water, followed by adding 25 µL of 1:10 diluted F-C reagent (Doshi; Braganza, 2018; Doshi; Braganza, 2019). The mixture was incubated for 6 min at room temperature, and then 40 µL of 75 g/L Na_2CO_3 was added to each well. The plate was then incubated in the dark for the time of 90 min at room temperature, and then the absorbance was calculated at 765 nm in the UV-visible spectrophotometer of Shimadzu. Total phenolics were measured relative to gallic acid (GA), and the outcomes have been reported as GA equivalent. For flavonoids, 5 µL of the sample was diluted with 100 µL of distilled water, followed by the addition of 10 µL of $NaNO_2$ (50 g/L), it was allowed to incubate for five minutes at room temperature, 15 µL of 100 g/L $AlCl_3$ has been then added, and the plate was incubated for 6 min at room temperature. Subsequently, 50 µL of 1M NaOH & 50 µL water were added to each well. After shaking for 30 s, absorbance was measured at 510 nm in the UV-visible spectrophotometer of Shimadzu. Rutin served as the standard for flavonoids, and outcomes were displayed as Rutin equivalent.

2.3 C. elegans strain and maintenance

The *C. elegans* strains N2 bristol and TJ375(gpls1) have been utilized in this research. N2 bristol was obtained from Dr. Sandhya Kaushika, who is working at TIFR. The Caenorhabditis Genetic Center provided the remaining strains. According to the protocol mentioned by Doshi S., *C. elegans* strains have been kept at 20 ºC on an NGM) (Nematode Growth Medium) plate seeded with *Escherichia coli* OP50. (Doshi; Braganza, 2018; Doshi; Braganza, 2019; Poupet et al., 2019).

2.4 Synchronization of C. elegans

For synchronization, *C. elegans* were collected by washing them off the NGM plate in 10 mL of distilled water, transferred into 15 mL conical tubes, and centrifuged at 1000 rpm for 1 min; the pellet was washed two times to eliminate the bacteria. Then, the pellet was dissolved in 2 mL of 200 mM NaOH and 500 µL of 30% hydrogen peroxide. This step dissolved adult worms but did not harm the eggs of *C. elegans*. The debris of adult *C. elegans* was eliminated by centrifugation in a tabletop centrifuge at 1500 RPM, and the egg pellet was then washed thrice with distilled water by repetitive centrifugation in a tabletop centrifuge at 3000 RPM for 1 minute. After removing the supernatant, the eggs were suspended in 5 mL of S-complete media and left to hatch at 20 ºC for the entire night (Porta-de-la-Riva et al., 2012).

2.5 Lifespan analysis of C. elegans

The *C. elegans* lifespan assay has been carried out in 96-well plates using a liquid S complete medium, following the methodology outlined by Solis G with a modification outlined by Doshi S. (Doshi; Braganza, 2018; Solis; Petrascheck, 2011). After being treated with sodium hypochlorite, synchronized populations of *C. elegans* were produced. Ten or so L1 stage *C. elegans* were then placed in each well, which contained 100 µl of S complete medium and OP50 as a source of food. The 96-well plate was kept at 20 ºC for 2 days. The matured worms were then treated with FUDR to stop progeny formation on day 0 of adulthood. An equal volume of (5 µL) plant extracts and DMSO (as a control) were added to the appropriate wells to evaluate the efficacy of the plant extracts at three different concentrations (Sen et al., 2019). These were then replenished every week along with OP50. In each experiment, the average number of *C. elegans* (*n*) was between 50 and 55. Three separate trials of the aforementioned experiment were conducted. The survival of the *C. elegans* was checked daily under the inverted microscope. Lifespan data analysis was performed through the Kaplein Meier statistical analysis (Park et al., 2017).

2.6 Assessment of resistance to thermal and oxidative stress

In a "96-well plate containing S-complete medium, L1 larvae of the wild-type N2 were subjected to varying concentrations of plant extracts or DMSO as a solvent. The 96-well plates contained 18–20 larvae" (n) in each well (Doshi; Braganza, 2018). Three additions of either DMSO or plant extracts were made. After 72 h of incubation at 20 °C, 50 mM paraquat was introduced to induce oxidative stress, and the survival of the larvae was assessed after 24 h of incubation at 20 °C (Possik; Pause, 2015; Doshi; Braganza, 2018). The thermal stress assay has been carried out as per Doshi S. (Doshi; Braganza, 2018). "In 96-well plates containing S-complete medium and OP50 as a source of food, L4 C. elegans treated with DMSO" or plant extracts have been then incubated for four hours at 37 °C and then recovered for 24 h at 20 °C (Chen et al., 2019). Subsequently, the *C.*

elegans were observed daily until all of them had died.

2.7 Fluorescence quantification and visualization

C. elegans strain TJ375 contains a GFP transcriptional fusion to the HSP-16.2 promoter. TJ375's synchronized culture was produced by treating it with sodium hypochlorite as mentioned above. From the L1 stage, *C. elegans* were grown for 72 h in a "liquid S medium supplemented with the extracts of plant or DMSO as control at 20 °C (Sen et al., 2019). Twenty *C. elegans* were then paralyzed by placing them in PBS comprising sodium azide on a glass slide, one from each group. The reporter protein GFP's fluorescence has been quantified to determine the HSP-16.2 expression level. Using a camera-equipped Zeiss microscope, the fluorescence intensity was monitored, and pictures were taken. ImageJ (NIH) software then calculated the GFP intensity from the pictures in terms of the fluorescence pixel density (Duangjan et al., 2019).

2.8 Data analysis

For statistical analysis, "GraphPad Prism version 7.00 for Windows (San Diego, CA)" was utilized (Jayarathne et al., 2020). Plotting the mean ± SEM of at least three different experiments was done to display the results. The survival curve was plotted using a Kaplan-Meier analysis and analyzed by calculating the mean survival time. The log-rank test has been utilized to compare survival under various conditions and P-values. Various doses of plant extract were compared concerning control by utilizing one-way ANOVA to calculate the statistical significance. The results were considered statistically significant if the *P* value obtained was less than or equal to 0.05 (Doshi & Braganza, 2018).

3. Results

3.1 Total polyphenol content and flavonoid content

E. alsinoides contain special metabolites like alkaloids, polyphenols, carbohydrates, amino acids, proteins, flavonoids, and tannins. Phenolics and flavonoids from the ethanol: water extract of *E. alsinoides* were quantified as they are potential antioxidants and free radical scavengers. Total phenolics and flavonoid contents were found to be 17.41 ± 1.35 mg of GA equivalent and 2.11 ± 0.04 mg of Rutin equivalent, correspondingly.

3.2 The lifespan-promoting activity of E. alsinoides

To find out the *E. alsinoides* impact on the longevity of *C. elegans*, ethanol: water extract prepared by cold maceration was used. *C. elegans* were subjected to 3 various concentrations of extracts (0.01 mg/mL, 0.1 mg/mL, and 1 mg/mL) in a liquid S- complete medium supplemented with *E. coli* OP50 (Sen et al., 2019). Control *C. elegans* was treated by DMSO as vehicle control. The impact on the *C. elegans* lifespan is depicted below in (Table 1). The result indicates a dose-dependent increase in the lifespan.

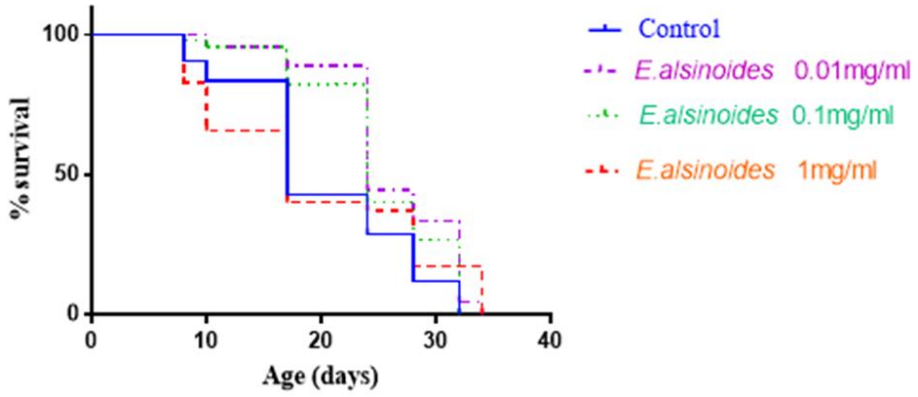

Figure 1. The survival curve showed effect of ethanol: water extract of *Evolvulus alsinoides* prepared by cold extraction method. Survival curve was plotted by Kaplein Meier analysis and analysed by Log-Rank (Mentel cox analysis). *E. alsinoides* treated *C. elegans* showed an increase in lifespan at 0.1 mg/mL concentration and 1 mg/mL. Source: Authors, 2024.

Table 1. Effect of *Evolvulus alsinoides* ethanol: water extract prepared by cold extraction method on the life span of *Caenorhabditis elegans*.

	***C. elegans* strain**	**No. of *C. elegans* (n)**	**Mean life span ± SE**	***P* value v/s control**
Control	Wild type	126	18.4 ± 0.9	
E. alsinoides 0.01 mg/mL	Wild type	105	18.28 ± 2.0	≤0.001
E. alsinoides 0.1 mg/mL	Wild type	135	25 ± 0.68	≤0.001
E. alsinoides 1 mg/mL	Wild type	135	25.26 ± 1.9	≤0.001

Note: The table depicts the mean survival in days; at 0.1 mg.ml and 1 mg/mL, the lifespan increased by 13% and 35%, respectively. Source: Authors, 2024.

3.3 Effect on oxidative stress and thermal stress

To investigate whether *E. alsinoides* ethanol: water extracts, in addition to its longevity effect, can modulate oxidative stress responses in *C. elegans*, we examined the *C. elegans* survival when treated with 0.01 mg/mL, 0.1 mg/mL and 1 mg/mL of plant extract after induction of oxidative stress by Paraquat (Sen et al., 2019). Oxidative stress was not alleviated by *E. alsinoides* treatment. In the control group, 28.16 % ± 076 *C. elegans* survived after 24 h of oxidative stress, while *C. elegans* treated with *E. alsinoides* ethanol: water extracts showed 24.47 % ± 2.82, 25.33 % ± 4.04, and 26.04 % ± 2.00 showed survival at all tested concentrations, which suggests a nonsignificant change in the *C. elegans* lifespan (Figure 2 A) (Sen et al., 2019).

To further examine the anti-thermal stress effect of *E. alsinoides* ethanol: water extract, *C. elegans* from the L1 larvae stage have been then treated with "0.01 mg/mL, 0.1 mg/mL, and 1 mg/mL of" plant extract. Heat stress was then induced by incubating the *C. elegans* at 37 ºC for four hours on the second day of adulthood. Survival was monitored after 24 hours till all the *C. elegans* were dead (Sen et al., 2019). Compared to the control group (*C. elegans* treated with vehicle control DMSO), plant extracts treated *C. elegans* showed increased tolerance to heat stress. Statistical data for the same is depicted in (Table 2 and Figure 2). In thermal stress assay, the mean lifespan of control *C. elegans* has been observed to be 48 ± 4.5 h, while that of treated *C. elegans* extended up to 72 ± 2.7 h. The statistical analysis is shown in (Table 2 and Figure 2 B).

Table 2. The protective effect of an ethanol-water cold extract of *Evolvulus alsinoides* on N2 Bristol *Caenorhabditis elegans* experiencing thermal stress.

	Thermal stress	**No. of *C. elegans*, n = 3**	**Mean life span± SE (in hours)**	***P* value v/s control**
Control	37 ºC for 4 h	132	48 ± 4.5	
E. alsinoides 0.01 mg/mL		142	60 ± 3	≤ 0.001
E. alsinoides 0.1 mg/mL		174	72 ± 1.2	≤ 0.001
E. alsinoides 1 mg/mL		155	72 ± 2.7	≤ 0.001

Note: The Mean survival time increased by 150% ($P \leq 0.0001$), with both mean and maximum survival significantly rising in treated *C. elegans*. Source: Authors, 2024.

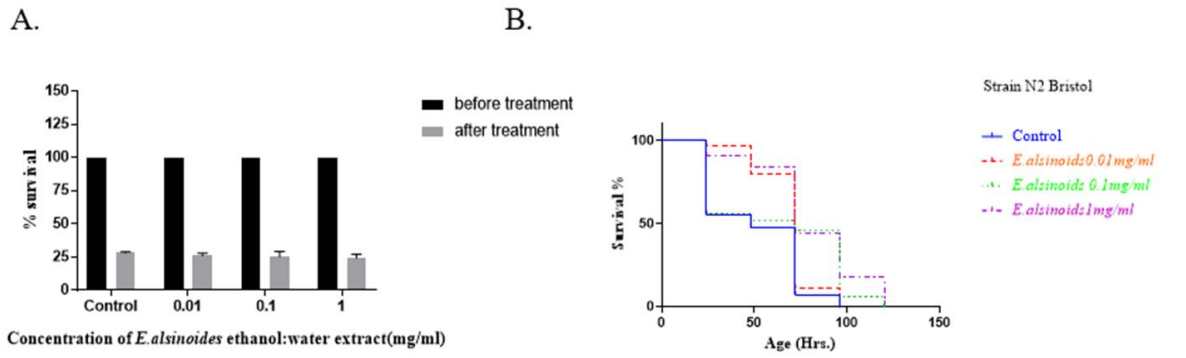

Figure 2. Effect of *Evolvulus alsinoides* ethanol: water extract prepared by cold extraction on (**A**) oxidative stress: Pretreatment of *Caenorhabditis elegans* with *Evolvulus alsinoides* did not promote stress resistance in *Caenorhabditis elegans*. (**B**) Thermal stress demonstrates a clear dose-dependent increase in lifespan with higher concentrations of the plant extract. Source: Authors, 2024.

3.4 Evolvulus alsinoides up-regulates HSP: GFP -expression

The TJ375 was utilized to substantiate the role of *E. alsinoides* in reducing heat stress. Worms of TJ375 strains were treated with solvent control or various concentrations of *E. alsinoides* ethanol: water extract. Worms were visualized under a fluorescent microscope, and images were captured and analyzed by utilizing Image J software. The intensity of untreated *C. elegans* was taken as 100 %. The intensity of treated worms was increased by 28 % ± 18.60, 24 % ± 11.59, and 69 % ± 15.76, respectively, for the three concentrations (Figure 3).

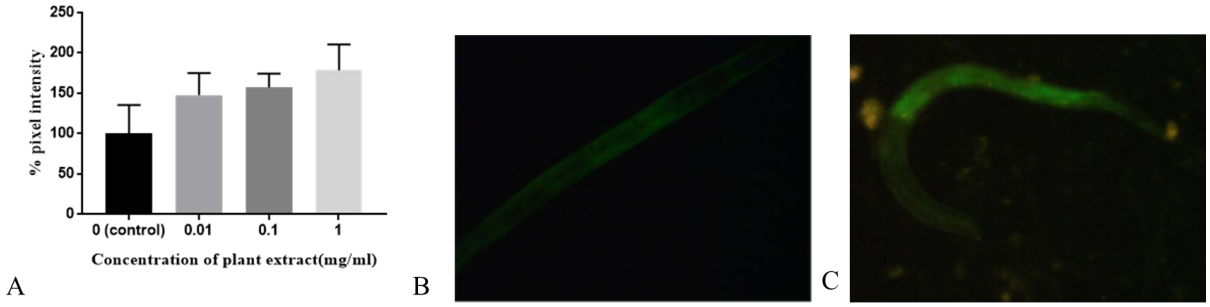

Figure 3. Effect of *Evolvulus alsinoides* et:w extract on the expression of HSP gene. (**A**) The hsp16.2 gene is upregulated by the treatment of plant extract at various concentrations. Statistical significance was calculated by one-way ANOVA. *P* value 0.001. All data are mean ± S.D. (**B**) Image of control worm. (**C**) Image of *Evolvulus alsinoides* ethanol: water-treated worm. Source: Authors, 2024.

4. Discussion

Plant-derived compounds are sources of natural antioxidants that are known to promote longevity (Salehi et al., 2020). Multiple lines of evidence indicate that polyphenols and flavonoids can alleviate age-associated cellular damage initiated by the metabolic generation of ROS (Reactive Oxygen Species) (Tollefsbol et al., 2010). Additionally, plant extracts rich in flavonoid and quercetin can extend the lifespan, decrease lipofuscin deposits in the intestine of *C. elegans*, and protect it against various stresses by reducing intracellular ROS and hence decreasing internal stress in *C. elegans* (Pietsch et al., 2009). Therefore, the polyphenol and flavonoid content of *E. alsinoides* was determined. We demonstrated that *E. alsinoides* possess a high concentration of polyphenols and flavonoids, which agreed with previously reported studies.

Previous studies have reported various medicinally important properties of *E. alsinoides* extracts, like use in treating amnesia and asthma, neurodegenerative diseases, and further antihaemorrhagic, antispasmodic, anti-inflammatory, and antioxidant effects (Yadav et al., 2019). However, to the best of our knowledge, the

anti-ageing properties of *E. alsinoides* have not been reported. Utilizing the short-lived nematode *C. elegans*, the effect of *E. alsinoides* upon longevity has been examined. The present study indicates that *E. alsinoides* significantly increases the life span of *C. elegans*. This is the first report to describe the potential anti-ageing effects of whole-plant aqueous ethanolic extracts of *E. alsinoides*. Several studies have demonstrated that increasing *C. elegans* resistance to stress complements extending its lifespan (Doshi et al., 2018; Duangjan et al., 2019).

It has been broadly accepted that aging may result from DNA, protein, and lipid damage caused by excessive ROS produced during normal metabolism or external stresses (Sadowska-Bartosz et al., 2014). Furthermore, heat shock and oxidative stress are significant risk factors for several age-related illnesses, including cancer, neurodegenerative diseases, and cardiovascular diseases (Slimen et al., 2014). In another set of experiments, the *E. alsinoides* impact extracts on oxidative stress and thermal stress were investigated. In our research, we found that extracts from *E. alsinoides* can help *C. elegans* withstand high temperatures, suggesting that these extracts may improve the worms' ability to survive in challenging conditions. This finding aligns with research by Zhang et al. (2019) which depicted that a polyphenol extract from *Rosa rugosa* tea affected both the resistance to heat stress and the average lifespan of the worms.

To substantiate the thermal stress resistance effect of *E. alsinoides in vivo*, gene expression for HSP-16.2 has been studied using transgenic strain TJ375. HSP 16.2 is a heat shock protein involved in restoring misfolded or impaired proteins and is important for the cell's recovery after a thermal shock (Min et al., 2017). The proteins' presence shields cells from stress-induced harm and readies them to withstand future cellular challenges. These proteins may also mend the gradual wear and tear associated with aging, potentially extending lifespan. This effect of lifespan increase after low doses of heat shock is known as hormesis. Therefore, it can be determined that the lifespan-enhancing effect of *E. alsinoides* may be achieved by harnessing hormesis mechanisms.

Further, in *C.elegans*, the Insulin/IGF signaling pathway plays a vital role in extending longevity. This pathway is regulated via three sets of transcription factors namely heat shock transcription factor (HSF-1), SKN-1, and DAF-16/FOXO (Zhu et al., 2019). HSF-1 is a transcription regulator of HSP-16.2, molecular chaperones, and HSP -70 which in turn extends the lifespan (Muñoz, 2003b). Future studies on various transgenic and mutant *C. elegans* will reveal whether the lifespan-extending ability of *E. alsinoides* is only due to the HSF-1-mediated pathway or whether other mechanisms are also involved. More assays are also needed to evaluate improvement in cognitive functions, muscle movement, and fecundity to establish the correlation between the increase in life span and quality health parameters.

5. Conclusions

Our results demonstrated that the *Evolvulus alsinoides* ethanol: water extracts encompass the *Caenorhabditis elegans* lifespan by mediating enhanced resistance against heat shock and in vivo ROS generation. These effects might be mediated through the Insulin/IGF-1 signaling pathway. Future studies are needed to decipher the mechanism of longevity and involvement of the other pathways. It can be studied in more complex organisms like mice models and humans.

6. Authors' Contributions

Shital Hemal Doshi: writing of the project, plant collection, extract production, laboratory analyses, article writing, submission, revisions, and publication.

7. Conflicts of Interest

No conflicts of interest.

8. Ethics Approval

Not applicable.

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