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Streblus asper Lour. exerts MAPK and SKN-1 mediated anti-aging, anti-photoaging activities and imparts neuroprotection by ameliorating Aβ in *Caenorhabditis elegans*

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Abstract.
BACKGROUND: *Streblus asper* Lour., has been reported to have anti-aging and neuroprotective efficacies *in vitro*.
OBJECTIVE: To analyze the anti-aging, anti-photoaging and neuroprotective efficacies of *S. asper* in *Caenorhabditis elegans*.
METHODS: *C. elegans* (wild type and gene specific mutants) were treated with *S. asper* extract and analyzed for lifespan and other health benefits through physiological assays, fluorescence microscopy, qPCR and Western blot.
RESULTS: The plant extract was found to increase the lifespan, reduce the accumulation of lipofuscin and modulate the expression of candidate genes. It could extend the lifespan of both *daf-16* and *daf-2* mutants whereas the *pmk-1* mutant showed no effect. The activation of *skn-1* was observed in *skn-1*::GFP transgenic strain and in qPCR expression. Further, the extract can extend the lifespan of UV-A exposed nematodes along with reducing ROS levels. Additionally, the extract also extends lifespan and reduces paralysis in Aβ transgenic strain, apart from reducing Aβ expression.
CONCLUSION: *S. asper* was able to extend the lifespan and healthspan of *C. elegans* which was independent of DAF-16 pathway but dependent on SKN-1 and MAPK which could play a vital role in eliciting the anti-aging, anti-photoaging and neuroprotective effects, as the extract could impart oxidative stress resistance and neuroprotection.

Keywords: *S. asper*; *C. elegans*, anti-aging, anti-photoaging, DAF-16, SKN-1, MAPK, neuroprotection

1. Introduction

Plants have always played a role in human health as a medicine or as an immune enhancer [1, 2]. The use of plants as medicines paved the way for unique treatments for various diseases including headache, dizziness, cold, wounds, cough, asthma and many more which is still being practiced in developing countries [3–5]. The advent of modern medicine was from the foundation laid by traditional medicinal practices [5]. However, the excess use of antibiotics and synthetic drugs has led to various unexpected side effects, leading many researchers to look back into the

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traditional knowledge for different medicines [1]. A recent report suggests that phytochemicals from plant sources can even act against the coronavirus [6].

Aging is a unique and constant process that eventually leads to age-related diseases, majorly neurodegeneration, and increased oxidative stress. In other words, aging and neurodegeneration are two sides of a coin as both are dependent on each other and are inevitable. Plants can synthesize large amounts of diverse bioactive substances, many of which have the potential to provide substantial antioxidant, neuroprotective and anti-aging benefits. Examples of herbal remedies include green tea, turmeric, cinnamon, ginger, and rosemary, which are widely used across the world due to their health benefits [7, 8]. Essential oils extracted from flowers, leaves, seeds, rhizomes, or barks, of different plants, mainly thyme, clove, eucalyptus, cinnamon leaf, juniper, basil, chamomile, coriander, cumin, could be used for their anti-aging, antioxidant and neuroprotective effects [9].

Streblus asper Lour. belongs to the family Moraceae and the subfamily of Moroideae with the 4th tribe *Strebleae* along with *Taxotrophis, Phyllochlamys,* and *Maillardia. S. asper* was used in traditional medicine in treating cardiac disorders, epilepsy, edema, leprosy, dysentery, elephantiasis, and tuberculous glands. Different parts of the plant including bark, roots, seeds, leaves, and latex, are used for their different medicinal properties, including fever, diarrhea, dysentery, disinfect wounds, toothache, filariasis, leprosy, snakebite, regulating blood pressure, stomach-ache, obesity and other skin disorders [10–12].

The plant has been identified to have different pharmacological and medicinal properties such as anti-cancer [13], antioxidant [14], antibacterial [15], anti-fungal [16], anti-diabetic [17] and anti-inflammatory effects [18]. We have previously reported the neuroprotective efficacies of *S. asper* in glutamate-induced oxidative toxicity in mouse hippocampal (HT22) cells. Liquid Chromatography-Mass Spectrometry (LC-MS) screening of the extract revealed the presence of andrographolide, carnosic acid, α-linolenic acid, and oleoyl oxazolopyridine along with known compounds such as (+)-3-O-β-D-fucopyranosylperiplogenin, strebluslignanol and magnolignan A, and taxifolin [19]. Additionally, when the extract was separated into acidic, basic and neutral fractions, the acidic fraction exhibited the strongest antioxidant and antibacterial potential, whereas the basic and neutral fractions exhibited neuroprotective effects. Gas chromatography-mass spectrometry (GC-MS) analysis of these fractions confirmed the presence of terpenoids, steroids, phenolics, fatty acids, and lipidic plant hormone [20].

Caenorhabditis elegans, the *in vivo* model system, has been widely used to study aging, development, reproduction, obesity, stress resistance, neuroprotection and many more diverse mechanisms [21–24]. Small size, short lifespan and life cycle, ease of handling and maintenance, completely sequenced genome are some of the advantages offered by this model, which attracts researchers towards it [23, 25]. The nematodes have been utilized to understand the different compounds or conditions that could impact aging, which could be analyzed using various physiological and molecular parameters [23, 26]. Further, the availability of mutant and transgenic strains allows the model to mimic the pathologies of neurological disorders, making them a preferred model [23].

A variety of plants such as *Bacopa monnieri* [21], *Sonneratia apetala* [27], *Betula utilis* [28], *Rehmannia glutinosa* [29], *Diplocyclos palmatus* [30], *Cleistocalyx nervosum* [31], *Caesalpinia mimosoides* [32] and *Glochidion zeylanicum* [33] have been recently identified to exert anti-aging effects in *C. elegans*. The present study aims to understand the anti-aging, antioxidant and neuroprotective mechanisms of *S. asper* in *C. elegans* and the mode of action exhibited by the plant to induce the effect.

2. Materials and methods

2.1. Chemicals, reagents, and equipment used

The chemicals and reagents utilized in the present study were obtained from Sigma-Aldrich (St. Louis, MO, USA) and HiMedia Laboratories (Mumbai, India). *C. elegans* strains were kept under UV transilluminator lamp, SANKYO DENKI (F20T10BL), for 4 h for UV-A exposure.

2.2. Plant collection and extraction

Leaves of *S. asper* were collected from the Princess Maha Chakri Sirindhorn Herbal Garden (Rayong Province, Thailand), authenticated [A013419 (BCU)] and deposited in Kasin Suvatabhandhu (Department of Botany, Faculty of Science, Chulalongkorn University, Thailand) herbarium. Dried

powdered leaves (35 g) were soaked in absolute ethanol for 48 h, and the resulting supernatant was collected and vacuum dried. The crude ethanol extract (100 mg) was dissolved in DMSO filtered (0.2 μm) and stored at –20 °C until use [19].

2.3. C. elegans strains used and culture conditions

Wild type strain N2 (Bristol), *daf-16* mutant CF1038, *daf-2* mutant CB1370, *pmk-1* mutant KU25, *skn-1*::GFP transgenic strain LG333, Aβ transgenic strain CL2006 and the bacterial food source *E. coli* OP50 were purchased from *Caenorhabditis* Genetics Center, (University of Minnesota, USA). All strains were grown, maintained and propagated in Nematode growth medium (NGM) at 15 °C as per the standard protocol [34]. Age synchronized young adult worms were used for conducting all the experiments.

2.4. Lifespan assay

Analysis of lifespan was performed in liquid media. Briefly, ten age synchronized young adult nematodes (wild type or mutants) were transferred into a 24 well microtiter plate with M9 buffer along with *E. coli* OP50 and 5-Fluoro-2′-deoxyuridine (FUDR). Different concentrations (1–100 μg/ml for wild type and 10–30 μg/ml for mutants) of *S. asper* extracts dissolved in DMSO were added to each well. The worms alive on the well were counted every 24 h. Nematodes were considered to be dead when they did not respond to gentle prodding using the platinum loop. Worms with no extract treatment but only with *E. coli* OP50 served as the control and DMSO as the vehicle control. The experiments were carried out in five independent trials [31].

2.5. Pharyngeal pumping assay

Pharyngeal pumping was measured in young adult staged wild type nematodes (∼10) grouped under control and extract treated, by monitoring the pharyngeal contractions for 30 seconds in every 24 h under a stereomicroscope (Motic SMZ-171) [31].

2.6. Fluorescence imaging

Accumulation of lipofuscin in the wild type nematodes and Nuclear factor erythroid 2-related factor 2 (NRF2) green fluorescent protein (GFP) transgenic strain LG333 were monitored using Confocal microscope. Briefly, worms treated with varying *S. asper* concentrations (10–30 μg/ml) consecutively for five days were monitored, with *E. coli* OP50 fed worms as control. After incubation, the worms were thoroughly washed using M9 buffer several times and then placed on a glass slide into a drop of sodium azide. Fluorescent imaging was performed using a ZEISS LSM 700 Confocal microscope under 10X magnification in 10 nematodes. The images were further analyzed using Image J software, and the relative fluorescence was represented as arbitrary units (AU). The experiment was carried out in three independent trials [31, 35].

2.7. Measurement of extracellular ROS using DCF

Briefly, two sets of wild type nematodes were taken for UV-A exposure for 4 h. In the first set, the worms were transferred to the microtitre plate and then treated with varying concentrations of *S. asper* extracts (10–30 μg/ml) and taken for UV-A exposure (pre-treatment). In the second set, the worms were transferred to the microtitre plate and then taken for UV-A exposure followed by treatment with varying concentrations of *S. asper* extracts (10–30 μg/ml) (post-treatment). In both cases, the extract treatment continued for five days under standard conditions and then the worms were washed thoroughly with M9 buffer. The worms were then incubated with DCFH-DA (5 μg/ml) for 20 minutes, followed by another wash using M9 buffer to remove the excess of DCFH-DA. The worms were placed in a glass slide and imaged under a confocal microscope. The images were analyzed using Image J software, and the relative fluorescence was represented as arbitrary units (AU). Worms exposed to UV-A for 4 h and did not receive any extract treatment were considered a positive control, whereas worms without UV-A exposure or extract treatment were considered negative control. The experiment was carried out in three independent trials [31].

2.8. Superoxide dismutase assay

SOD activity was measured using the protein lysate from control and treated worms obtained by crushing the worms, and the protein concentration was measured using the Bradford method. Protein (50 μg) from each sample is mixed with 100 mM Triethanolamine-Diethanolamine buffer (pH-7.4), 2 mM NADPH, 100 mM EDTA-$MnCl_2$ and the reaction was initiated by the addition of 10 mM

Table 1
List of Primers used

Gene Name	Forward Primer	Reverse Primer
daf-2	TCGAGCTCTTCCTACGGTGT	CATCTTGTCCACCACGTGTC
age-1	ATAGAGCTCCACGGCACTTT	ATAGAGCTCCACGGCACTTT
utx-1	GCAGAACACCAGCTCATCAG	ATCAACGCCATTCTTCTCGC
col-19	CACACAAATGCTCCACCAAC	CTGGATTTCCCTTCTGTCCA
egl-8	CGTATCGTTGCGCTTCTCA	AGTAGTGACACAGCGGTTG
egl-30	TCAGAAAGGCGGAAGTGGAT	GGTTCTCGTTGTCACACTCG
dgk-1	GTTGGGGAAGTGGTGCAAAT	GCGAGCTTGGATTGGATGAG
goa-1	TGTTCGATGTGGGAGGTCAA	TCGTGCATTCGGTTTGTTGT
skn-1	ATCCATTCGGTAGAGGACCA	GGCGCTACTGTCGATTTCTC
sir-2.1	CGGGGAAGTGCAAGAAATAA	GAGTGGCACCATCATCAAGA
act-2	ATCGTCCTCGACTCTGGAGATG	TCACGTCCAGCCAAGTCAAG

β-mercaptoethanol. The decrease in absorbance was measured for 5 min at 340 nm, and the enzyme activity was expressed as Units/mg of protein [36].

2.9. Analysis of photoaging effect

This experiment was done according to Prasanth et al. [37] with slight modifications. As explained in the previous section, worms were treated with *S. asper* (10–30 μg/ml) before and after UV-A exposure and their lifespan was monitored. Worms exposed to UV-A alone and did not receive any treatment of extracts was used as control. The number of worms alive on the individual wells was scored and counted every 24 h and was plotted, similar to the lifespan assay.

2.10. Paralysis assay

The Aβ transgenic strain was treated with different doses of *S. asper* extract (10–30 μg/ml) and closely monitored under the microscope. The worm was considered paralyzed if it does not show any body movements when touched with a platinum loop. Imaging was done using differential interference contrast (DIC) microscopy [38].

2.11. Western blotting

The Aβ transgenic strain was treated with different doses of *S. asper* extract (10–30 μg/ml), and then the total protein was isolated using 1 mM PMSF in PBS buffer. Total protein (50 μg) was separated using 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after it was mixed with a 2 × Laemmli buffer (ratio 1:1) and heated at 95 °C for 5 min. The separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes, which were then blocked with 5 % nonfat milk for 1 h at room temperature. Membranes were incubated with primary antibodies (Aβ (1:2000) or β actin (1:2000)) overnight at 4 °C. After incubation, membranes were washed three times with 1×TBS-Tween 20 (TBST) for 5 min each, then incubated with secondary antibodies (anti-rabbit IgG, HRP-linked antibody) for 45 min at room temperature and washed three times with TBST for 5 min each. Protein bands were visualized by adding an enhanced chemiluminescence detection reagent and exposure to autoradiography film. Each band was normalized against β actin as an internal control [39].

2.12. Total RNA isolation and real-time PCR analysis

The nematodes were treated with varying concentrations of *S. asper* extracts (10–30 μg/ml), and after the treatment period total RNA was isolated using Trizol kit (Invitrogen, Carlsbad, CA, USA). From this, 1000 ng was reverse transcribed to cDNA using Accupower RT Premix (Bioneer, Korea) and oligo dT primers through following the manufacturer's protocol. Gene-specific primers were designed (Table 1) to carry out Real-time PCR using SYBR Green. The Green Star PCR Master Mix (Bioneer, Korea) was used in the Exicycler Real-Time Quantitative Thermal Block (Bioneer, Korea). The expression data were normalized to the internal control actin and then represented as upregulated or downregulated by normalizing with untreated control [31].

2.13. Statistical analysis

One-way ANOVA (SPSS 17) was used to compare each treatment's mean values in every experiment unless otherwise specified. The data were represented as the average of at least three independent experi-

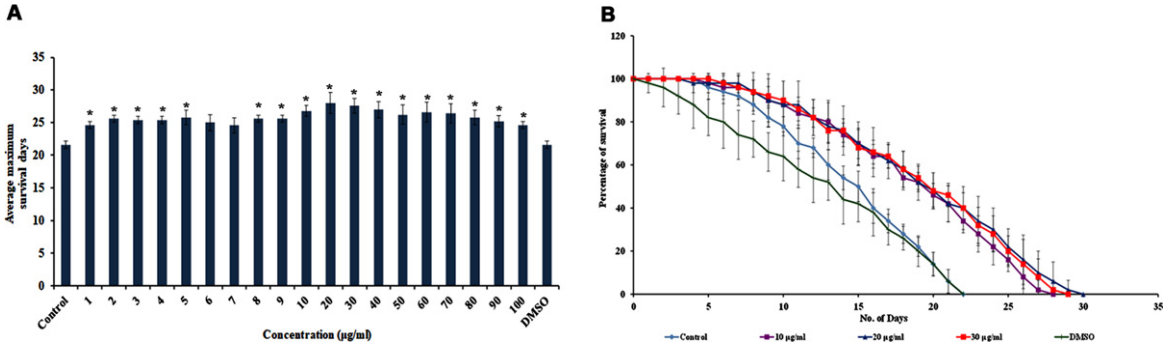

Fig. 1. *S. asper* extract could extend the maximum lifespan in *C. elegans.* One-way ANOVA (SPSS 17) was used to compare each treatment's mean values in every experiment, and the data were represented as the average of the independent experiments. (A) Wild type nematodes treated with varying concentrations of *S. asper* extract (1–100 µg/ml) could significantly ($p < 0.05$) extend the maximum lifespan of the nematodes. Control worms that did not receive any extract treatment and the vehicle control DMSO treated worms survived up to 22 days (B) Doses ranging from 10–30 µg/ml that exhibited maximum extension of lifespan were represented wherein they survived up to 28, 30 and 29 days respectively which were used for the further experiments.

ments. Significant differences between the means of parameters were determined by using Duncan's test ($P < 0.05$) comparing the groups' control vs treated [37].

3. Results

3.1. S. asper extended the lifespan of C. elegans

The lifespan extension activity of *S. asper* extract was tested for varying concentrations ranging from 1–100 µg/ml. Almost all the tested concentrations were able to extend the lifespan of wild type *C. elegans* (Fig. 1) significantly ($p < 0.05$), in which the doses ranging between 10–70 µg/ml exhibited maximum extension of lifespan which was up to 28, 30, 29, 28, 28, 28 and 28 days respectively (Fig. 1). The control worms were fed with *E. coli* OP50 alone, and the vehicle control, the worms were treated with DMSO. Both the control and vehicle control exhibited similar lifespan as they survived up to 22 days (Fig. 1). This indicates that the solvent-induced no change in mediating lifespan extension by the extract. The concentrations 10–30 µg/ml showed the maximum survival rate and were used for further experimental analysis.

3.2. S. asper improved the healthspan of C. elegans

Pharyngeal pumping was analyzed in *C. elegans* treated with 10–30 µg/ml of *S. asper* extract. It was observed that the extract did not alter the pharyngeal pumping of the nematodes when compared to the untreated control, which was consistent up to 10 days (Fig. 2A).

The level of autofluorescent protein, the "age-pigment" lipofuscin, was monitored inside the nematodes after treatment with 10–30 µg/ml of *S. asper* extract. All the tested concentrations showed a reduction in the fluorescence levels and a significant ($p < 0.05$) reduction was observed in 20 µg/ml concentration when compared to control (Fig. 2B), indicating that the extract was successful in slowing down the aging process, as it could reduce the accumulation of lipofuscin.

Further, qPCR analysis was performed on candidate genes that could mediate healthspan. Candidate genes of the Diacylglycerol (DAG) pathway were monitored, namely *egl-8* and *egl-30* that positively regulate healthspan and *dgk-1* and *goa-1,* negatively regulate healthspan. Additionally, the level of expression of an adult-specific marker, *col-19* was also observed. The expression of *egl-8* and *egl-30* were observed to be upregulated significantly ($p < 0.05$) whereas the expression of *col-19*, *dgk-1* and *goa-1* were downregulated significantly ($p < 0.05$) in the worms treated with 10–30 µg/ml of *S. asper* extract when compared to control (Fig. 2G). The results indicate that *S. asper* extract could positively improve the healthspan of the nematodes.

3.3. S. asper mediated extension of lifespan and healthspan is independent of DAF-16 pathway

Some of the key players of the DAF-16 mediated pathway were analyzed in nematodes treated with

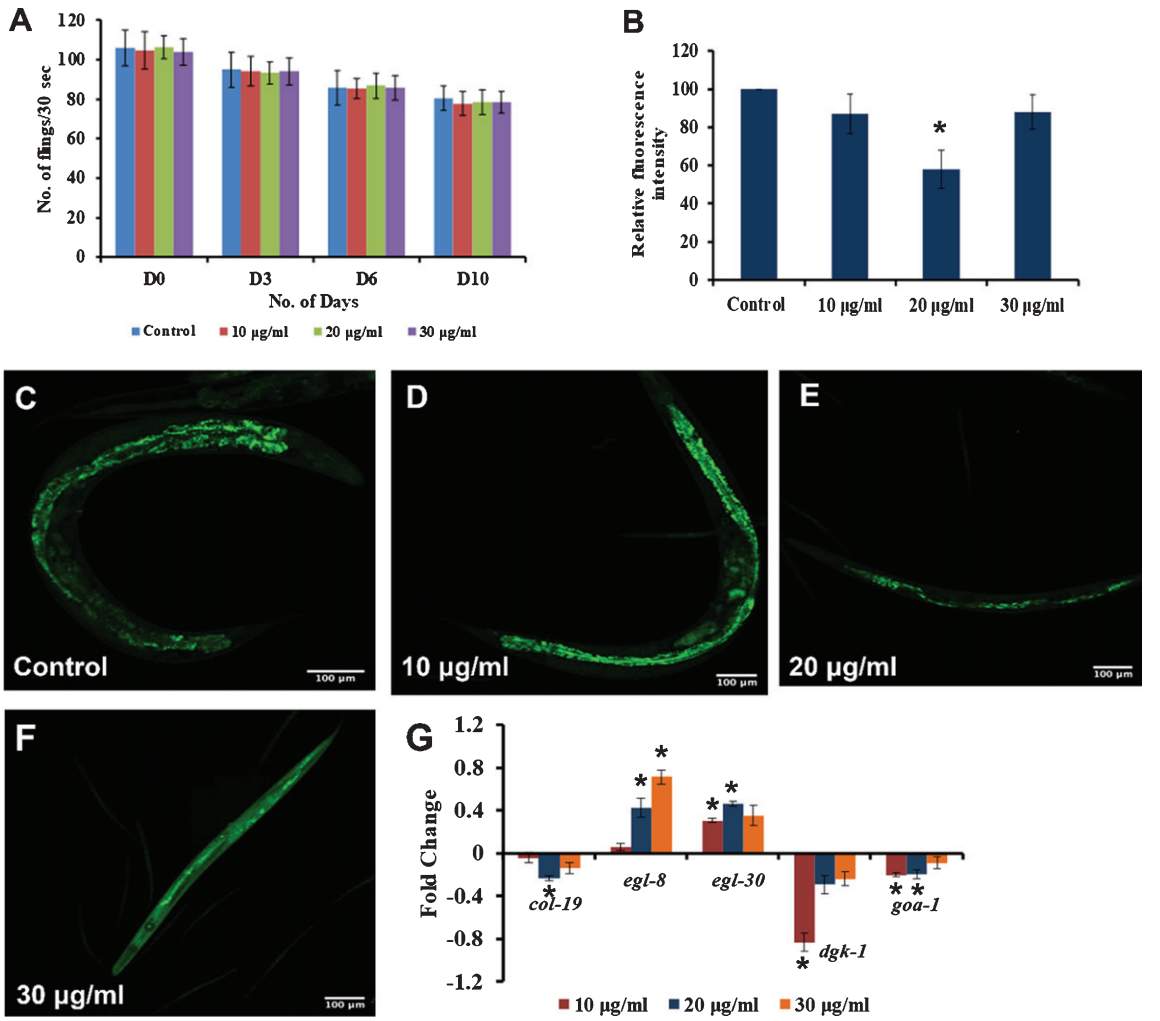

Fig. 2. *S. asper* could extend the healthspan of *C. elegans* (A) Pharyngeal pumping was observed in wild type nematodes fed with 10–30 µg/ml of *S. asper* extract. No significant changes were observed in the feeding pattern of worms treated or untreated with *S. asper* extract (B) Analysis of the relative fluorescence intensity of nematodes treated with 10–30 µg/ml of *S. asper* extract in which 20 µg/ml concentration showing significant ($p < 0.05$) reduction in fluorescence when compared to control ($n = 10$) (C) Representative image of wild type nematode with no extract treatment (control) with the level of lipofuscin accumulation (D–F) Representative images of wild type nematodes with 10–30 µg/ml of *S. asper* extract with the level of lipofuscin accumulation (G) Real-Time PCR analysis of *col-19, egl-8, egl-30, dgk-1* and *goa-1* was done in nematodes treated with 10–30 µg/ml of *S. asper* extract. The expression of *col-19, dgk-1* and *goa-1* were observed to be downregulated, whereas the expression of *egl-8* and *egl-30* was upregulated compared to untreated control normalized to the X axis.

10–30 µg/ml of *S. asper* extracts using qPCR analysis. It was observed that the expression of *daf-2*, *age-1* and *utx-1* were upregulated significantly ($p < 0.05$) in a concentration-dependent manner when compared to control, suggesting that the DAF-16 mediated pathway may not play a role in *S. asper* mediated extension of lifespan (Fig. 3A).

To further confirm this, mutants of DAF-16 and DAF-2 were treated with 10–30 µg/ml of *S. asper* extract, and the survival rate was monitored. It was observed that there was a significant ($p < 0.05$) increase in the lifespan in both DAF-16 mutants at 10 and 20 µg/ml as they survived up to 19, 21 and 20 days respectively when compared to 16 days survived by control (Fig. 3B-C) as well as in DAF-2 mutants at 20 µg/ml as they survived up to 52, 53 and 49 days respectively when compared to 48 days survived by control (Fig. 3D-E) worms. This suggests that the lifespan extension exhibited by *S. asper* extracts was not mediated by DAF-16 pathway, and there could be some other mechanism mediating the effect.

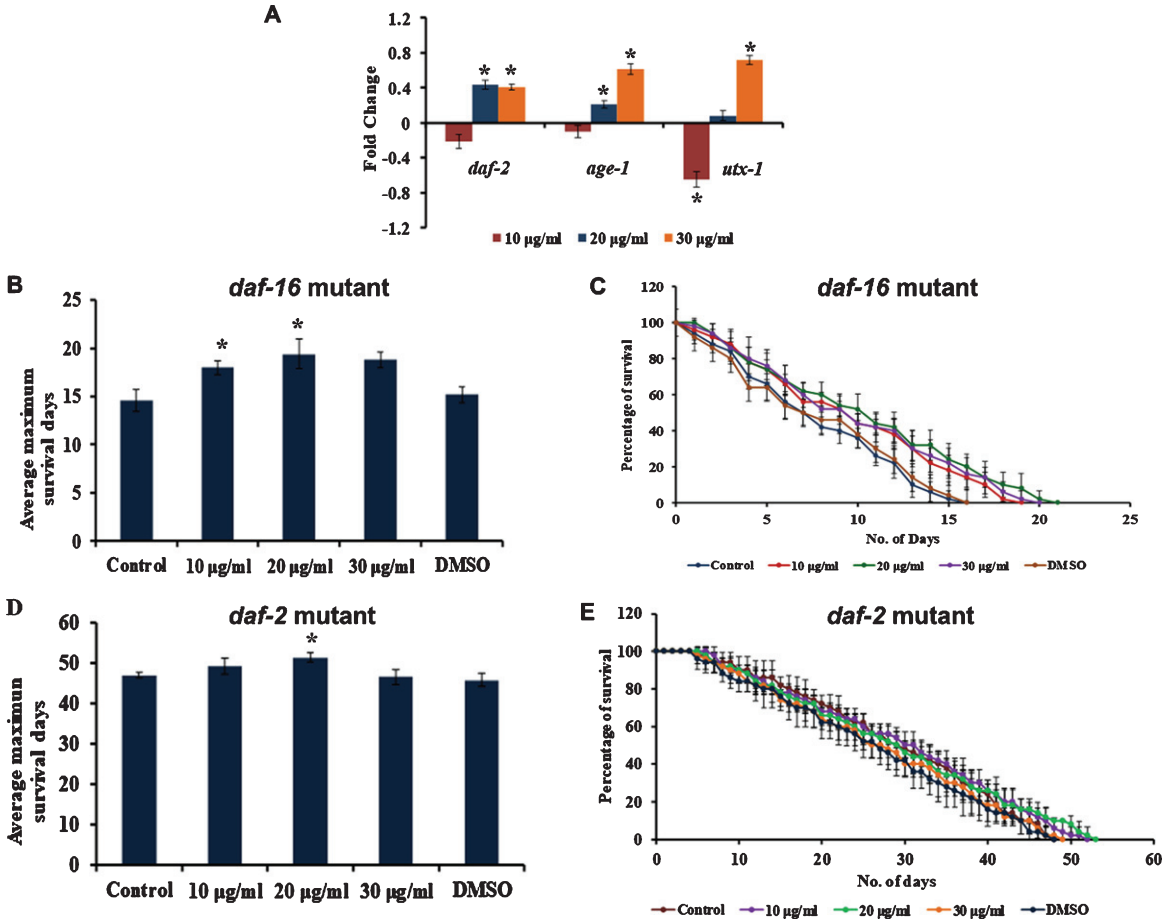

Fig. 3. *S. asper* mediated lifespan extension is independent of DAF-16 pathway (A) Real-Time PCR analysis of *daf-2, age-1* and *utx-1* was done in nematodes treated with 10–30 µg/ml of *S. asper* extract. All the genes were upregulated in a dose-dependent manner when compared to untreated control which was normalized to the X-axis (B) Maximum lifespan of *daf-16* mutants (mu86) were significantly ($p < 0.05$) extended when treated with 10–30 µg/ml of *S. asper* extract at 15 °C (C) Graph representing the average of maximum lifespan extension of *daf-16* mutants when treated with 10–30 µg/ml of *S. asper* extract (D) Maximum lifespan of *daf-2* mutants (e1370) were significantly ($p < 0.05$) extended when treated with 10–30 µg/ml of *S. asper* extract at 15 °C (E) Graph representing the average of maximum lifespan extension of *daf-2* mutants when treated with 10–30 µg/ml of *S. asper* extract.

3.4. *S. asper mediated lifespan extension, and stress resistance is mediated by MAPK pathway*

Mitogen-Activated Protein Kinase (MAPK) pathway plays a major role in stress resistance and extending the nematodes' lifespan. To analyze whether *S. asper* has a role in activating MAPK signalling, mutants of PMK-1 were treated with *S. asper* extracts. It was observed that the extract could not induce any significant change in the lifespan of the mutant strains (Fig. 4), indicating the importance of PMK-1 in mediating the effect of *S. asper* extracts in the nematodes.

3.5. *S. asper can induce antioxidant potential in C. elegans*

Since *S. asper* has been reported to possess antioxidant potential *in vitro* [19], the extract's ability to induce the same under *in vivo* conditions was analyzed through qPCR analysis of candidate genes, *skn-1* and *sir-2.1*, that are responsible for the antioxidant mechanism in *C. elegans.* The significant ($p < 0.05$) upregulation of both the genes at 10 and 20 µg/ml upon treatment with 10–30 µg/ml of *S. asper* extract treatment suggest that the extract could activate the antioxidant mechanism in the nematode (Fig. 5).

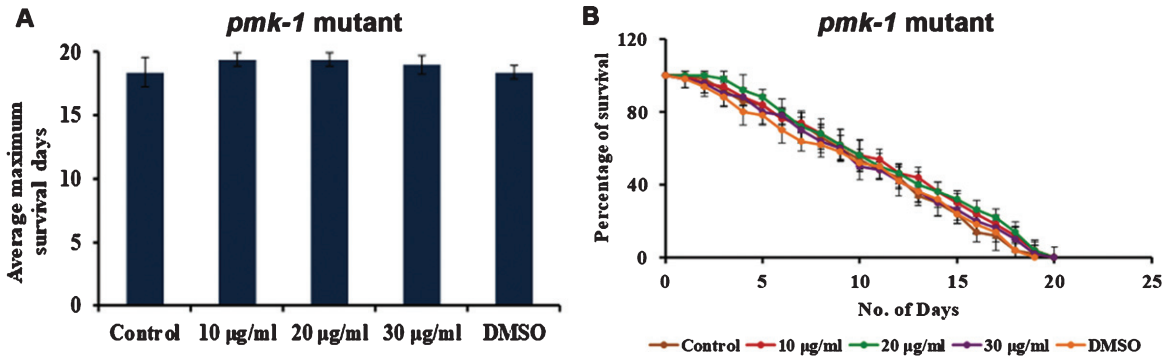

Fig. 4. *S. asper* mediated lifespan extension is dependent of MAPK pathway (A) Maximum lifespan of *pmk-1* mutants were showing no significant change when treated with 10–30 µg/ml of *S. asper* extract (B) Graph representing the average of maximum lifespan extension of *pmk-1* mutants when treated with 10–30 µg/ml of *S. asper* extract.

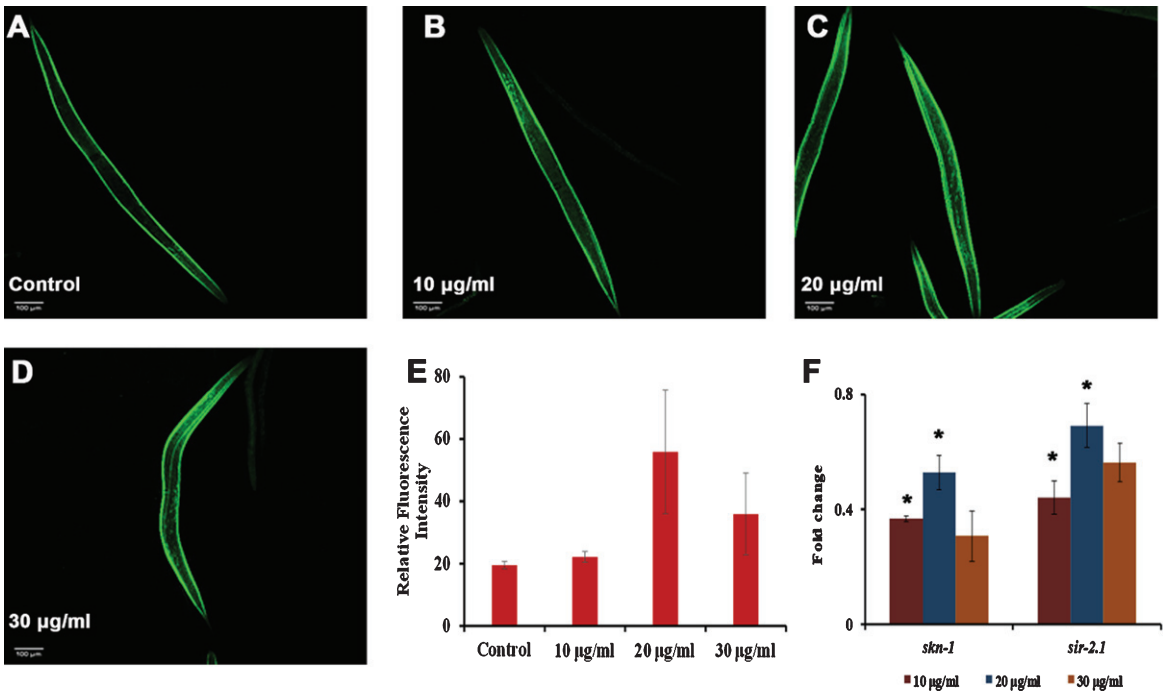

Fig. 5. S. asper extract can activate antioxidant potential in C. elegans. (A) Representative image of skn-1::GFP transgenic strain (Control) (B-D) Representative image of skn-1::GFP transgenic strain treated with 10–30 µg/ml of S. asper extract (E) Quantification of fluorescence wherein 20 µg/ml showed maximum expression (F) Real-Time PCR analysis of skn-1 and sir-2.1 was done in nematodes treated with 10–30 µg/ml of S. asper extract that showed significant ($p < 0.05$) upregulation at 10 and 20 µg/ml concentrations when compared to untreated control which was normalized to the X-axis.

Additionally, to analyze the level of *skn-1* in the nematode after *S. asper* treatment, *skn-1*::GFP transgenic strains were monitored for their fluorescence intensity. It was observed that the extract treatment improved the fluorescence intensity when compared to the control wherein 20 and 30 µg/ml of *S. asper* extract showed maximum effect (Fig. 5).

3.6. *S. asper can reduce the level of photoaging in C. elegans*

The anti-photoaging effect was monitored by analyzing the effect of *S. asper* extracts on the lifespan of UV-A exposed worms. The extract was analyzed for its protection and repair effects by treating

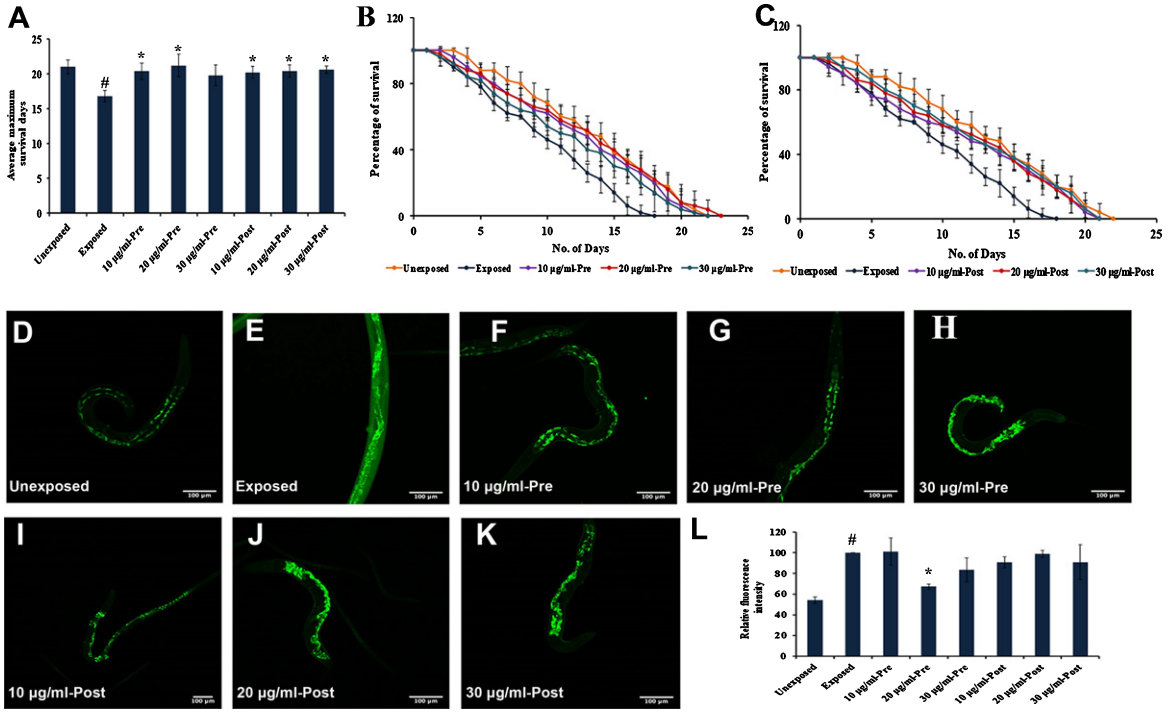

Fig. 6. *S. asper* extract could activate antioxidant and anti-photoaging potential (A) Wild type nematodes were exposed to UV-A. *S. asper* extract was treated before (pre-treatment) or after (post-treatment) UV-A exposure. Significant ($p < 0.05$) extension of lifespan was observed in both kind of treatments when compared to nematodes exposed to UV-A without any treatment (B) Graph representing the average maximum lifespan of nematodes pre-treated with UV-A exposure (C) Graph representing the average maximum lifespan of nematodes post-treated with UV-A exposure (D) Representative image of worm showing the level of ROS that was not exposed to UV-A and did not receive any treatment (negative control) (E) Representative image of worm showing the level of ROS that was exposed to UV-A and did not receive any treatment (positive control) (F–H) Representative image of worm showing the level of ROS that was exposed to UV-A and was treated with 10–30 µg/ml of *S. asper* extract before exposure (pre-treatment) (I–K) Representative image of worm showing the level of ROS that was exposed to UV-A and was treated with 10–30 µg/ml of *S. asper* extract after exposure (post-treatment) (L) Analysis of the relative fluorescence intensity of nematodes pre and post-treated with 10–30 µg/ml of *S. asper* extract ($n = 10$).

the extracts before and after the exposure to UV-A individually. There was a significant ($p < 0.05$) extension of lifespan in worms pre-treated and post-treated with the extracts when compared to the worms which were exposed to UV-A without any treatment (Fig. 6A-C). This suggests that the extract possesses anti-photoaging activity along with antioxidant potential.

As UV-A causes ROS induced photoaging, oxidative stress was induced in *C. elegans* by exposing to UV-A for 4 h [40]. The extract was analyzed for its protection and repair effects by treating the extracts to nematodes before and after UV-A exposure individually. A significant ($p < 0.05$) reduction in the level of ROS was observed, which could be evident from the reduction in fluorescence intensity in the extract pre-treated with 20 µg/ml of *S. asper* extract (Fig. 6L). The post-treated group also showed a reduction in fluorescence which is not as significant as the pre-exposed ones. The results indicate that the extract exhibits substantial antioxidant properties and could protect the nematodes from UV-A induced photoaging.

Additionally, the superoxide dismutase (SOD) level was analyzed in the whole protein of *C. elegans* exposed to UV-A pre- and post-treated with *S. asper* extract as described above. It was observed that UV-A triggered the level of SOD inside the nematode, which was reduced by the treatment of *S. asper* wherein 20 µg/ml pre-treatment exhibited significant reduction (Fig. 7).

3.7. *S. asper imparts neuroprotection in transgenic C. elegans*

As antioxidants are known to impart neuroprotection, the neuroprotective effect of *S. asper* extract

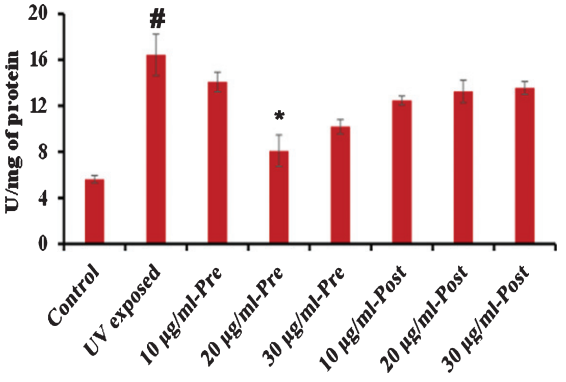

Fig. 7. *S. asper* extract can regulate the level of SOD in *C. elegans*. Wild type nematodes were exposed to UV-A. *S. asper* extract was treated before (pre-treatment) or after (post-treatment) UV-A exposure. Analysis of the relative fluorescence intensity of worm lysate (50 µg) pre- and post-treated with 10–30 µg/ml of *S. asper* extract. 20 µg/ml pre-exposed concentration showed a significant ($p < 0.05$) reduction in the level of SOD (# control vs UV exposed; *UV exposed vs extract treated).

was analyzed using a transgenic strain of *C. elegans,* CL2006 that expresses $A\beta_{1-42}$ constitutively by treating with 10–30 µg/ml of *S. asper* extract to monitor its survival. The extract was observed to significantly ($p < 0.05$) extend the lifespan of the transgenic nematodes at 20 and 30 µg/ml as they survived up to 25, 26 and 26 days when compared to control which survived up to 23 days. Additionally, the extract was able to protect the worms from being paralyzed due to the accumulation of Aβ inside the worm. Western blot analysis further suggests a significant reduction in Aβ load inside the worm after the treatment with 20 µg/ml *S. asper* extract (Fig. 8) suggesting the neuroprotective potential of the extract.

4. Discussion

Aging is an irreversible, progressive and degenerative process causing damage to tissues and organs, leading to an increased level of oxidative stress coupled with different age-associated diseases, eventually leading to death [23, 26]. This has always led researchers to strive hard to identify molecules that can target the molecular pathways to delay aging and its associated diseases, which further improves the overall health of the organism, thereby improving lifespan [41]. Since many natural sources such as plants and their derivatives pose limited to negligible side effects, they are gaining more attention to be a strong contender for anti-aging research [9, 42]. *S. asper,* a plant used in traditional medicine, has been identified to have neuroprotective effects against glutamate-induced cytotoxicity in HT22 cells by reducing ROS level via activation of Nrf2. Additionally, the extract was also able to extend the lifespan of the larval stages of *C. elegans,* indicating its anti-aging potential [19].

Extension of lifespan and healthspan is essential for any formulation to claim its anti-aging potential. *S. asper* has already been reported to be used in Thai traditional formula for longevity and antioxidant potential [43]. As in previous reports, *C. elegans* fed with 1–100 µg/ml of *S. asper* extract were observed to have extended the nematode's lifespan (Fig. 1). The extension in lifespan may be due to the phytochemicals andrographolide, carnosic acid, α-linolenic acid, and oleoyl oxazolopyridine and other phytochemicals in the extract [19, 44, 45]. Carnosic acid and α-linolenic acid have been reported to elicit anti-aging potential in *C. elegans,* which could be dependent on SKN-1 [46, 47].

Dietary restriction is a critical mechanism that can also extend lifespan in different models, including *C. elegans* by mediating signalling pathways and maintaining the cellular energy balance [48]. A pharyngeal pumping assay was performed to confirm that the worms feed on *S. asper* and the lifespan extension was not because of dietary restriction. It was observed that the worms were able to feed upon the extract in a similar way to that of its *E. coli* OP50 and there was no significant change in the pumping rate between the control and extract-treated group (Fig. 2A), indicating that the worms could consume the extract and that the extension of lifespan was not induced by dietary restriction process.

The autofluorescent age pigment, lipofuscin gets accumulated in *C. elegans* during the aging process; wherein, the accumulation will initially start in the intestinal tract and uterus area and further develop into other parts of the body [49, 50]. A reduction of the level of lipofuscin by small molecules indicates the reversal of aging [51, 52]. It is understood that during aging, an increase in lipofuscin is further involved in limiting the removal of oxidized proteins and induce the formation of ROS and leading to apoptosis [53]. Recent studies of plant extracts also correlate the reduction of lipofuscin level to anti-aging potential [31, 54, 55]. Corroborating to the previous reports, *S. asper* extract was found to exhibit anti-aging potential in *C. elegans,* as it could significantly reduce the level of lipofuscin inside the nematode (Fig. 2B-F).

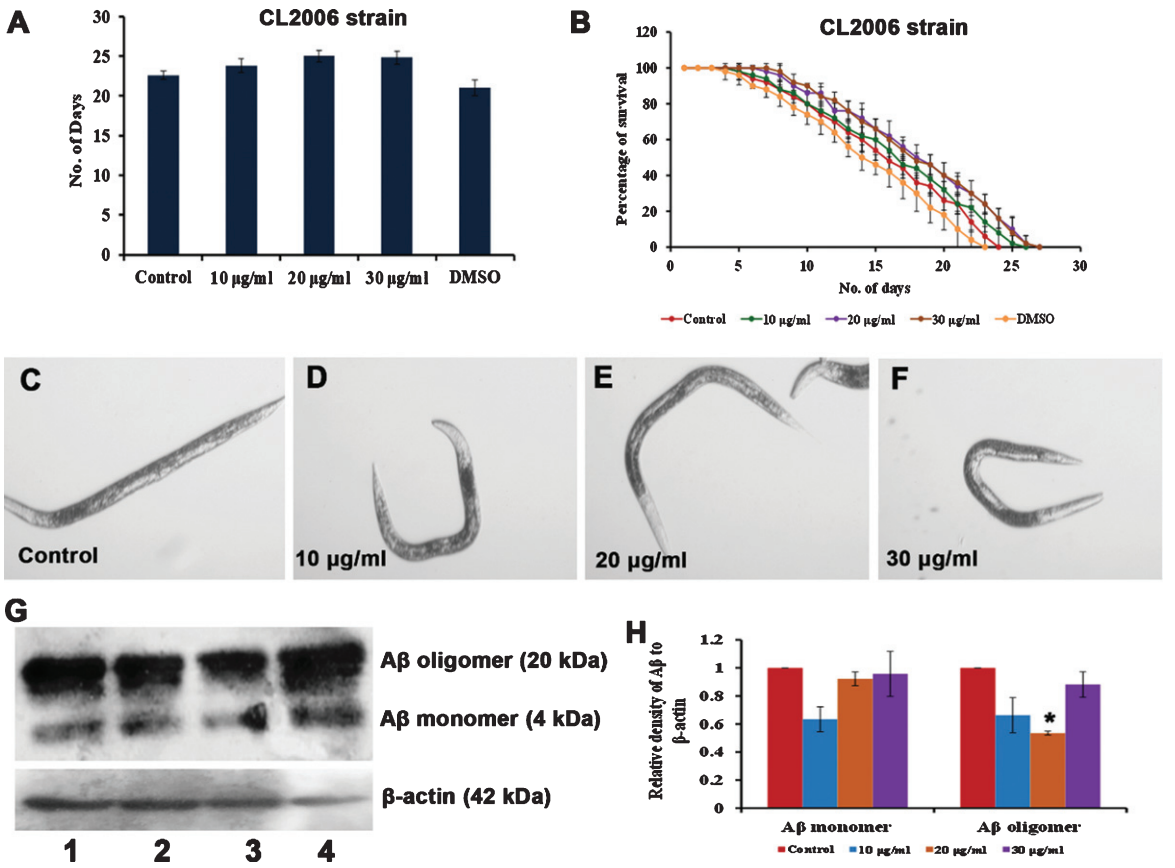

Fig. 8. *S. asper* extract can impart neuroprotection in *C. elegans* (A) Maximum lifespan of Aβ transgenic strain were significantly ($p < 0.05$) extended at 20 and 30 µg/ml when treated with 10–30 µg/ml of *S. asper* extract (B) Graph representing the average of maximum lifespan extension of Aβ transgenic strain when treated with 10–30 µg/ml of *S. asper* extract (C) Representative image of Aβ transgenic strain which is paralyzed (control) (D-F) Representative image of Aβ transgenic strain treated with 10–30 µg/ml of *S. asper* extract which is not paralyzed (Paralysed worm – straight line; non-paralysed worm – "C" or "S"- shaped) (G) *S. asper* extract significantly reduced the expression of Aβ oligomer. Lane 1 (control) Lane 2 (10 µg/ml *S. asper* extract) Lane 3 (20 µg/ml *S. asper* extract) Lane 4 (30 µg/ml *S. asper* extract) (H) Quantitative measurement of the relative density of Aβ to β-actin expression ($^*P < 0$ .05 compared between control and *S. asper* treated group).

Further, to validate the activation of healthspan by *S. asper* extract, qPCR analysis of candidate genes that could mediate the nematode's overall health was monitored. The adult-specific marker of *C. elegans, col-19* [24, 56] was observed to be downregulated significantly ($p < 0.05$) (Fig. 2G) suggesting the delay in the aging process. Candidate genes of the Diacylglycerol (DAG) pathway that mediates healthspan, including pharyngeal pumping, locomotion and egg-laying [24, 57] were monitored. The significant ($p < 0.05$) upregulation of *egl-8* and *egl-30* along with the significant ($p < 0.05$) downregulation of *dgk-1* and *goa-1* (Fig. 2G) in line with their role in the aging mechanism. Both *egl-8* and *egl-30* are essential for the activation of healthspan, whereas *dgk-1* and *goa-1* negatively regulate healthspan [58–62]. This suggests the activation of healthspan inside the nematodes treated with *S. asper*, which goes in parallel to a recent report from our group [31].

Further, the study focused on understanding the molecular mechanism by which *S. asper* mediates the anti-aging effects. The conserved insulin/IGF-1 signalling (IIS) pathway, which is generally known as DAF-16 pathway in *C. elegans* is a well-known pathway to mediate anti-aging and stress resistance in nematodes [28, 54, 55, 63]. The regulation of candidate genes of the pathway, *daf-2, age-1* and *utx-1*, which could negatively regulate anti-aging and stress resistance in *C. elegans* [24, 54, 64] were analyzed through qPCR approach. Both *daf-2* and *age-1* are negative regulators of lifespan and the histone demethylase *utx-1* acts upstream of *daf-2* and

negatively regulates lifespan [25, 64, 65]. It was observed that all three genes were upregulated significantly ($p < 0.05$) when treated with *S. asper* extract in a dose-dependent manner (Fig. 3A) indicating that the anti-aging mechanism exhibited by *S. asper* could be independent of the pathway.

The qPCR analysis results were further confirmed with studies using *daf-16* and *daf-2* mutants short-lived and long-lived, respectively [65]. The significant ($p < 0.05$) increase in lifespan of both the mutants treated with *S. asper* (Fig. 3B-E) further confirm that the anti-aging effect could be independent of DAF-16 pathway. A traditional uyghur medicine, aiweixin, was identified to extend lifespan independent of DAF-16 [66]. Another report suggests that lifespan extension by tryptophan in *C. elegans* was independent of DAF-16 pathway [67].

To identify the alternate pathway through which *S. asper* exerts its anti-aging effects, the MAPK pathway in *C. elegans* was analyzed using mutants of *pmk-1*, the downstream effector of MAPK pathway. It was recently reported that the MAPK pathway could elicit anti-aging effect in *C. elegans* exposed to UV-A [68]. Several extracts and phytochemicals have already been reported to extend longevity in nematodes by acting through MAPK pathway. Mulberry anthocyanins and sesamin were observed to extend lifespan in *C. elegans,* dependent on MAPK mediated by *pmk-1* and *skn-1* [69, 70]. In a similar fashion to these reports, the extract could not induce any significant change in the lifespan of the nematode (Fig. 4) indicating the role of MAPK in exerting the anti-aging properties for *S. asper* in *C. elegans.* Andrographolide, the antioxidant present in *S. asper* has been previously reported to activate the JNK MAPK pathway in human neuroblastoma cells against Parkinson's disease [71].

The mammalian Nrf-2 acts as a transcription factor of MAPK. On that note, qPCR analysis of the genes *skn-1* and *sir-2.1*, the mammalian orthologs of Nrf-2 and SIRT-1 respectively were analyzed. Previous reports suggest that SKN-1 acts to extend longevity and oxidative stress resistance [72–74], whereas *sir-2.1* was crucial in eliciting antioxidant potential by green tea [37] and black tea [75]. It was observed that both the genes were significantly ($p < 0.05$) upregulated (Fig. 5), suggesting the activation of the antioxidant mechanism. Additionally, the *skn-1*::GFP strains showed an increase in fluorescence when treated with *S. asper* extract, indicating the activation of *skn-1* by the extract, which can reduce the level of oxidative stress [35, 76]. It is also important to note that MAPK aids in the nuclear localization of SKN-1 during oxidative stress response [77]. Previously, *S. asper* extract has been reported to induce the expression of Nrf-2 in HT22 cells and protect cells from glutamate-induced oxidative stress [19]. Further the bioactive compounds in *S. asper* including, carnosic acid and taxifolin were also found to activate Nrf-2 [78–80]. In the present study, the activation of *skn-1* could have played a key role in preventing the ROS formation and increasing the nematodes' lifespan.

C. elegans are prone to a spike in oxidative stress when exposed to UV-A, leading to photoaging, which is dependent on MAPK and SKN-1 [40, 68]. To monitor *S. asper* extract's efficacy in protecting and treating the nematode from oxidative stress, *C. elegans* were treated with the extracts before and after exposure individually. A significant ($p < 0.05$) reduction of fluorescence in the nematodes pre-treated with the extract was observed (Fig. 6D-L) suggesting that the extract could protect the nematode from oxidative stress. Similarly, another study showed that a water-soluble form of andrographolide suppressed the harmful effects of UV exposure in mice skin and protected the accumulation of oxidative stress [81]. Taxifolin in black rice extract reduced the UV induced ROS level and collagen damage, which is mediated by JNK in HaCaT cells [82].

Parallel to this, nematodes exposed to UV-A and treated with the extract before and after exposure individually were monitored for their overall lifespan. It was observed that the nematodes treated with the extract before or after the exposure to UV-A could significantly ($p < 0.05$) extend the lifespan of the nematode when compared to the worms exposed to UV-A without any treatment (Fig. 6A-C). Interestingly, the worms pre-treated with the extract exhibited longer lifespan than the worms that received post-treatment, in line with the previous data, indicating that the protective effect of *S. asper* against oxidative stress was much better than the treatment effect.

The increase in the level of oxidative stress led to an increase in the level of SOD inside the worms. This could be attributed to the host's counteractive mechanism to reduce the level of oxidative stress [36, 83]. In the present study, the UV-A exposed worms showed an increase in the level of SOD, which was significantly reduced by the 20 μg/ml pre-treated *S. asper* extract (Fig. 7). A recent study in *C. elegans* suggests a decrease in the level of SOD could be because of oxidative stress detoxification [84].

Aging dependent oxidative stress poses one of the major threats for neurodegenerative diseases such as Alzheimer's disease. The use of antioxidant-based therapies is thus gaining prominence in treating these diseases [85]. A recent study in Neuro2a cells highlights the importance of Nrf2 in reducing the toxicity induced by Aβ [86]. Nutritional interventions using antioxidants in diet could positively affect the health of older people susceptible to age-related neurodegenerative diseases [87]. Neuroprotective efficacy of *S. asper* against glutamate-induced oxidative toxicity has already been reported *in vitro* [19]. In the present study, a transgenic strain of *C. elegans*, with neurological defects induced by Aβ were treated with *S. asper* extracts. It was observed that the extract could significantly ($p < 0.05$) extend the lifespan of the transgenic strain and reduce the rate of paralysis. The transgenic strain for the Alzheimer's disease CL2006 expresses and accumulates intracellular Aβ leading to enhanced paralysis [38, 88]. *S. asper* extract downregulated the expression of Aβ in the nematodes thereby delaying the paralysis contributing to the neuroprotective effects and increased lifespan. The lifespan extension property of the extract can be attributed to its antioxidant activity, as the antioxidants identified in *S. asper* such as andrographolide, α-linolenic acid and strebluslignanol were able to impart neuroprotection in different models [71, 89–91]. Moreover, andrographolide was also able to reduce the total Aβ load and astrogliosis and interleukin-6 levels in the brains of aged *Octodon degus* [92].

The present study can be of potential interest in considering *S. asper* as an emerging candidate for anti-aging and neuroprotection in higher model systems and humans. However, the overall pharmacokinetic activity, including the absorption and metabolism of the compounds in the plant also needs to be analyzed. Available literature suggests the activity of some of the compounds present in *S. asper* in different systems, which could help in a better understanding of the role of the compounds inside the host.

Andrographolide was quickly and almost completely absorbed into the blood in rats following the oral administration of *Andrographis paniculata* which was dose dependent [93]. Among this, the highest concentration was observed in kidney, followed by heart, lungs, brain and plasma [94]. Carnosic acid is absorbed into the bloodstream of rats after oral administration which makes it bioavailable in intestine, liver and muscle tissues of abdomen and legs and was eventually eliminated through fecus [95]. The naturally enriched goat diary fat containing alpha-linoleic acid when administered orally in rats, the maximum bioavailability was observed in liver followed by plasma and erythrocytes [96]. Taxifolin can be absorbed into the blood of rats after oral administration, however hepatic fibrosis affects the pharmacokinetics of taxifolin indicating personalized dosage must be followed for individuals with liver diseases [97]. From the available reports, it can be said that the phytochemicals of *S. asper* are rapidly absorbed and distributed throughout various tissues via circulation to exert systemic effects. Further, earlier reports on the acute (2000 mg/kg body weight) and sub-chronic toxicity (400 mg/kg body weight) studies have revealed that *S. asper* extract is devoid of any toxicities indicating its safety [98].

Overall, the results indicate that *S. asper* can exert multi-faceted effects and play a major role in extending lifespan and healthspan apart from improving stress resistance and neuroprotection in *C. elegans* from photoaging and Aβ induced toxicity. A recent study in *C. elegans* reported that carnosic acid, a major phytochemical in *S. asper*, can mediate lifespan, healthspan, stress resistance and neuroprotection via SKN-1 and SEK-1 (another player in MAPK), but independent of DAF-16 pathway [46]. Another polyphenol, α-linolenic acid, was also reported to extend lifespan and extend neuroprotection which was dependent on both NHR-49 and SKN-1 transcription factors [47, 99] suggesting the importance of SKN-1 in *S. asper* mediated health benefits.

5. Conclusions

To summarize, *S. asper* was observed to be non-toxic and could extend lifespan and healthspan in *C. elegans.* It also protected the nematodes from aging and photoaging via MAPK pathway and SKN-1. Also, the extract offered neuroprotection against Aβ induced toxicity in nematodes. Our results suggest that *S. asper* acts as a multi-factorial agent and can be considered as a promising lead in anti-aging and neuroprotective studies. Further studies in this area will shed more light on the mechanisms through which *S. asper* acts in extending health benefits to the host.

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Conflict of interest

The authors declare no conflict of interest.

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