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Cleistocalyx nervosum var. *paniala* seed extracts exhibit sigma-1 antagonist sensitive neuroprotective effects in PC12 cells and protects *C. elegans* from stress via the SKN-1/NRF-2 pathway

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Abstract.
BACKGROUND: *Cleistocalyx nervosum* var. *paniala* (*C. nervosum*) is a plant that produces berries that are popular as a health food in Thailand. In previous studies we have identified *C. nervosum* fruit extracts to have anti-aging and anti-stress properties in *C.elegans*
OBJECTIVE: Evaluate the neuroprotective properties of *C. nervosum* seed extracts and investigate the mechanisms behind their neuroprotective properties.
METHODS: PC12 cells were differentiated using NGF and treated with amyloid-β $(Aβ)_{(25–35)}$ creating a model of neurodegeneration, in which *C. nervosum* seed extracts were tested for neuroprotective properties. Cell viability was measured after 24 hours of using the MTT assay. We also measured the lifespan and "health span" of various *C.elegans mutants* and the wild type.
RESULTS: *C. nervosum* seed extract protects PC12 cells from $Aβ_{(25–35)}$ toxicity, as well as potentiating NGF induced neurite outgrowth in the same cell line potentially under the control of the sigma-1 receptor. *C. nervosum* seed extracts have anti-aging properties in *C. elegans,* causing a significant increase in lifespan of Aβ expressing *C. elegans* and wild type worms. This appears to be regulated by SKN-1/NRF-2, possibly via the sigma-1 receptor. The DAF-16 pathway is also involved in the lifespan extension of *C. elegans*.
CONCLUSIONS: *C. nervousum* has been often used as a functional food. We have shown its neuroprotective properties which are sensitive to sigma-1 receptor antagonism in PC12 cells, and involve the SKN-1/NRF-2 and DAF-16 pathway in *C.elegans.* Further research into the active compounds may lead to future drugs for preventing or treating neurodegenerative disease.

Keywords: Natural products, *C. elegans*, ageing, Alzheimer's disease, sigma-1 receptor, neurodegenerative disease

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1. Introduction

The world's population has grown significantly over the past century. Furthermore, better nutrition and better access to health care has resulted in an increase in the average age of the population. Aging is a major risk factor for many neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's disease (PD) [1]. Neurodegenerative diseases can cause a reduction in life quality not only for the patient, but also for the family and friends who are left to care for the individual. It is estimated that there are 44 million people currently living with AD worldwide, and with no cure it is expected that this figure could rise to 135 million by 2050 [2]. The cost of caring for these patients is equivalent to 1 % of the worlds gross domestic product (GDP) which can put a strain of healthcare recourses [3].

Neurodegenerative diseases like dementia and AD are characterized by the loss of neurons in the brain as well as brain atrophy and the extracellular deposition of Aβ that results in the formation plaques. There is also hyperphosphorylation of tau protein leading to neurofibril tangles [4]. All of which results in loss of efficiency in energy production within the neurons, leading to oxidative stress and a disruption in calcium ion homeostasis, which in turn lead to cell death. There are many risk factors involved in the development of AD, such as age, diet [5] and the average amount of sleep an individual obtains per night [6]. While it is not possible to control the passage of time, and not easy to sleep more, as people age, they tend to sleep less [7, 8], it is possible for people to take control of their diets. The 'Mediterranean diet' and other similar diets have long been touted for its healthy ageing benefits, with its low fat and high antioxidant content. There have been a small number of studies which suggest such a diet (low fat, high fruits, berries and vegetables) may reduce the risk of AD and slow the decline of cognitive performance as people age [9–11].

The sigma-1 receptor is a ligand operated chaperone protein, which is a target for neurodegenerative disease as well as cancer [12–15]. It has been shown that in studies using positron emission tomography (PET) scanning that there is a decrease in sigma-1 receptors [16], whereas in normal aging there is no sigma-1 loss, in fact, studies in primates and rodents indicate an increase in sigma-1 receptor expression [17, 18]. The sigma-1 receptor is involved in preventing mitochondrial and endoplasmic reticulum (ER) stress [15, 19–22] as well as having a role in potential role in autophagy [23] and neurite growth [15, 24–27]. These properties as well as others such as the sigma-1 receptors role in tau phosphorylation and compartmentalization [28], make it an interesting target for the treatment of AD.

Cleistocalyx nervosum var. *paniala* (*C. nervosum*) is a berry bearing plant indigenous to northern Thailand. The berries are used in Thai traditional medicine and often used as a 'functional food' in health drinks due to its appealing sweet and sour flavor [29]. *C. nervosum* has been implicated as beneficial in a wide number of disease states, including caner, immune disease and neurodegenerative diseases [30]. The berries have a distinctive dark red color, produced by anthocyanins as well as other antioxidants and phenolic compounds [31]. There have been a number of studies investigating neuroprotective properties of C. *nervosum*, including its ability to protect against oxidative stress, calcium ion dysregulation, glutamate toxicity and ER stress [32–35]. It has been suggested that targeting multiple pathways either by using multiple drugs or a single less specific drug could be a strategy for the treatment of many neurodegenerative diseases, particularly AD and PD that have multiple causes and many pathways involved [13]. Naturally occurring phytochemicals derived from plant extracts could well provide the synergism required to prevent or treat neurodegenerative disease [36].

Caenorhabditis elegans are a widely used model for studying ageing, stress resistance and neurological disorders [37–39]. Their short life cycle and lifespan along with the ability to generate single gene mutants and the ease of handling make them an ideal model organism for studying aging and age-related diseases [40]. It has been observed that many plants or plant products including green tea [41], *Streblus asper* [42], *Bacopa monnieri* [43], *Paullinia cupanna* [44], and mulberry [45] can extend the lifespan and health-span and protect against stress in *C. elegans* models of disease. Previously, we have shown that the fruit pulp of *C. nervosum* is able to extend the 'health-span' and lifespan of wild type and an Aβ transgenic strain of *C. elegans* [29].

The aim of this study was to investigate the neuroprotective effects of *C. nervosum* seed extracts *in vitro* and *in vivo and* identify the pathways involved. We used cell culture to measure sigma-1 receptor sensative neurite outgrowth and Aβ toxicity, and *C. elegans* (wild type and mutants) to investigate the mechanisms involved in neuroprotection and lifespan increasing activity.

2. Materials and methods

2.1. Chemicals, reagents and equipment

Chemicals were purchased from Sigma-Aldrich. Tissue culture media materials were purchased from GibThai. *C. elegans* culture materials were purchased from HiMedia Laboratories (Mumbai, India) and the bacterial food source *E. coli* OP50 was purchased from *Caenorhabditis* Genetics Center, (University of Minnesota, USA). For ultra-violate (UV)-A exposure of *C. elegans* a UV transilluminator lamp, was used SANKYO DENKI (F20T10BL).

2.2. Plant collection and extraction

C. nervosum was collected from the Plant Genetic Conservation Project initiated by Her Royal Highness Princess Maha Chakri Sirindhorn in Lampang (Thailand) and the seeds were collected, ground up and dried. The powdered seeds were extracted in ethanol using Soxhlet for 2 days. The resulting extract was concentrated at 50 °C using a rotary evaporator until all the ethanol was removed leaving a dry extract. The dried extract was then dissolved in dimethyl sulfoxide (DMSO) at 10 mg/ml and stored at –20 °C until further use. From here onwards *C. nervosum* extract is referred to as LMKS.

2.3. Cell culture

PC12 cells were purchased from Japanese Collection of Research Bioresources (Tokyo, Japan) and maintained in Roswell park memorial institute (RPMI) -1600 medium supplemented with 5 % fetal bovine serum (FBS), 10 % horse serum (HS), penicillin (50 I.U./ml) and streptomycin (50 μg/ml). The cells were incubated at 37 °C in a humidified 5 % CO_2 atmosphere, with the media being replaced every 2–3 days and the cells passed approximately every 7 days. To remove large clumps of cells the cells were centrifuged at 500 *g* resuspended in fresh growth media and passed through a 24-gauge needle approximately 20 times; this was carried out prior to counting and plating cells.

Neuro2A expressing the sweedish mutant of the amyloid precursor protein ($APP^{(Swe)}$), Neuro2A $APP^{(Swe)}$ cells [46], were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10 % FBS and incubated at 37 °C in a humidified 5 % CO_2 atmosphere, with the medium being replaced every 2–3 days and the cells passed approximately every 7 days.

2.4. Potentiation of neurite outgrowth in PC12 cells

For the differentiation of PC12 cells, the cells were plated on poly-d-lysine (PDL) plates (3,000 cells/cm^2) and allowed to adhere overnight. The following day the medium was replaced with RPMI-1600 supplemented with 2 % HS and a submaximal concentration of nerve growth factor (NGF) (2.5–10 ng/ml). The cells were treated with LMKS (0.01–1 μg/ml) and incubated for 5–7 days with the medium and LMKS being replaced every 2–3 days. The cells were imaged using a Zeiss Axio Observer A1 Inverted Fluorescence Phase Contrast Microscope, and Image J was used to count the number of cells with neurites (defined as cellular extensions double the width of the cell) and the lengths of the neurites were measured using Neuron J (an Image J plugin) [47].

2.5. Protection against beta-amyloid toxicity in differentiated PC12 cells

PC12 cells were left to adhere to precoated PDL plates (30,000 cells/cm^2) overnight in RPMI-1600 supplemented with 10 % HS and 5 % FBS. Subsequently the medium was replaced with RPMI-1600 supplemented with 2 % HS and NGF (50 ng/ml). Cells were then incubated for 5–7 days to allow differentiation in a humidified 5 % CO_2 atmosphere at 37 °C, with the medium being replaced every 2–3 days. Once the PC12 cells were differentiated (5–7 days after the addition of NGF), the cells were pretreated with LMKS (1–0.1 μg/ml) for 24 hours prior to the addition of 10 μM $A\beta_{(25–35)}$. Treated and control cells were incubated for a further 18 hours with 10 μM $A\beta_{(25–35)}$, before the cell survival was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [15, 48]. $A\beta_{(25–35)}$ was selected over the $A\beta_{(1–40)}$ and $A\beta_{(1–42)}$ fragments as $A\beta_{(25–35)}$ shows a much faster fibril formation rate than the longer amyloid peptides and retains the toxicity of the full length $A\beta_{(1–40/42)}$ peptides [49].

2.6. Dopamine toxicity in neuro2A $APP^{(Swe)}$ cells

Dopamine toxicity in Neuro2A $APP^{(swe)}$ was carried out as described previously [15, 46]. Briefly,

Neuro2A APP$^{(swe)}$ cells were plated at approximately in 2.5×10^4 cells/cm^2, and allowed to adhere overnight in DMEM containing 10 % FBS at 37 °C (humidified 5 % CO_2 atmosphere). The following day the cells were treated with varying concentrations of LMKS and 150 μM dopamine. The cells were then incubated for further 18 hours and cell viability was measured using the MTT assay as described previously [15, 48].

2.7. *C. elegans strains used and culture conditions*

The Wild type strain N2 (Bristol), *daf-16* mutant (CF1038) and Aβ transgenic strain (CL2006) were obtained via the *Caenorhabditis* Genetics Center, (University of Minnesota, USA), and maintained in Nematode growth medium (NGM) at 15 °C unless otherwise specified. Experiments were carried out using age synchronized young adult worms.

2.8. *Lifespan assay*

The *C. elegans* life span assay was carried out as described previously [29, 43]. Briefly a known number of worms (usually 10 age synchronized worms per treatment) were place in a 24 well plate containing M9 buffer along with *E. coli* OP50 and 5-Fluoro-2′-deoxyuridine (FUDR) to prevent progeny worms. The worms were treated with a range of LMKS concentrations (between 1–100 μg/ml) and the total number of live worms were counted every 24 hours. Dead worms were identified as worms which did not respond to a gentle touch from the platinum loop. The experiment was carried out in the same way for the wild type and mutant worms. Each experiment was carried out in 5 independent trials, and data is presented as mean ± Standard error (SEM).

2.9. *Pharyngeal pumping assay*

Pharyngeal pumping was assessed as described previously [37]. A known number of worms (usually 10 per treatment) were transferred to nematode growth medium (NGM) plates treated with varying concentrations of LMKS. Pharyngeal pumping was observed using a stereo microscope (Motic SMZ-171) for 30 seconds once every 24 hours. NGM plates treated with *E. coli* OP50 was used as the control.

2.10. *Florescence imaging*

Reactive oxygen species (ROS) staining with H_2 DCFDA, Lipofuscin (autofluorescence) and Nuclear factor erythroid 2-related factor 2 (NRF_2) green fluorescent protein (GFP) imaging were carried out as described previously [29, 43]. Worms were treated with varying concentrations of LMKS or OP50 as the control. For imaging the worms were washed thoroughly in M9 buffer and placed on a glass slide along with a drop of sodium azide to immobilize the worms. Images were taken with ZEISS LSM 700 confocal microscope using 10× magnification at the objective lens.

2.11. *Total RNA isolation and real-time (RT) Polymerase chain reaction (PCR) analysis*

RNA was extracted from the worms using the TRIZOL method as described previously [29]. RNA was converted to cDNA using Accupower RT Premix (Bioneer, Korea) with oligo dT primers following the manufacturer's instructions. SYBR Green, Green Star PCR Master Mix (Bioneer, Korea) was used for the real time PCR with the specific primes detailed in Table 1. The expression data was normalized to β-actin and then represented as up or downregulated by normalizing to the untreated control.

2.12. *Statistical analysis*

Unless otherwise stated data is presented as the mean ± SEM from three independent experiments. Statistical analysis was carried out using GraphPad Prism version 8.0 for Mac. Analysis of variance (ANOVA) was used to compare the means of the treatments to the controls, Dunnett's *post hoc* test was used unless otherwise stated in the results. The P values obtained are reported in the results section. P values less than 0.05 were considered significant.

2.13. *Data availability*

Data can be made available to any who require via reasonable request to the corresponding author.

3. Results

3.1. *In vitro Aβ toxicity*

Here we used PC12 cells differentiated with 50 ng/ml NGF over 5–7 days (Fig. 1A) as the model for

Table 1
Primers used in PCR analysis of gene expression

Gene name	Forward primer	Reverse primer
act-2	ATCGTCCTCGACTCTGGAGATG	TCACGTCCAGCCAAGTCAAG
age-1	ATAGAGCTCCACGGCACTTT	ATAGAGCTCCACGGCACTTT
col-19	CACACAAATGCTCCACCAAC	CTGGATTTCCCTTCTGTCCA
daf-16	TGGTGGAATTCAATCGTGAA	ATGAATATGCTGCCCTCCAG
daf-2	TCGAGCTCTTCCTACGGTGT	CATCTTGTCCACCACGTGTC
dgk-1	GTTGGGGAAGTGGTGCAAAT	GCGAGCTTGGATTGGATGAG
egl-30	TCAGAAAGGCGGAAGTGGAT	GGTTCTCGTTGTCACACTCG
egl-8	CGTATCGTTGCGCTTCTCA	AGTAGTGACACAGCGGTTG
goa-1	TGTTCGATGTGGGAGGTCAA	TCGTGCATTCGGTTTGTTGT
sir-2.1	CGGGGAAGTGCAAGAAATAA	GAGTGGCACCATCATCAAGA
skn-1	ATCCATTCGGTAGAGGACCA	GGCGCTACTGTCGATTTCTC
utx-1	GCAGAACACCAGCTCATCAG	ATCAACGCCATTCTTCTCGC

neuron damage by $A\beta_{(25–35)}$ (Fig. 1B). The treatment with 10 μM $A\beta_{(25–35)}$ caused damage to the cells (shown by the white arrows indicating the shrunken cells) and damage to the neurites (blue arrows indicated broken and tangled neurites). Pretreatment with 0.01–10 μg/ml LMKS appeared to have a protective effect on the cells (Fig. 1C-F) preventing cell shrinkage and damage to the neurites.

We also endeavored to quantify the effect of Aβ on the viability of NGF differentiated PC12 cells using the MTT assay (Fig. 2A). We found that Aβ reduced the metabolism of MTT by almost 60 % compared to the control (statistically significant ANOVA followed by Dunnett's multiple comparison *post hoc* test $P < 0.0001$). Furthermore, we found that LMKS pretreatment was able to reduce they cytotoxic effect of Aβ on NGF differentiated PC12 cells with statistical significance observed at 0.1 μg/ml (ANOVA followed by Dunnett's multiple comparison *post hoc* test $P = 0.041$)

3.2. Potentiation of neurite outgrowth in PC12 cells

For the potentiation of neurite outgrowth, the PC12 cells were exposed to a sub maximal dose of NGF (10 ng/ml). After seven days of treatment the average neurite length of the control cells was measured at 68.3 ± 23.0 μm. Whereas the treatment with LMKS (1 μg/ml) along with 10 ng/ml NGF resulted in an average neurite length of 181.3 ± 37.5, which was statistically significant (ANOVA, followed by Dunnett's *post hoc* multiple comparison test $P = 0.043$). Potentiation of neurite outgrowth has been shown to involve the sigma-1 receptor [25–27, 50, 51]. When we introduced the sigma-1 receptor antagonist BD1047, the average neurite length was of 24.0 ± 24.1, confirming the mechanistic role of the sigma-1 receptor (Fig. 2B).

3.3. Dopamine toxicity in neuro2A APP cells

Treatment of Neuro2A cells with 150 μM dopamine causes ROS induced cell death, resulting in only 22 ± 12 % cell viability measured with the MTT assay. Treatment with LMKS (0.03 –1 μg/ml) had no significant effect on the cell viability of the dopamine treated cells, with the highest being 0.03 μg/ml which had a cell viability of 28 ± 18 % (Fig. 3).

3.4. LMKS effect on C. elegans lifespan: Wild type and mutants

LMKS was found to be non-toxic (Fig. 4A) at all the concentrations tested except for 100 μg/ml, which reduced the median survival time (Fig. 4B) from 17.5 ± 1.0 days (control) to 13.5 ± 0.5 days (LMKS 100 μg/ml), and this was statistically significant (ANOVA followed by Dunnett's *post hoc* multiple comparison test $P = 0.002$). LMKS also affected the maximal life span with 10 and 20 μg/ml extending the maximal life span (Fig. 4C) from 25.3 ± 0.3 days to 30 ± 0.4 and 29.0 ± 0.9 days respectively. These changes in maximal lifespan were statistically significant (ANOVA followed by Dunnett's *post hoc* multiple comparison test $P < 0.001$). The maximal lifespan was also cut short by 100 μg/ml reducing the life span to 23.0 ± 0.4 days, and this was statistically significant (ANOVA followed by Dunnett's *post hoc* multiple comparisons test $P < 0.05$).

To confirm that the lifespan extension seen is not a result of calorie restriction in the worms, we measured the pharyngeal pumping of the worms treated with

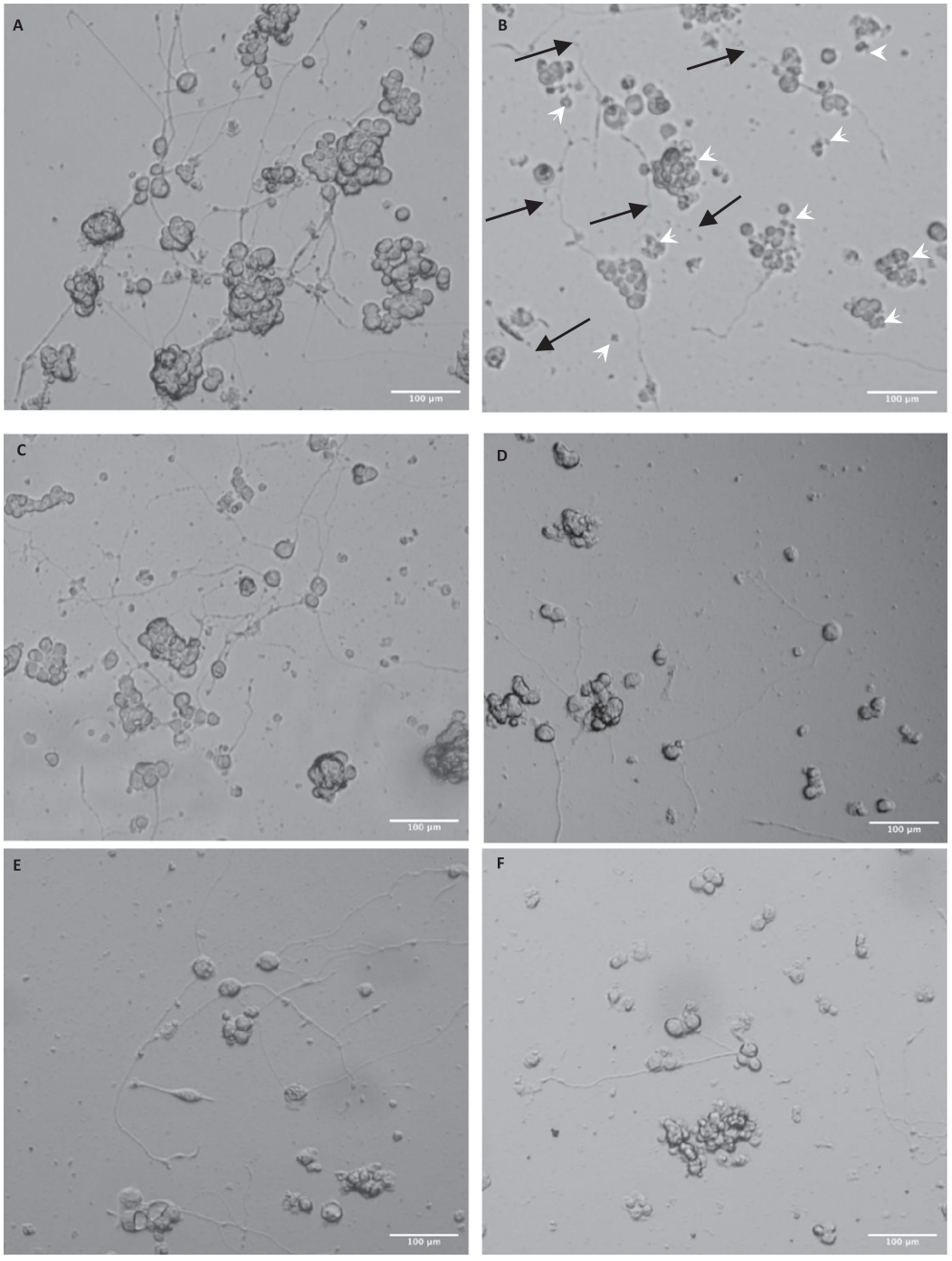

Fig. 1. Micrographs of differentiated PC12 cells exposed to $A\beta_{(25–35)}$. A) Control PC12 cells (differentiated in 50ng/ml NGF). B) Differentiated PC12 cells treated with 10 µM $A\beta_{(25–35)}$. The image shows damaged neurites (black arrows) and shrunken cells (white arrow heads). Differentiated PC12 cells treated with 10 µM $A\beta_{(25–35)}$ plus (C) LMKS (0.01 µg/ml) (D) LMKS (0.1 µg/ml) (E) LMKS (1 µg/ml) (F) LMKS (10µg/ml).

LMKS (Fig. 4D). No effects on pharyngeal pumping were observed when treated with LMKS at 10 or 20 µg/ml.

Transgenic *C. elegans* strains expressing Aβ have a reduced lifespan (Fig. 4E); however, LMKS treatment of the Aβ mutant (10–40 µg/ml) increased the

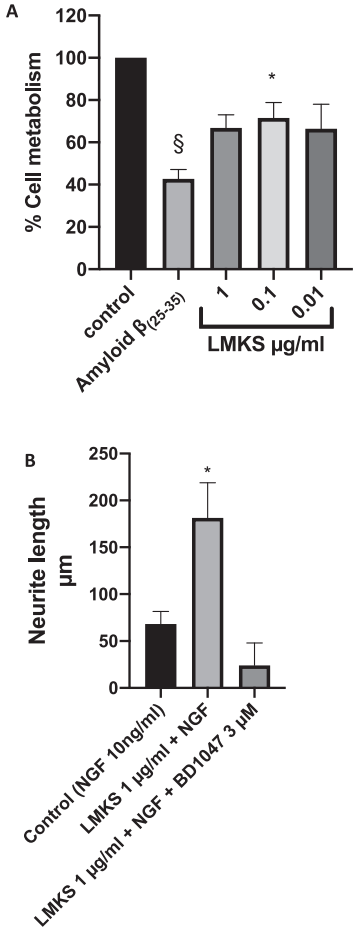

Fig. 2. A) MTT Assay of differentiated PC12 cells treated with LMKS and/or 10 μM $A\beta_{(25-35)}$. §Significant difference in MTT metabolism between control and $A\beta_{(25-35)}$ treated PC12 cells (ANOVA followed by Dunnett's *post hoc* multiple comparison test $p < 0.0001$). *Significant difference in MTT metabolism between $A\beta_{(25-35)}$ treated PC12 cells and LMKS 0.1 μg/ml $A\beta_{(25-35)}$ treated PC12 cells (ANOVA followed by Dunnett's *post hoc* multiple comparison test $p = 0.04$). B) The effect of LMKS on neurite outgrowth in PC12 cells. *Significant increase in neurite growth compared to the control ANOVA followed by Dunnett's *post hoc* multiple comparison test $p = 0.04$).

median lifespan to 20.3 ± 1.2 days and 19.2 ± 1.3 days (10–20 μg/ml), respectively, from the untreated control (13.2 ± 0.7 days) (Fig. 4F) and increased the maximal lifespan to 28.3 ± 0.3 days (10 μg/ml), compared to 13.2 ± 0.7 days of lifespan in untreated control (Fig. 4G).

The sole *C. elegans* ortholog of the Forkhead box protein O1 (FOXO) family of transcription factors is *daf-16*. It is responsible for activating genes involved in longevity and survival of oxidative stress [52]. We tested LMKS in the *daf-16* mutant strain of *C. elegans*

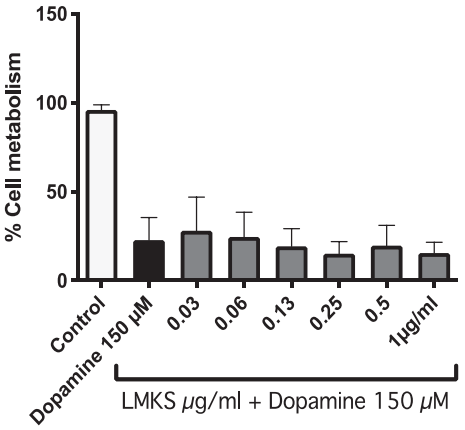

Fig. 3. MTT assay of N2A APP^{swe} cells treated with LMKS and/or dopamine (150 μM).

to monitor if it could extend the lifespan in *C. elegans* independently of *daf-16* (Fig. 4H). The *daf-16* mutant had a median and maximum lifespan of 10.3 ± 0.4 and 14.3 ± 0.3 days, respectively. LMKS at 10 and 20 μg/ml increased the median lifespan (Fig. 4I) to 14.8 ± 0.6 and 14.8 ± 0.4 days, respectively. LMKS 10–40 μg/ml also increased the maximum lifespan (Fig. 4J), with 10 μg/ml being the most effective (21.7 ± 0.3 days).

3.5. *LMKS effect on lipofuscin, ROS and NRF2 expression*

We investigated whether LMKS affected the deposition of lipofuscin in wild type *C. elegans* (Fig. 5A-E). LMKS at 10 and 40 μg/ml was able to reduce the deposition of lipofuscin (Fig. 5F).

ROS damage to cells is a key component of aging and cellular damage in neurodegenerative disease. We initiated ROS expression in *C. elegans* using exposure to UV-A (Fig. 6A-B). LMKS treatment at the same time as the UV-A exposure and post UV-A exposure was able to reduce ROS expression within the worms (Fig. 6C-F). These protective and repair effects of LMKS were statistically significant (Fig. 6G) (ANOVA followed by Dunnett's *post hoc* multiple comparison test $P < 0.001$) indicating the antioxidant potential of LMKS.

NRF2 is a mammalian transcription factor and ortholog of the *C. elegans* transcription factor SKN1. NRF2/SKN1 regulate the expression of antioxidant proteins in response to injury and inflammation [53, 54]. We used a transgenic strain of *C. elegans* (*skn-1*-GFP) to investigate whether LMKS treatment

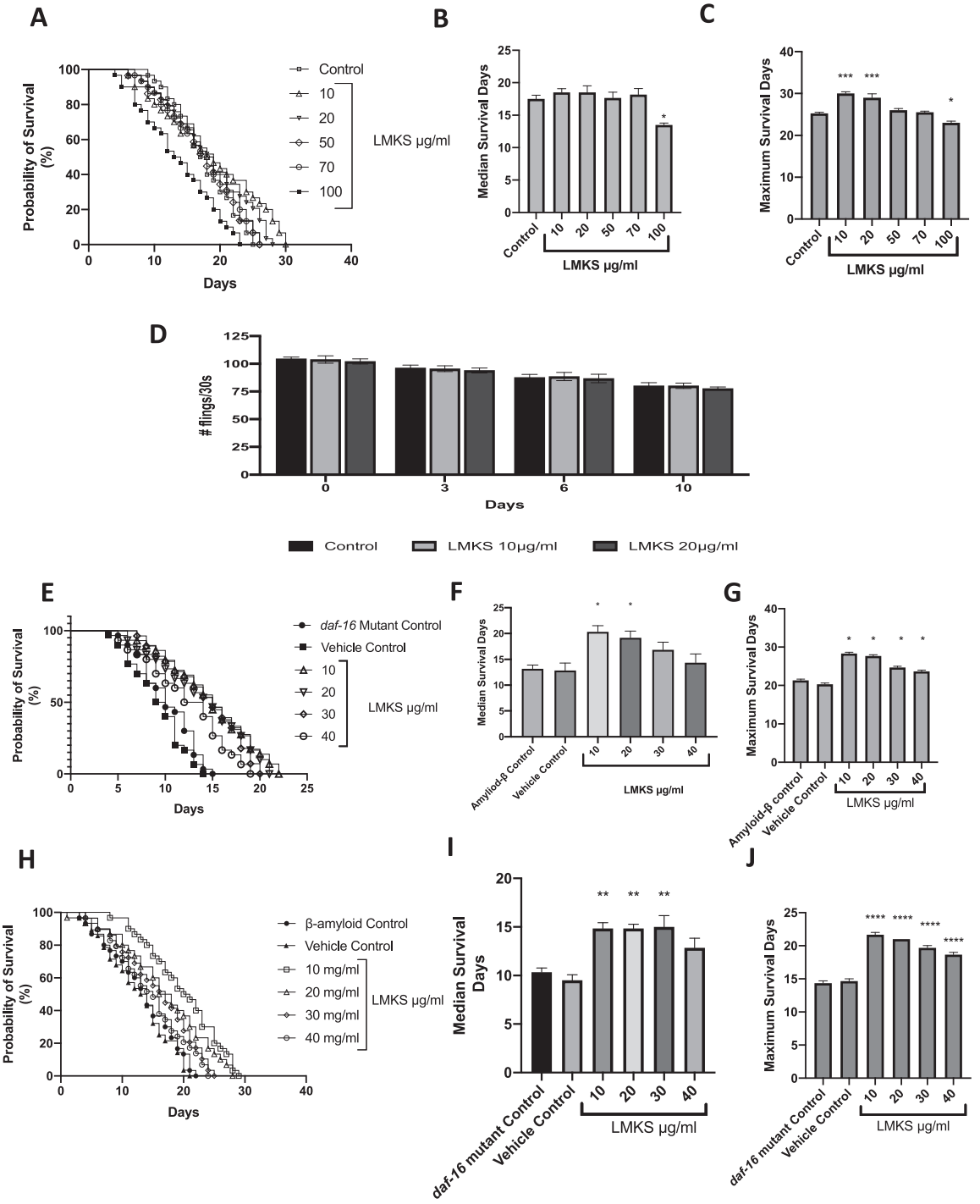

Fig. 4. The effect of LMKS on the lifespan of wild type *C.elegans*. A) Survival curve for increasing concentrations of LMKS compared to the control. B) The median survival in days of the worms treated with LMKS (mean ± SEM; $n = 4$). C) The maximal life span of worms treated with LMKS compared to the control (mean ± SEM; $n = 4$). D) The effect of LMKS on pharyngeal pumping. E) Survival curve for increasing concentrations of LMKS compared to the Aβ controls. F) The median life span of Aβ expressing worms treated with LMKS (10–40 µg/ml) (mean ± SEM; $n = 4$). G) The maximum lifespan of wild type and Aβ expressing worms treated with LMKS (10–40 µg/ml) (mean ± SEM; $n = 4$). H) Survival curve for increasing concentrations of LMKS compared to *daf-16* mutant controls. I) The median life span of *daf-16* mutant worms treated with LMKS (10–40 µg/ml) (mean ± SEM; $n = 4$). J) The maximum lifespan of *daf-16* mutant worms treated with LMKS (10–40 µg/ml) (mean ± SEM; $n = 4$). *Statistically significant change from control measured by ANOVA followed by Dunnett's *post hoc* multiple comparison test. $P < 0.05$. **$P < 0.01$. ***$P < 0.001$.****$P < 0.0001$.

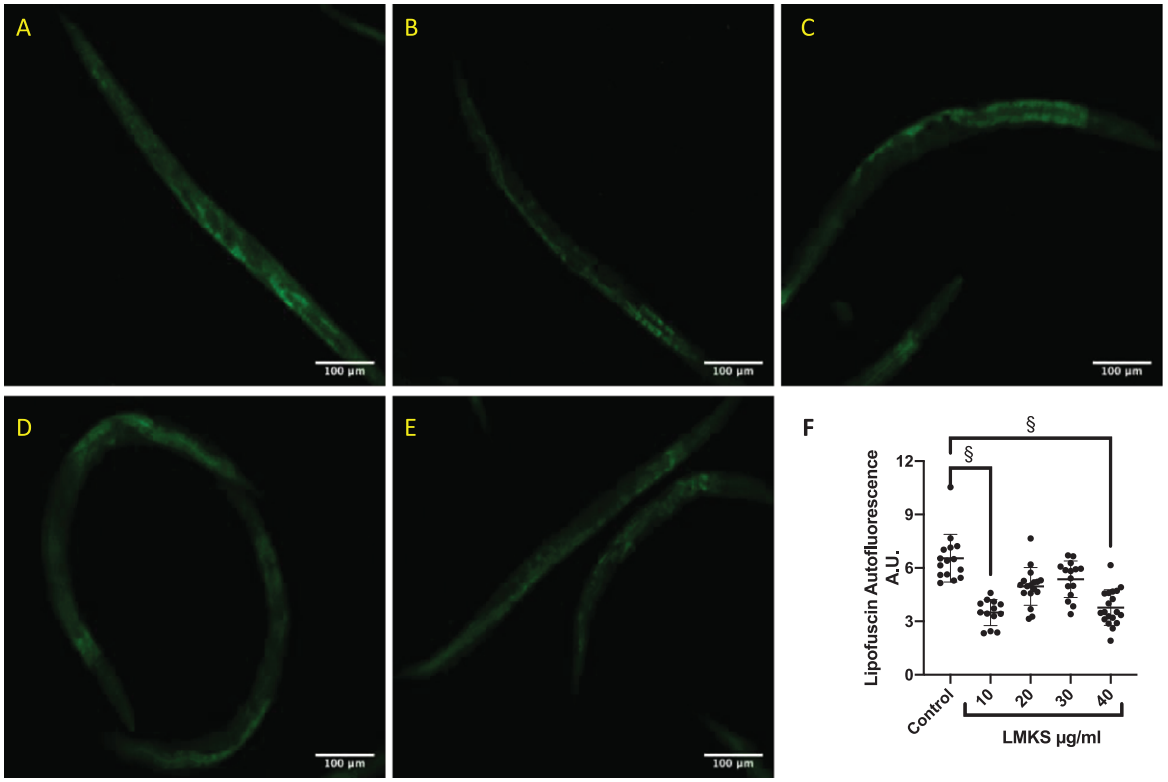

Fig. 5. Representative micrograph of lipofuscin expression in worms (A) without LMKS, (B) with 10 µg/ml LMKS, (C) 20 µg/ml LMKS, (D) 30 µg/ml LMKS, (E) 40 µg/ml LMKS, (F) Analysis of individual worms from 3 independent experiments. [§] Statistically significant difference compared to control (ANOVA followed by Dunnett's *post hoc* multiple comparison test $p = 0.01$ (10 µg/ml) and $p = 0.02$ (20 µg/ml)).

activated the NRF2/SKN1 pathway (Fig. 7). Exposure to UV-A for 4 hours caused a small but not statistically significant increase in GFP signal (Fig. 7A-B). However, treatment with LMKS (10 µg/ml) subsequent to UV exposure increased NFR2-GFP expression compared to the UV treated worms (Fig. 7C), although LMKS (20 µg/ml) did not (Fig. 7D) (ANOVA followed by Dunnett's *post hoc* multiple comparisons test $^{**}P = 0.0095$). These differences are quantified in Fig. 7E.

3.6. LMKS effect on gene worm gene expression

We investigated the effect of LMKS on genes involved in the extension of lifespan (*daf-16, daf-2*, *age-1* and *utx-1*), antioxidant mechanisms (*skn-1* and *sir-2.1*) and genes involved in health-span (*egl-8* and *egl-30 col-19*, *dgk-1* and *goa-1*) (Fig. 8). We observed a small but significant down regulation of *daf-2, age-1* and *utx-1* (Wilcoxon one-sample *t*-test $P < 0.05$, $P < 0.05$, and $P < 0.01$ respectively). Whereas there was an aproximate 2-fold increase (LMKS 10 µg/ml) and an approximate 5-fold increase (LMKS 20 µg/ml) in *daf-16* expression (Wilcoxon one-sample *t*-test $P < 0.01$ and $P < 0.01$ respectively). The expression of *egl-8* (LMKS 10 µg/ml) sees an approximate 1-fold increase and *egl-30* sees approximately a 2-fold increase in expression while *col-19*, *dgk-1* and *goa-1* all see small decreases in expression. Both *skn-1* and *sir-2.1* see an approximate 2-fold increase (both 10 and 20 µg/ml).

4. Discussion

Oxidative stress induced activation of glial cells, neuroinflammation and mitochondrial dysfunction lead to various molecular events in brain leading to neurodegenerative disorders [55, 56]. Novel molecular peptides inhibiting the production of Aβ show promising effect in *in vitro* and animal model for AD [57]. However, majority of the drugs used will only reduce the symptoms of these diseases and cannot stop the process of neurodegeneration [58]. In this regard, plant-based therapies are gaining more focus in dealing with neurodegenerative disorders

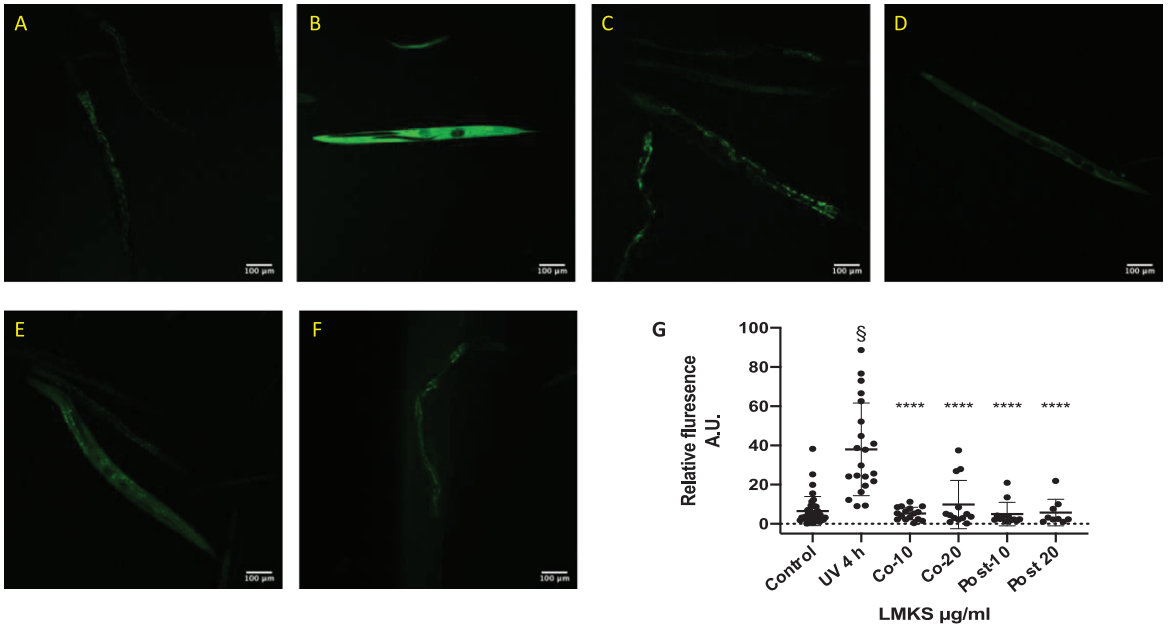

Fig. 6. ROS production in worms exposed to UV-A for 4 hours (representative micrographs). A) Control worms with no UV-A exposure. B) Worms exposed to 4 hours of UV-A. C) Worms cotreated with 10 μg/ml LMKS and exposed to 4 hours of UV-A. D) Worms cotreated with 20 μg/ml LMKS and exposed to 4 hours of UV-A. E) Worms exposed to 4 hours of UV-A and subsequently treated with 10 μg/ml LMKS. F) Worms exposed to 4 hours of UV-A and subsequently treated with 20 μg/ml LMKS. G) Analysis of the florescence of each individual worm over 3 independent experiments (mean ± SD $n = 3$). §Significant difference compared to the control (non-UV exposed) (ANOVA followed by Dunnett's *post hoc* multiple comparison test $P < 0.0001$). ****(Statistically significant difference compared to UV 4h ANOVA followed by Dunnett's *post hoc* multiple comparison test $P < 0.0001$).

which can shed light on the mechanism of action also. LMKS was able to significantly extend the lifespan of transgenic Aβ strains indicating its neuroprotective potential.

Here we have shown that the seed extract of *C. nervosum* (LMKS) is neuroprotective against Aβ toxicity in PC12 cells (Fig. 1). The $A\beta_{(25–35)}$ fragment is derived from the Aβ protein by proteolytic cleavage of the $A\beta_{(1–40)}$ fragment [59]. It lacks the N-terminal domain and has a β-sheet structure [49] The $A\beta_{(25–35)}$ fragment can rapidly aggregate [60] and is involved in age dependent neurotoxicity and the pathogenesis of AD [61]. The $A\beta_{(25–35)}$ fragment has been used previously to induce neurotoxicity in cortical cultures [62] induce AD like symptoms in rats [63] and induce cell death in cultured PC12 cells [64]. Aβ treatment causes oxidative stress in neuronal cell lines [65], and previous work from this laboratory has shown that *C. nervosum* has neuroprotective effects via antioxidant and ER stress preventing mechanisms [29, 34]. Previous studies have shown that Neuro2A cells expressing APP or APP Swedish variant are susceptible to dopamine toxicity as the result of ROS production leading to apoptosis [15, 46]. Neuro2A cells expressing muscarinic receptors (M2) [66] are able to undergo cholinergic dependent differentiation with neurite elongation [67], and in the right conditions express tyrosine hydroxylase for the production of dopamine and L-DOPA [68], thus making these cells a useful tool in studying drug effects on parkinsonian symptoms in AD [15, 46]. However, LMKS did not provide any protection against dopamine toxicity in $APP^{(swe)}$ expressing Neuro2A cells (Fig. 3). The mechanism of cell death in this model is also ROS mediated [46], which suggests that LMKS is not acting purely as an antioxidant rather it is activating an antioxidant pathway. We and others have previously shown that activation of the sigma-1 receptor is neuroprotective and can activate a ROS-dependent mechanism [15, 21, 69, 70]. Furthermore, we have shown that LMKS is able to potentiate NGF induced neurite outgrowth (Fig. 2), which itself is regulated by the sigma-1 receptor [25–27, 51]. Moreover, using a sigma-1 receptor antagonist (BD1047) prevented the potentiation of the NGF induced neurite outgrowth (Fig. 2).

Both the sigma-1 receptor and amyloid peptides are able to regulate ERK phosphorylation, which

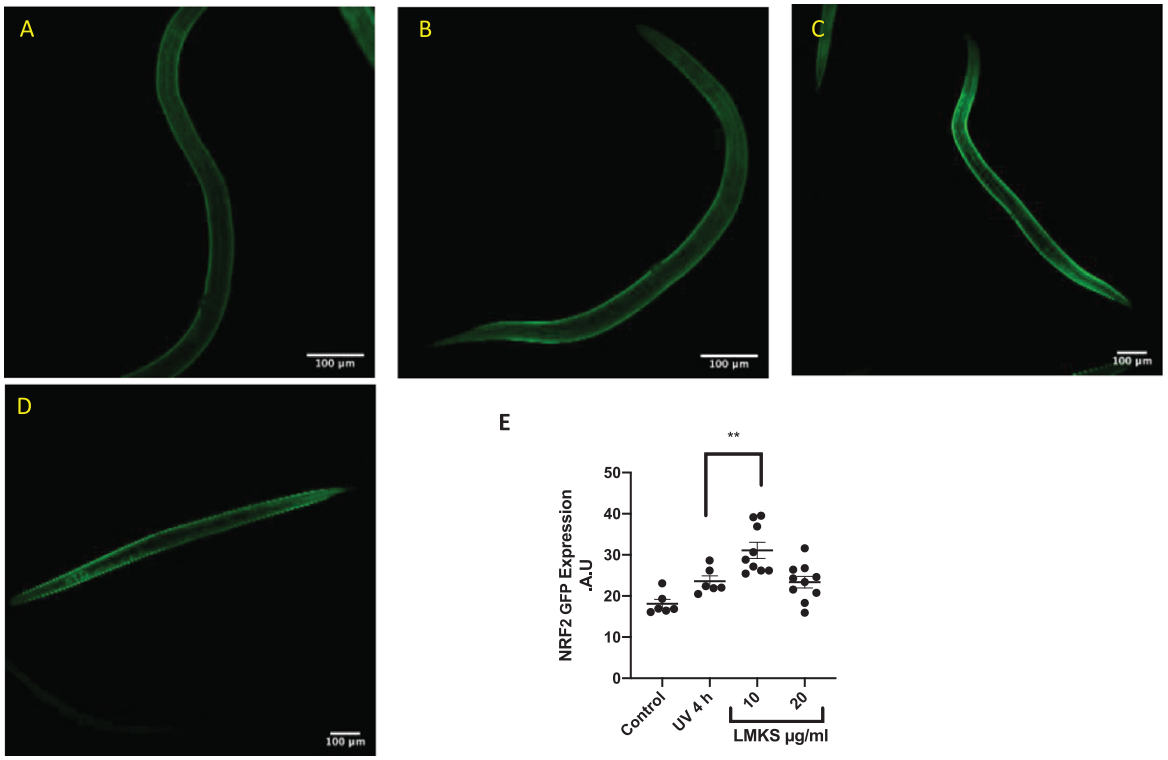

Fig. 7. SKN-1-GFP expressing *C.elegans* exposed to UV-A for 4 hours. A) Control. NRF2-GFP expressing worms with no UV-A exposure. B) NRF2-GFP worms exposed to 4 hours of UV-A. C) NRF2-GFP worms treated with 10 µg/ml LMKS after exposure to 4 hours of UV-A D) NRF2-GFP worms treated with 20 µg/ml LMKS after exposure to 4 hours of UV-A. E) Quantification of fluorescence of the individual worms. Significant difference compared to UV 4h using ANOVA followed by Dunnett's *post hoc* multiple comparisons test $^{**}P = 0.0095$.

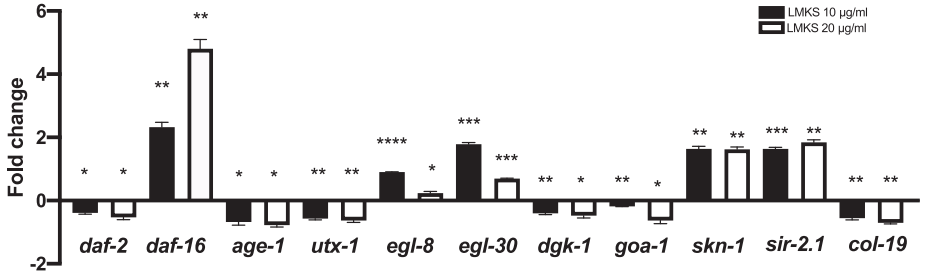

Fig. 8. Gene expression of worms exposed to LMKS (10 and 20 µg/ml) expressed as fold change compared to control. Statistically significant changes measured using one sample Wilcoxon *t*-test $^{*}p < 0.05$ $^{**}p < 0.01$ $^{***}P < 0.001$ $^{****}P < 0.00$.

has a role in neuron cell survival [71] as well as neurite outgrowth [72, 73]. Furthermore, the sigma-1 receptor [74] and Aβ proteins [75] are involved in the phosphorylation of tau protein, and neurons with reduced sigma-1 receptor exhibit an increase in Tau hyperphosphorylation [28]. Tau functions to stabilize microtubules [74], and its hyperphosphorylation results in neurofibril tangles [76]. Also, amyloid peptides, via ERK phosphorylation, modulate the phosphorylation of tau and its subcellular compartmentalization, an event that may lead to the formation of neurofibrillary tangles and to cell death in post-mitotic neurons. If LMKS contains a compound acting as a sigma-1 receptor agonist and activates the sigma-1 receptor it is possible that it is acting to reduce Tau hyperphosphorylation. While we cannot affirm conclusively, from the data presented, that the sigma-1 receptor is involved with the LMKS neuroprotective properties, it does appear to fit some of the qualities seen with sigma-1 receptor ligands.

The LMKS extract contains a wide range of compounds, and there is a likelihood that one of those may well be a sigma-1 receptor agonist especially considering the number of different compounds that the sigma-1 receptor interacts with [12].

A number of studies have implicated the sigma-1 receptor in the mediation of the NRF2 antioxidant pathway [70, 77–79], with sigma-1 receptor agonists increasing the expression of NRF2 protein. Furthermore, a recent study investigating the effects of Cyanidin-3-glucoside (C3G) (one of the major compounds found in *C. nervosum*) showed that C3G could protect HT-22 cells from glutamate induced oxidative stress via the upregulation of NRF2 [80]. Using an *in vivo C. elegans* model of *skn-1*-GFP transgenic strain that expresses a ortholog of NRF2 we were able to show a statistically significant increase in *skn-1*-GFP expression in response to LMKS treatment (at 10 μg/ml but not 20 μg/ml) when the worms were exposed to UV-A to induce oxidative stress (Fig. 7). Moreover, with the qPCR analysis of *skn-1* in wild type worms, we show that LMKS induces an approximate 2-fold increase in *skn-1* expression (Fig. 8), which further suggests that LMKS is able to activate oxidative stress resistance pathway via the NRF2/SKN-1 protein pathways. It is interesting that the NRF2-response does not appear to be dose dependent, with the possibility of a bell-shaped dose response curve, again another feature of sigma-1 receptor agonism [13].

Previously, we have reported that *C. nervosum* fruit extract could extend lifespan and health-span along with extending neuroprotection in *C. elegans* [29]. The fruit pulp with its immense health benefits has been explored by the researchers. However, there has been limited research about the seeds which also has reported to have antioxidant and anti-cancer properties [81, 82]. In this regard, the seed extracts were analyzed in *C. elegans* for their anti-ageing and neuroprotective efficacies. LMKS was able to extend the lifespan of wild type *C. elegans* (Fig. 4A-D), and this was not due to calorie restriction based on the pharyngeal pumping assay. Lipofuscin is the name given to a lipid containing pigment whose accumulation is considered to be a sign of aging [83]. It is formed by the oxidation of unsaturated fatty acids and may be a sign of membrane or mitochondrial damage [84]. LMKS was also able to reduce the accumulation of lipofuscin inside the worms (Fig. 5), indicating the improvement in health span. The gene expression of *col-19*, *egl-8*, *egl-30*, *dgk-1* and *goa-1* also supported this view (Fig. 8). Interestingly, the extract was able to significantly extend the lifespan of transgenic strains expressing Aβ indicating the neuroprotective potential of the extract. Several plant extracts have been reported to exhibit neuroprotective efficiency in *C. elegans* [85, 86]. ROS plays a key role in enhancing neurodegeneration. In this regard, the antioxidant effect of LMKS was analyzed in wild type worms challenged with ROS by exposing to UV-A. The significant reduction in the level of ROS in the worms co-treated and post-treated with LMKS confirmed the antioxidant potential of the extract (Fig. 6). Additionally, the expression of genes that mediate the antioxidant mechanism like *skn-1* and *sir-2.1* also supports this view (Fig. 8). Antioxidants such as green tea could mitigate oxidative stress via *sir-2.1,* the mammalian ortholog of Sirtuins [87]. Recent studies suggest that different plant extracts elicit antioxidant mechanisms, which thereby impart neuroprotection to the host [86–88].

DAF-16 is known to mediate longevity and reduce the level of oxidative stress, thus its activation may be useful in the prevention of AD. In the present study, LMKS was able to upregulate the lifespan of *daf-16* negative mutants, indicating that LMKS can exert its protective properties independent of the *daf-16* pathway. Interestingly, the extract was also able to upregulate the expression of *daf-16* along with downregulating the expression of *daf-2, age-1* and *utx-1* indicating that the extract works both dependently and independently of the *daf-16* pathway. We have observed similar pattern in our previous study with the C. nervosum fruit pulp extract [29]. As DAF-16 and SKN-1 are known to elicit neuroprotection [86], this could explain neuroprotective activity of LMKS.

5. Conclusions

LMKS is able to provide significant protection against neurodegeneration, particularly against AD as it protected the Aβ treated PC12 cells and extended the lifespan of the *C. elegans* transgenic strain that expresses Aβ. The neuroprotection is likely to follow an NRF2/SKN-1 antioxidant pathway, which may be under the control of the sigma-1 receptor. LMKS also provides its protection both dependently and independently of *daf-16*. Further studies into the constituent compounds found within LMKS could provide new lead compounds for the treatment of AD. This study highlights the importance of seed extract that could

impart health benefits along with the pulp extract, thereby confirming its possible use as 'functional food'.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

JMB, CI and TT conceived the research idea, MS carried out the collection and extraction of the herb. MIP carried out all the *C. elegans* work and JMB carried out the Cell culture. JMB MIP and TT drafted the manuscript. CI provided the N2A APP cell lines and edited the final draft manuscript. All authors have seen and approved the final draft of the manuscript.

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