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Original Research Article (Experimental)

Ameliorative effect of *Argyreia boseana Sant. & Pat.* on stress in *C. elegans*

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ABSTRACT

Background: *Argyreia boseana Santapau and Patel* commonly known as Kumbharao belongs to the family of Convolvulaceae. The plant is rare in distribution and found chiefly in the dediapada region of Gujarat. Traditionally it is used by the tribals of south Gujarat forest region in the treatment of various diseases of the nervous system. In order to study the scientific basis of the plants effect we set out to investigate the effect of the plant on ageing organisms and used *Caenorhabditis elegans* as a model.
Objective: To evaluate the effect of crude extract of leaves, prepared from *A. boseana* on oxidative stress, thermal stress, longevity and *in vivo* gene expression of *C. elegans*.
Material and Methods: Plant extracts was prepared by sonication based method using solvent ethanol:water. Longevity experiments were carried out in liquid S media. Oxidative stress was induced by paraquat.
Result: Results indicate no increase in the normal life span of *C. elegans*. However, *A. boseana* significantly induces stress tolerance and increased the mean lifespan of worms during thermal and oxidative stress. Additionally *A. boseana* was also able to up regulate the stress associated gene gst-4.
Conclusion: Thus the present study, for the first time, unravels the anti-stress and ROS modulating effect of *A. boseana*.

1. Introduction

Stress in its acute and chronic form has become an integral part of life. If left untreated it can lead to a variety of diseases like hypertension, heart diseases and anxiety. Extreme stress situations can be life threatening [1]. The drug therapies for stress management involves use of beta blockers or benzodiazepines which may lead to serious side effects and have been found to be ineffective against numerous stressors like stress induced by radiation, oxidative stress or emotional stress [2]. Plant adaptogens are metabolic regulators of natural origin that increase the ability of organisms to fight against a broad spectrum of physical, chemical and biological stressors [3]. Isolates of plants, such as *Eleutherococcus senticosus* and *Rhodiola rosea* are well-known to elicit stress tolerability and improved physical performances in various mammalian models [4]. Nevertheless the mechanism by which adaptogens stimulate stress resistance is not clear, but several studies have shown involvement of pathways leading to synthesis of heat shock proteins and development of oxidative resistance. [5,6], We undertook this study to test the efficacy of the plant *Argyreia boseana* as an adaptogen on the model system *C.elegans*.

A. boseana Sant. and Patel(A.boseana) also known as *Argyreia Festiva Wall*, belongs to the family of Convolvulaceae. It is an extensive climber with the stem which is grooved, glabrous or slightly pubescent. Previous studies have identified ferulic acid, caffeic acid, rutin as biological active components present in the plant. *Argyreia* species have been traditionally used as drugs to upkeep mental as well as physical health. [7,8], It is also used to treat diseases of the nervous system. Despite its traditional use studies regarding its *in v ivo* effects are limited, more particularly for *A. boseana*. Keeping these facts in mind, we set out to evaluate performance of *A. boseana* as adaptogen.

In the present study, we determined the total phenolic, flavonoids and *in vitro* antioxidant capacity of the plant. Thereafter we used *C. elegans* as a model to test the efficacy of *A. boseana* as an

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in vivo longevity promoting agent. Along with the lifespan, response of *C. elegans* to environmental stressors like thermal stress and oxidative stress was monitored in normal and mutant models. The effect of *A. boseana* on stress responsive genes was determined using Green Fluorescent Protein (GFP) expressing transgenic strains.

2. Material and methods

2.1. Collection of plant material and preparation of extract

Plant of *A. boseana* were collected from the Dediapada region, planted, maintained and propagated at the Loyola Center for Research and Development, Ahmedabad for further use. Their leaves were collected and washed under running tap water carefully and dried under the shade for ten days. The dried material was powdered and extracts were prepared by sonication using Ethanol: Water (70:30) as solvent. The extract after evaporation of solvent was dissolved in molecular biology grade DMSO purchased from sigma-aldrich (D4818) to prepare stock solution of 100 mg of extract/ml.

2.2. Quantification of phenolics and flavanoids

The total phenolic and total flavonoids of *A. boseana* was estimated according to the *Folin –Ciocalteu's* (F-C) method and $AlCl_3$ method respectively with the modification adapted from Herald T et al. [9]. For total phenolic, in each well of 96 well plate 75 μL of distilled water, followed by 5 μL of standard or sample and 25 μL of F-C reagent was added. The mixture was allowed to incubate for 6 min and then 100 μL of 75 g/L Na_2CO_3 was added to each well. The plate was incubated in dark for 90 min and then absorbance was measured at 765 nm. For flavonoids, 100 μL of distilled water, 10 μL of $NaNO_2$(50 g/L), and 5 μL of sample was added to each well of 96 well plate. After 5 min 15 μL of 100 g/L $AlCl_3$ was added and plate was allowed to incubate for 6 min. Then 50 μL of 1M NaOH and 50 μL water was added to each well. The plate was shaken for 30s and then the absorbance as measured at 510 nm. Gallic acid was taken as a standard for total phenolic and the results are expressed in Gallic acid equivalent. For flavonoids, rutin was taken as standard and results are expressed in Rutin equivalent.

2.3. Total antioxidant activity assay and reducing power assay

The total antioxidant capacity (TAC) of *A. bosiana* was determined by the phosphomolybdenum assay as per the method described by Kasangana PB [10] TAC results were expressed as Ascorbic acid equivalents (mg Ascorbic acid/g of dry sample). Reducing power of the plant extract was determined as per the method by Oyaizu and Jayanthi P [11].

2.4. Caenorhabditis elegans(C. elegans) strains, maintenance and synchronization

The *C. elegans* strains used in the experiments where N2 bristol,TJ375 (gpls1), CL2166 (*dvIs19 [pAF15 (gst-4:gfp-nls)]* III), and TK22 (*mev-1*). N2 bristol were gift by Dr. Sandhya Kaushika from TIFR. All of the other strains were obtained from Caenorhabditis genetic Centre, which is funded by NIH office of Research infrastructure program (P40 OD010440). *C. elegans* strains were maintained at 20 °C on Nematode Growth Medium (NGM) plate seeded with *E.coli* OP50. Age synchronized worms were obtained by sodium hypochlorite treatment.

2.5. Life span assay

Life span assay of *C. elegans* was performed in 96 well plates in a liquid S complete media as per the method described by Solis G [12].Synchronized populations of worms were obtained by sodium hypochlorite treatment. Around 10 L1 stage worms were dispensed in each well of 96 well plate containing 100 μL of S complete medium along with OP50 as food source. Worms were allowed to reach till adulthood. After that to prevent progeny formation they were treated with 5-fluoro-2′-deoxyuridine (FUDR) on day 0 of adulthood. Thereafter plant extracts and DMSO as control was added in respective wells. Plant extracts and OP50 were supplied to worms once in a week to maintain effects. The worms were maintained at 20 °C throughout the experiment. Average number of worms(n) in each experiment was 50–55. Three such experiments were performed. Survival of animals was monitored under inverted microscope every day. Life span analysis was done using Kaplan Meier statistical analysis.

2.6. Oxidative and thermal stress resistance assay

L1 larvae of wild type N2 were subjected to different concentration of plant extracts or DMSO as solvent control in S complete medium in a 96 well plate. Each well of 96 well plates contained 18–20 larvae(n). The respective plant extracts or DMSO was added in triplicate in each of the experiment. After 72 h 50 mM parquet was added to each well to induce oxidative stress and vitality was checked after 24 h.

The thermal stress assay was performed as described by Asthana J [13] Briefly plant extracts or DMSO treated L4worms were incubated at 37 °C for 4hrs in 96 well plate in S-complete medium along with OP50 as food source and then allowed to recover at 20 °C for 24 hrs. After which worms were monitored daily till all worms were dead. The total number of worms were as described in table number 3.

2.7. In vivo gene expression and quantification

Worms of CL2166 strain contains transcriptional fusion of GFP to the *gst-4* promoter. Synchronized culture of CL2166 was obtained by sodium hypochlorite treatment. Starting from L1 stage worms were grown in liquid S medium supplemented with plant extracts or DMSO as control for 72hrs. At the end of the incubation period twenty worms per group were placed on a glass slide in PBS containing sodium azide as a paralyzing agent. The expression of gst-4

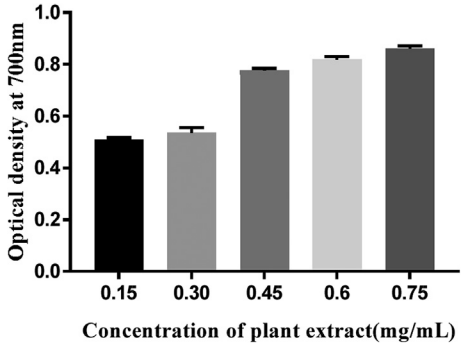

Fig. 1. Reducing ability of *A. boseana* was measured in terms of ability of plant extract to reduce ferric chloride to ferrous chloride. The data represents the dose dependent increase in reduction of ferric chloride as the concentration of plant extracts increases.

Table 1
Effect of *A. boseana* on life span of Wild type N2 Bristol and *mev-1* worms.

	C.elegans strain	No. of worms(n)	Mean life span± S.E.	P value V/S control
Control	N2 Bristol.	165	19.98 ± 1.2	
A. boseana 1 mg/mL	N2 Bristol.	153	20.83 ± 1.5	0.4848
A. boseana 0.1 mg/mL	N2 Bristol.	177	19.22 ± 0.75	0.2098
A. boseana 0.01 mg/mL	N2 Bristol.	189	18.9 ± 1.1	0.0129
Control	Tk-22 (*mev-1*)	147	13.95 ± 0.63	≤0.001
A. boseana 1 mg/mL	Tk-22 (*mev-1*)	174	19.62 ± 0.60	≤0.001
A. boseana 0.1 mg/mL	Tk-22 (*mev-1*)	132	18.58 ± 0.72	≤0.001
A. boseana 0.01 mg/mL	Tk-22 (*mev-1*)	150	13.26 ± 0.85	≤0.001

was measured by quantifying the fluorescence of the reporter protein GFP. The fluorescence intensity was visualized through Zeiss microscope and images of worms were captured by camera attached to microscope. Intensity of GFP was quantified by imageJ (NIH) software in terms of pixel density of fluorescence [14].

2.8. Measurement of fat accumulation in C. elegans

Fat accumulation in *C. elegans* was observed by the Nile red staining. Age synchronized *C. elegans* from day zero of adult hood were exposed to plant extract or DMSO as vehicle control for seven days on NGM plate containing FUDR. After seven days 20 worms(n) from each group were stained with Nile red o dye as per the protocol described by Pino E [15]. Images were observed with a Zeiss florescent microscope, and fluorescent intensity was quantified by Image J (NIH) software.

2.9. Data analysis

Statistical analysis was performed using GraphPad Prism version 7.00 for Windows (San Diego, CA). The results were plotted as the mean SEM (standard error of the mean) of at least three individual experiments. All survival curves were analyzed by the Log-rank (Mantel–Cox) test. The statistical significance was determined as p˂0.05.

3. Results

3.1. In vitro antioxidant and reducing ability of activity A. boseana

Plant polyphenols promote free radical scavenging activity while flavonoids are known to confer stress resistance on organism by inducing antioxidant enzymes [16]. Determination of content of polyphenols and flavonoids in *A. boseana* leaves indicated that it has total phenolic 0.931 ± 0.023 mg/g of dry plant material in terms of gallic acid equivalence while flavonoids are 9.2881 mg/g of dry plant material in terms of rutin equivalence.

The total antioxidant capacity of the plant extract was determined in terms of ascorbic acid equivalent, which was found to be 58.839ug/g. *A. boseana* also showed dose dependent increase in its ferric reducing ability (Fig. 1).

3.2. A. Boseana did not prolong life span under standard condition

We examined the effect of *A. boseana* extracts on lifespan of wild type *C. elegans* at three different concentrations. Worms were checked for survival daily. Worms which did not move or showed pharynx pumping were considered dead. The mean life span of wild type was found to be 19.28 ± 1.2 days. The life span of treated worms were 20.83 ± 1.5 days, 19.22 ± 0.75 days and 18.9 ± 1.1 for the 1 mg/mL, 0.1 mg/mL and 0.01 mg/mL treatments respectively. The result shows that none of the treatments showed any significant increase in the mean life span of the worms (Table 1).

To investigate whether *A.boseana* supplement can increase lifespan under oxidative stress condition a mutant strain of *C. elegans, mev-1* was employed. The strain has short lifespan and oversensitivity to oxidative stress because of mutation in subunit of complex II of mitochondrial respiratory chain [17]. The age synchronized *mev-1* worms were subjected to the identical three concentrations of *A. boseana* as per the method described above. In this experiment all the tested concentrations were able to increase the life span of worms. The 1 mg/mL concentration of *A. boseana* showed 40% increase (p value ≤ 0.001) in mean lifespan compared to control, which is maximum (Fig. 2).

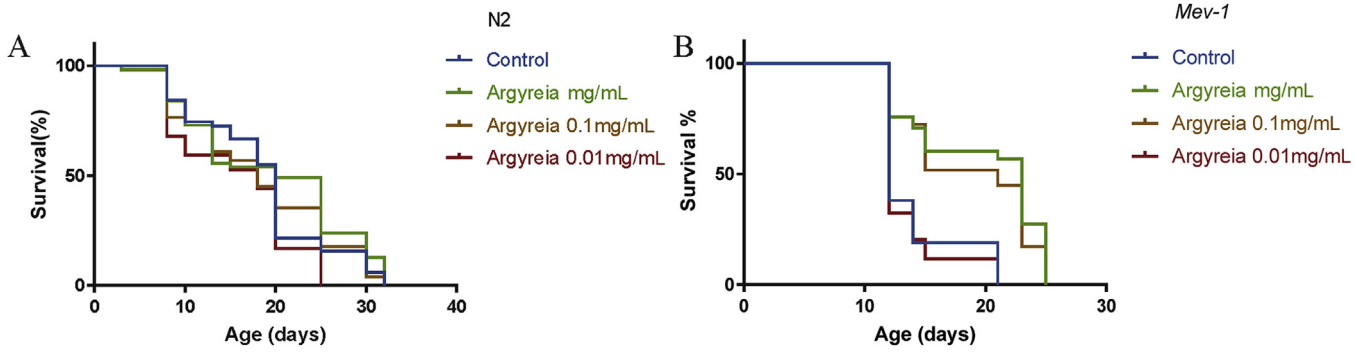

Fig. 2. Effect of *A. boseana* on longevity of *C. elegans*. Survival curve were plotted as per the Kaplan Meier survival assay. The worms were fed heat killed OP50 every fifth day along with plant extracts as supplements. (A) *A. boseana* could not augment lifespan of wild type worms. (B) Substantial increase of lifespan by 40% ($p < 0.0001$),33.18% ($p < 0.0001$) was observed at 1 and 0.1 mg/mL concentration of *A. boseana*. However 0.01 mg/mL concentration of *A. boseana* did not prolong lifespan of mev-1 mutants.

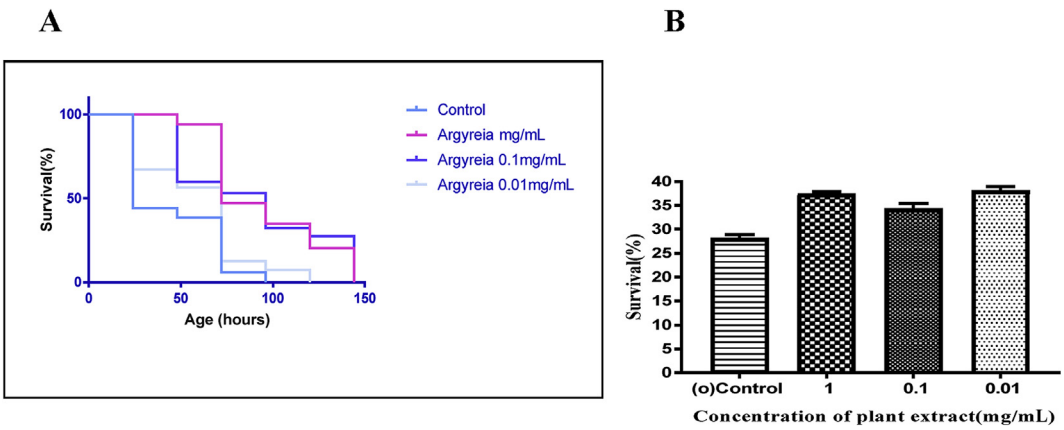

Fig. 3. Effect of *A. boseana* on (A) thermal stress: There is a dose dependent increase in life span as concentration of plant extract increases. The lifespan of control worms is 24 ± 1.2 while of treated worms extended up to 96 h (B) oxidative stress. Pretreatment of worms with PE significantly promote stress resistance in *C. elegans*.

3.3. A.boseana increases stress resistance in C. elegans

As *A. boseana* has shown considerable *in vitro* antioxidant activity, its influence *in vivo* was evaluated by oxidative stress assay. The result and the statistical analysis (Fig. 3 and Table 2) showed that under the oxidative stress generated by paraquat, *A. boseana* treatment rescued the worms from thermal stress. *A. boseana* pretreatment results in increase in survival by 29%.

3.4. Fat accumulation

Fat deposition in *C. elegans* predisposes them to accelerated aging and reactive oxygen species (ROS) accumulation. To investigate the effect of *A. boseana* on fat accumulation, *C. elegans* were pretreated with 1 mg/mL concentration of *A. boseana*. Fluorescent images of worms were captured under 40× magnification in a Leica fluorescent microscope (Fig. 4). The treated worms showed 51.91 ± 2.612 % decrease in fat deposition.

Table 2
Protective effect of *A. boseana* on wild type N2 *C.elegans* undergoing thermal stress.

	Thermal stress	No. of worms	Mean life span± S.E.	P value V/S control
Control	37 °C for 4 h.	140	24 ± 1.2	
A.boseana 1 mg/mL	37 °C for 4 h.	153	72 ± 1.5	≤0.0001
A.boseana 0.1 mg/mL	37 °C for 4 h.	186	96 ± 0.75	≤0.0001
A.boseana 0.01 mg/mL	37 °C for 4 h.	165	72 ± 1.1	≤0.0001

3.5. A. boseana up regulates gst-4:GFP -expression

Glutathione S-transferase (gst-4) gene codes for an enzyme involved in the detoxification procedure induced by insulin/IGF-1 signaling pathway and is a known molecular marker of intracellular oxidative stress, while small heat shock proteins are induced upon exposure to thermal shock or to pro-oxidant such as paraquat. In the present study, the CL2166 was employed to validate the role of *A. boseana* in mediating oxidative stress. The results indicate that *A. boseana* upregulates gst-4 gene at 1 mg/mL and 0.01 mg/mL doses (Fig. 5).

4. Discussion

Plant based diets contain a variety of phytomolecules like polyphenols, which are known to have protective effect. The mechanism by which they exert their protective effect depends upon their metal reducing and antioxidant capacity. Polyphenols amongst them are more important as their bioavailability is also high; they can exert antioxidant activity and are also good metal chelators[18,19], Therefore polyphenol and flavonoids content of *A.boseana* was determined *in vitro*. We demonstrated that *A.boseana* has polyphenols and showed metal reducing activity in a dose dependent manner. It has *in vitro* antioxidant capacity too.

C. elegans is a well-established model system for studying longevity improving and stress modulating effects of natural

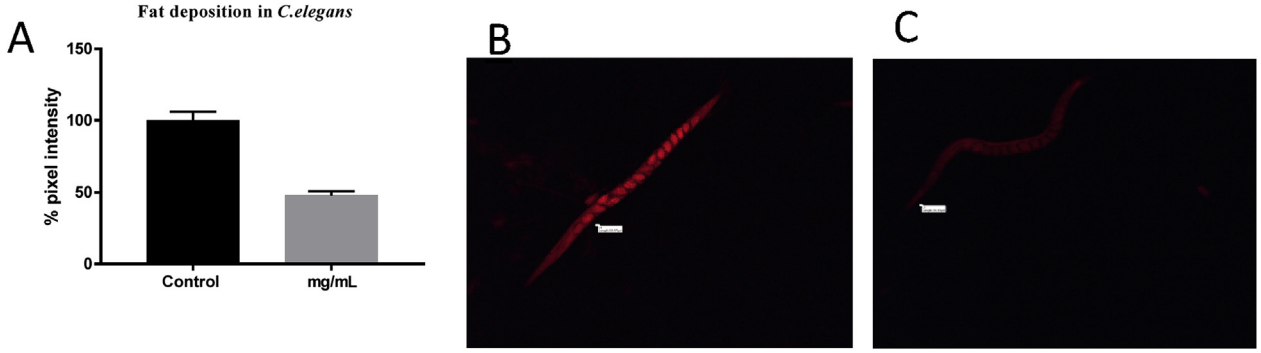

Fig. 4. (A) Effect of *A. boseana* extract on fat accumulation. Fluorescent microscopy images of *C. elegans* treated either with (B) DMSO or with (C)1 mg/mL *Argyreia boseana* are shown here. Fluorescent intensity was analyzed by image J and compared with DMSO. Control worms showed 42.66% ± 6 of fat accumulation while plant extract treated worms showed 22.146 ± 2.14 of fat accumulation.

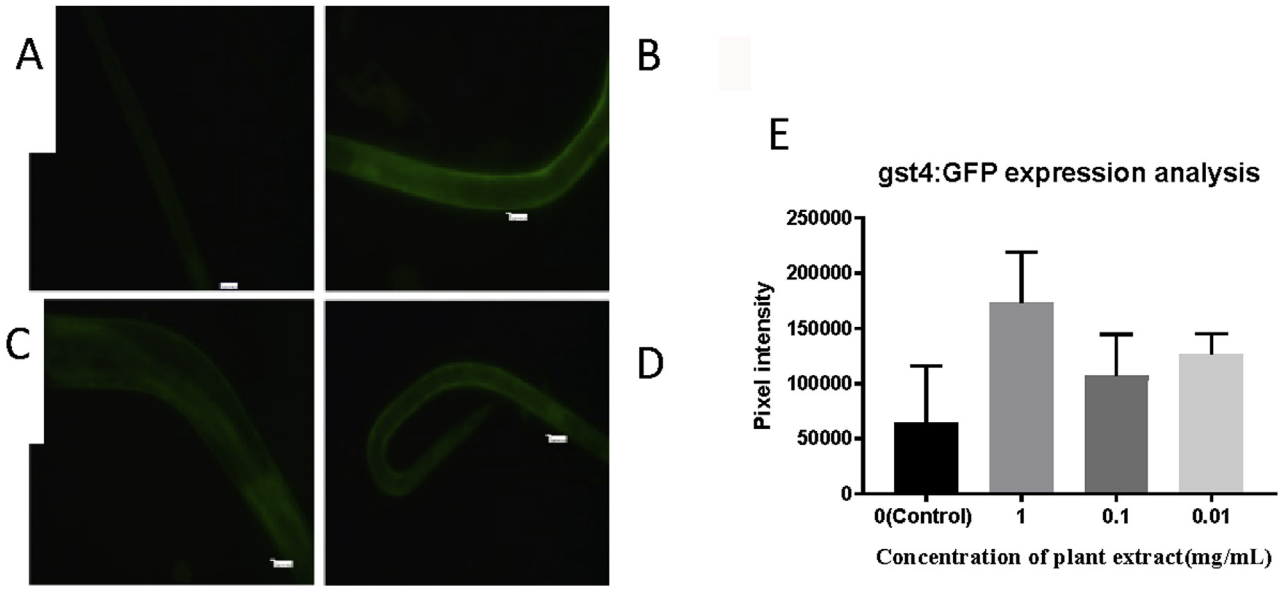

Fig. 5. Gst4 gene up reguation by *A. boseana*. CL2166 strain expresses GFP fused with promoter of GST. (A) Intensity of fluorescence of control worms. (B) Fluorescence intensity of *A. boseana* treated worms at 1 mg/mL. (C) *A. boseana* treated worms at 0.1 mg/ml. (D) *A. boseana* treated worms at 0.01 mg/mL. (E) The intensity of fluorescence was analyzed in terms of pixel intensity by imageJ software and presented graphically.

compounds and their isolates. Active principles of many natural compounds are reported to improve stress resistance and have shown antiaging effect in *C. elegans.* The majority of these compounds are anti-oxidants and free radical scavengers [20–23]. These studies prompted us to investigate the stress modulating and life span improving effect of *A.boseana* in *C. elegans.*

The present study indicates that *A.boseana* did not increase the mean life span of wild type worms but did significantly increase the life span of mev-1 mutant worms. *mev-1* mutant has a lifespan because of over accumulation of Reactive oxygen species [24]. *A.boseana* could increase the lifespan of these worms by 25–40% in comparison to DMSO treated control worms. This finding indicates good *in vivo* antioxidant capacity of *A. boseana*.

In *C. elegans* increased fat deposition is associated with increased production of reactive oxygen species and decrease in life span [24,25]. Therefore, the effect of *A. boseana* on fat accumulation in wild type worms was examined. The treated worms showed significant decrease in fat accumulation. Next wild type worms pretreated with plant extract, experiencing thermal and oxidative stress were examined. In the present study, we demonstrated that *A. boseana* significantly improved stress tolerance and increased the mean lifespan of the worms during thermal stress and paraquat induced oxidative stress. To investigate the effect of *A. boseana* on gene expression, its effect on gst-4:GFP strain was examined. Oxidative stress in *C. elegans* is mediated by SKN-1, a transcription factor. One of the target genes for this transcription factor is gst-4, which encodes glutathione dependent prostaglandin synthase. Up regulation of this gene is known to mediate oxidative stress response in *C. elegans* [26]. Our results indicating that Worms treated with *A. boseana* enhanced expression of gst-4 in comparison to untreated worms are consistent with the above fact. Future studies can be done to identify the individual active ingredient responsible to modulate stress resistant genes and can explore the entire pathway.

5. Conclusion

In the present study we showed for the first time that *A. boseana* augment stress resistance in *C. elegans* against environment stress like heat and oxidative stress. It does so by modulating the expression of stress resistive gene gst-4. However *A. boseana* could not increase the longevity.

Sources of funding

None.

Conflict of interest

None.

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