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ORIGINAL ARTICLE **OPEN ACCESS**

Effect of *B.pinnatum* Extract on Longevity, Health span and Stress Resistance in *Caenorhabditis elegans*

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ABSTRACT
Currently, research on longevity is receiving much attention. The focus is to enhance the lifespan and support a healthy aging process. Herbal products are being investigated in order to identify molecules. In the present investigation, Bryophyllum pinnatum (hydromethannolic)(BP-HM)leaf extract was selected to evaluate its longevity, healthspan and stress resistance efficacy. We have used Caenorhabditis elegans as a model in our experiments. Our results suggest that B. pinnatum hydromethanolic (BP-HM) (100 µgmL^{-1}) treatment of wild-type (N2) C. elegans prompts extension in the mean lifespan of the worms. In addition, the daf-2 anddaf-16mutants treated with the same dose also manifested a prolonged lifespan when compared to control untreated worms. Pharyngeal pumping and locomotion rate also indicate that BP-HM treated worms show enhanced activity as compared to untreated animals of the same age. Moreover, results of the present study also signify the potential of BP-HM extracts to enhance the tolerance against thermal stress in N2 C. elegans. Improved lifespan of C. elegans might be due to phytomolecules present in the Bryophyllum pinnatum (BP) which mimics the proteins of daf-2 and daf-16 mutants and their mode of action is probably independent from daf-2-daf-16 signaling pathway. Hence, the findings of this study reveal the in vivo antioxidant activity of hydromethannolic leaf extract of BP in C. elegans. Further, the present study also proves the efficacy of BP as an anti-aging compound and its role as a drug/medicine to reduce age related detrimental changes.
KEYWORDS: *Bryophyllum pinnatum, Longevity, Healthspan, Stress Resistance, Caenorhabditis elegans.*

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INTRODUCTION

A major challenge from a global perspective is to discover a herbal product or drug to delay the aging process and the subsequent commencement of age-linked diseases. Aging reduces the functional activities in an individual and hence has received attention in scientific research throughout human history. With the progression of aging several physiologically characteristic changes occur such as infertility, uncoordinated slow movement, gradual shrinkage of muscular tissue and accumulation of lipofuscin [1]. In addition to this, diminished immunity and loss of physiological integrity lead to impaired metabolic efficiency and consequent chronic disease [2]. Although the aging process is irreversible, the pace of aging can be delayed. A recent report indicates that aging has a conserved genetic pathway and hence identifying an effective natural compound that can impinge the pathway would be crucial to reduce the pace of aging process and improve the health indices [3]. Experimental studies have revealed many herbal products which can delay the onset of aging and age-related diseases. One such investigation has shown that the aqueous extract of green tea can prolong the lifespan and efficiently combat oxidative stress [4]. Recently, Zheng *et al.* [5] have also reported that pomegranate juice extracts prolong the lifespan of *C. elegans*. In another study, Anthocyanin-rich purple wheat was found to enhance the lifespan and stress tolerance in *C. elegans* by activating the daf-16/FOXO transcription factor[6].Similarly, improved lifespan and stress resistance has also been observed in *C. elegans* through regulation of daf-16 and sir-2.1 when treated with *Polygonum multiflorum* aqueous extract [7]. These investigations add to the pool of research to identify the particular genes involved in the mechanism of aging to discover potent natural products readily available in our surroundings and which is easy to cultivate, and that may reduce the age-linked adverse effects.

C. elegans is an ideal experimental model in anti-aging research which can be utilized to analyze the mechanisms involved in aging and age-associated diseases. Herndon et al. [8] have emphasized that the short lifespan, rapid reproduction and growth, feasible culture and well-known genetic pathways found in *C. elegans*are the advantages of this organism as a valuable research model. Furthermore, *C. elegans* is

an evolutionarily conserved organism forming a connection between nematodes and mammals including human beings[9]. Additionally, some genetic factors and pathways have also been recognized to regulate lifespan and oxidative stress in *C. elegans*.

Harman [10] proposed the 'Free radical theory of aging' which led to exploring the action of antioxidants to scavenge reactive oxygen species (ROS) and ultimately delay the age-linked changes and enhance longevity. Several studies have revealed the beneficial effects of exogenous antioxidants in combating the events of aging [11-13].On the other hand, some reports have ruled out the role of oxidative stress as the sole cause of aging due to the failure of some antioxidants in regulating the process of aging[14,15].

Heat shock response (HSR) is responsible for control of protein damage and regulation of protease activity upon being exposed to various stressors such as thermal stress [16]. HSR is highly conserved among different species and its regulation is controlled by the transcription factor, heat shock factor 1 (HSF-1). During particular stress condition, heat shock factor 1 (HSF-1) activates the expression of important proteins as well as molecular chaperones which are required for maintenance of proteostasis [17,18].Additionally, Kenyon [19] has indicated daf-16 dependent expression of heat shock protein in *C. elegans* by HSF-1.

Recent reports suggest that innate protective mechanisms of *C. elegans*against aging might be either due to the downregulation of daf-2-age-1-daf-16 insulin signaling pathway or by curbing reactive oxygen species [19-23].

Moreover, reports also suggest that the insulin/IGF signaling (IIS) pathway and the TOR signaling pathway can regulate lifespan in various organisms ranging from nematodes and insect to mammals. Guarente and Kenyon [24] have reported that dietary restriction and mitochondrial respiratory chain reactions can affect lifespan and stress tolerance in *C. elegans*. daf-16 is a forkhead transcription factor, FOXO which acts as an important downstream regulator that integrates various signaling pathways and plays a vital role in aging and longevity[25]. A recent report has indicated that daf-16 is a highly conserved transcription factor in different organisms ranging from flies, worms, rodents to humans and is responsible for regulating metabolism, lifespan and stress tolerance[26]. In addition to this, Li et al. [27]have reported that several gene mutations like daf-2, age-1 and daf-16 can prolong or shorten lifespan and alter stress resistance level in *C. elegans*.

In the present study, we have used BP-HM to evaluate two major aspects of aging;(a) downregulation of insulin signaling pathway (b)scavenging free radicals. Further, due to the mutants of *C. elegans* and comparatively easier knockout approach for different regulators of insulin signaling pathway, *C. elegans* provides the strategy to track the mechanism of action for any drug. Moreover, we have used mutants of daf-2 (DR1572) and daf-16 (TJ356)genes to determine the target point of action of *Bryophyllum pinnatum*in the insulin signaling pathways of *C. elegans*.

Bryophyllum pinnatum(Lam.) Kurz. (Crassulaceae) is a perennial herb growing all over the world and used in folkloric medicine in tropical India, Africa, tropical America, Australia, andChina. *B. pinnatum* (BP) is the source of many herbal products containing wide range of active compounds such as alkaloids, triterpenes, glycosides, flavonoid, steroids, bufadienolides, lipids and organic acids. Moreover, *B. pinnatum* is widely used in traditional medicine to cure a variety of diseases and is a well-known for its haemostatic and anti-coagulating properties. Besides this, *B. pinnatum* has also been found to contain several pharmacological activities such as immunomodulator, central nervous system depressant, analgesic, anti-allergic, anti-microbial, anti-bacterial, anti-viral, anti-fungal, anti-histamine, anti-inflammatory, anti-anaphylactic, anti-ulcer, anti-tumorous, anti-leishmanial, febrifuge, gastroprotective, immunosuppressive, insecticidal and muscle relaxant[28]. However, no studies have being done on such a medicinally rich plant to study its antiaging effect.

Hence, in the current study we have intensively explored the anti-aging activity of BP using *C. elegans* as a model. The study also provides a basis to understand the role of BP in regulation of possible pathways through which BP might be exhibiting its effects in prevention of age-associated outcome and diseases.

MATERIAL AND METHODS

Plant Material

Leaves of *Bryophyllum pinnatum* (BP)were collected from Gujarat University campus. The plant material was washed thoroughly under running tap water and was dried in an oven at 37 °C for one week. The dried plant material was ground to a coarse powder and used for extract preparation.

Extraction

Dried powder (10 g) of *Bryophyllum pinnatum* (BP) was kept for defatting with petroleum ether (100 ml) for 24 hours on a magnetic stirrer. Further, it was extracted with Hydromethanolic (60:40)solvent using

the Soxhlet extraction method until the solvent become colourless. The filtrate was concentrated using rotary evaporator and the crude extract was stored at 4 °C[29].

Strains and growth conditions

All strains were maintained at 20 °C on nematode growth medium (NGM) seeded with *Escherichia coli* OP50 feeding strain. One hundred microliters of OP50 was spotted in the center of the 60-mm NGM plates, which were allowed to dry overnight before the culture assays were carried out. Strains used in this study were: N2 (wild type), daf-16 (TJ356), daf-2 (DR1572). All the strains were obtained from the Caenorhabditis Genetics Center, University of Minnesota, USA.

Stress resistance assay

Stress tolerance of the wild type (N2) worms was determined as described by Wilson etal. [30]with minor modifications. The life cycle of *C.elegans* is comprised of an embryonic stage, four larval stages (L1-L4) and adulthood. Day 0 stands for L4 stage, and the adulthood days are counted consequently as day 1, day 2, day 3, etc[30]. Adult day 2 reflects the worm that is 2 days older to L4 and it is preferred for stress assays. Therefore, adult day 2 worms with and without BP-HM (100 µg/ml) were placed at 37°C for 12 hours for thermal stress and survival of worms was recorded after every two hour by touch provoked method.

Health span Assay

Pharyngeal pumping assay

N2 worms were grown on NGM plates, treated with BP-HM from L1 stage till L4stage. Pharyngeal pumping of the worms was recorded as the movement of the pharynx terminal bulb. The rhythmic pharyngeal contractions per minute were counted to express pharyngeal pumping rate. It was calculated on day1, 5 and 10 post transfer of L4 animals to BP-HM plates. Assay was performed on BP-HM treated and untreated worms[31].

Locomotion assay

Locomotion assay was performed on the 1st, 5thand 10th day of adult stage, respectively by counting the number of body bends observed under a stereo microscope during 30 second interval [32]. A body bend was defined as a change in the reciprocating motion of bending at the mid-body. Assay was performed on BP-HM treated and untreated N2 worms.

Lifespan assay

Age-synchronized worms of N2 wild type, daf-2(DR1572) and daf-16(TJ356) worms were used for the lifespan assay. Hydromethanolic extract of *B.pinnatum*(100 µg/ml) was spotted over the bacterial lawn and the isolated eggs wereallowedto hatch. L4 worms were transferred to fresh plates containing 50 µM 5-fluoro-2'-deoxyuridine (Sigma-Aldrich) which block progeny development. Worms were transferred to fresh plates in every 3–4 days to assure the presence of the extract throughout the experiment. Survival of the worms was scored daily till death.Worms not responding to mechanical stimuli were considered as dead[33].

Statistical analysis

Significant differences between lifespan of treated and control worms under normal/stressed conditions were determined using Kaplan-Meier survival assay in SPSS Version 20. The results are presented as the mean lifespan ± standard error (S.E.). Data other than lifespan were statistically analysed using t-test and ANOVA taking significance at $p<0.05$ level by using GraphPad Prism 8.0 software.

RESULTS

Stress tolerance enhancement by BP in *C. elegans*

To explore the protective role of BP-HM against thermal stress, we assayed heat stress resistance in BP-HM treated and untreated worms. Assay plates were prepared by culturing N2 young adult worms in BP-HM treated (100 µg/ml) and untreated plates for 4 days at 20 °C. For thermo tolerance assay, both BP-HM treated and untreated worms were shifted to 35 °C for 12 hours and numbers of dead worms were scored at every 2 h interval. Worms pre-exposed with 100 µg/ml BP-HM showed significantly higher survival rate compared to untreated worms (Fig.1). Mean survival time of untreated control worms was 62.9±9.29% while BP-HM treated (100 µg/ml) worms exhibited 73.3±8.07% mean survival rate. The mean survival percentage at 35 °C in a time span 72 hours was found to be significantly higher for BP treated (100 µg/ml) worms.

Health span Assay

Pharyngeal pumping assay

To check the efficacy of BP-HM in delaying aging, we have measured alternate physiological markers of aging like pharyngeal pumping rate and rate of worm locomotion(Body bend) in BP-HM treated (100 µg/ml) and untreated condition in the time course of aging (1st, 5th and 10th day post adulthood). The data is represented as Mean± Standard error. The rate of pharyngeal pumping for BP-HM treated animals was

found to be 209.4±6.27, 170.8±6.90 and 61.6±4.62 compared to control animals with respective values of 199.8±5.81, 125.2±4.15 and 36.2±1.63 for 1st, 5th and 10th day respectively (Fig.2).

Locomotion assays

Effect of BP-HM (100 µg/ml) treatment on worm's locomotory behaviour was assayed by counting the number of body bends per minute.

The rate of Body bend for BP-HM treated animals was found to be 115.2±5.51, 105.2±4.25 and 36.6±2.53 compared to control animals with values of 112.8±4.22, 87.4±5.87 and 21.8±2.65 for 1st, 5th and 10th day respectively (Fig. 3).

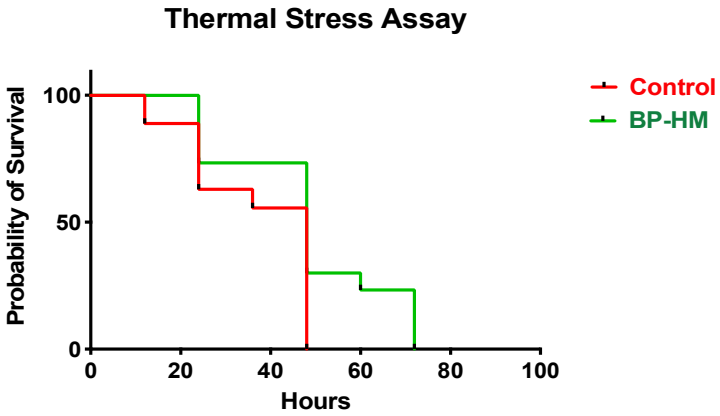
Extension of lifespan by BP-HMon N2 (Wild type), daf-2, and daf-16 mutants

Insulin/insulin-like growth factor signalling (IIS) pathway is a key determinant of aging and lifespan in *C. elegans*[34]. We have investigated the effect of BP-HM on IIS signalling by checking its effect on the downstream (daf-16) as well as upstream (daf-2/age-1) targets of IIS pathway. daf-16 is the chief activator of genes responsible for lifespan extension as well as stress tolerance [23]. Daf-2/age-1 are the phosphorylation-based controllers of daf-16, limiting the nuclear entry of daf-16 by phosphorylation and thus controlling the aging process. To check whether the *Bryophyllum pinnatum* acts via IIS pathway, we have tested the effect of BP-HM on lifespan by using N2 (wild type), daf-2 and daf-16 mutant.

BP-HM extended the mean lifespan of N2 (wild type) treated group-17.5±1.10 when compared to Control-14.91±0.79 days, ($p<0.033$, log-rank test) (Fig.4; Table 1).

BP-HM extended the lifespan irrespective of the absence of daf-16. (Fig. 5: Table.2);Mean lifespan (TJ356): BP-HM treated-10.34±0.85 days, Control-8.28±0.75 days, $p<0.002$, log-rank test (Fig. 5; Table 2).

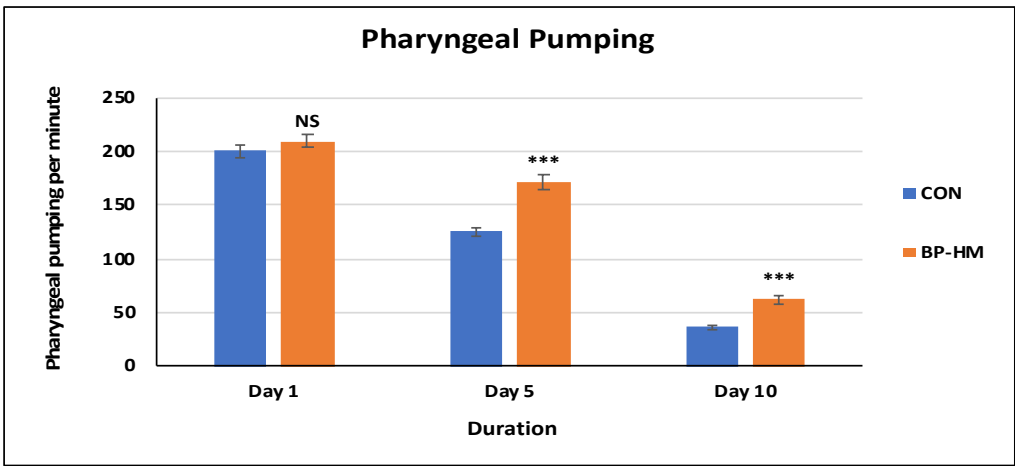
Similarly, BP-HM also extended the lifespan in daf-2 (DR1572) (Fig. 5: Table.3);Mean lifespan (DR1572): BP-HM treated-12.17±1.22 days, Control-11.36±0.94 days, , $p<0.033$, log-rank test(Fig. 6; Table 3).



C-Control; BP-*Bryophyllumpinnatum*

Figure 1 Thermal stress on N2 (Wild type) *C. elegans*

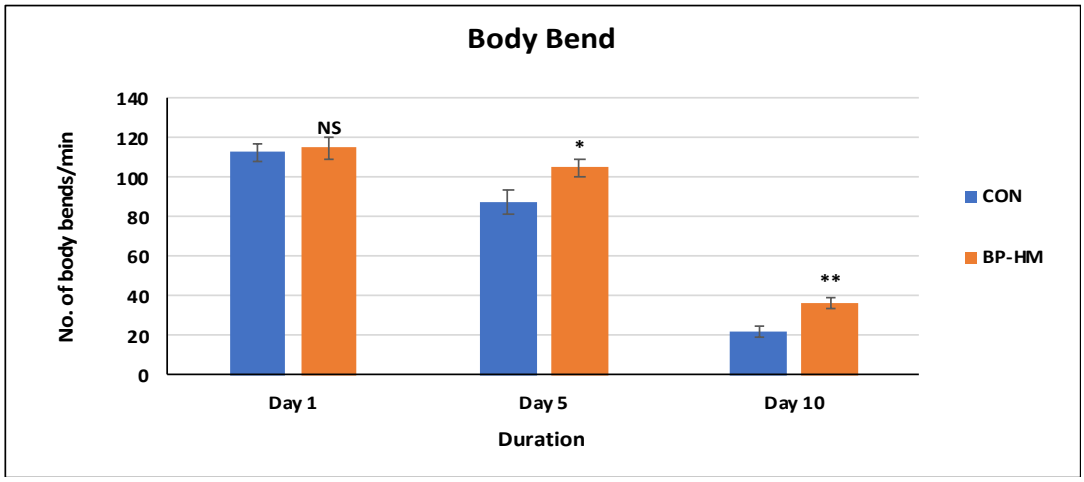
BP-HM treated worms showed significant increased survival time ($p<0.001$) compared to control worms.



CON-Control; BP- *Bryophyllumpinnatum*

Figure 2 Pharyngeal pumping assay on N2 (wild type) *C. elegans*

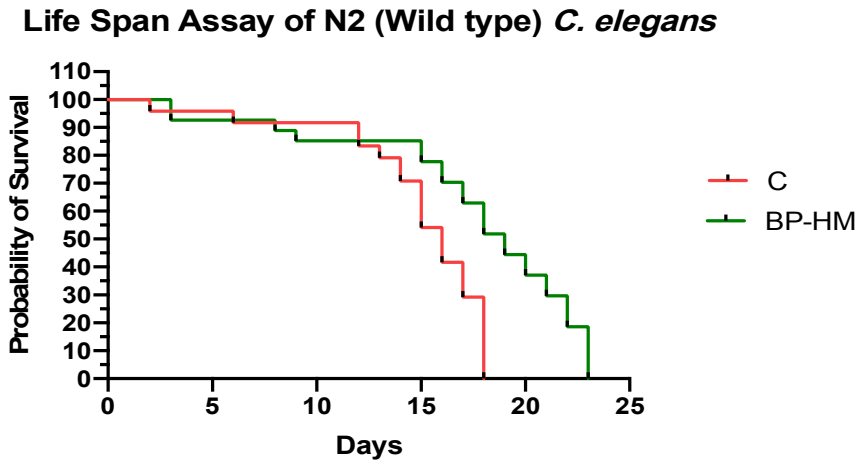
BP-HM treated worms showed significantly increased pharyngeal pumping after 5 days and 10 days ($p<0.001$) when compared to control worms.



CON-Control; BP-*Bryophyllum pinnatum*

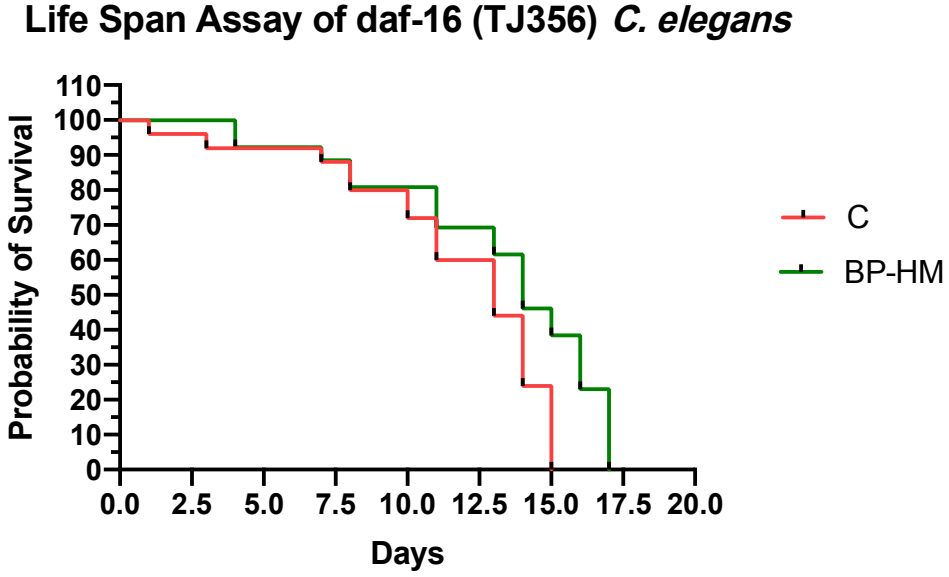
Figure 3 Locomotion assay on N2 (wild type) *C. elegans*

As seen in the figure, the rate of body bend was found to be significantly enhanced after 5 days ($p<0.033$) and 10 days ($p<0.002$) in BP-HM treated worms compared to control worms.



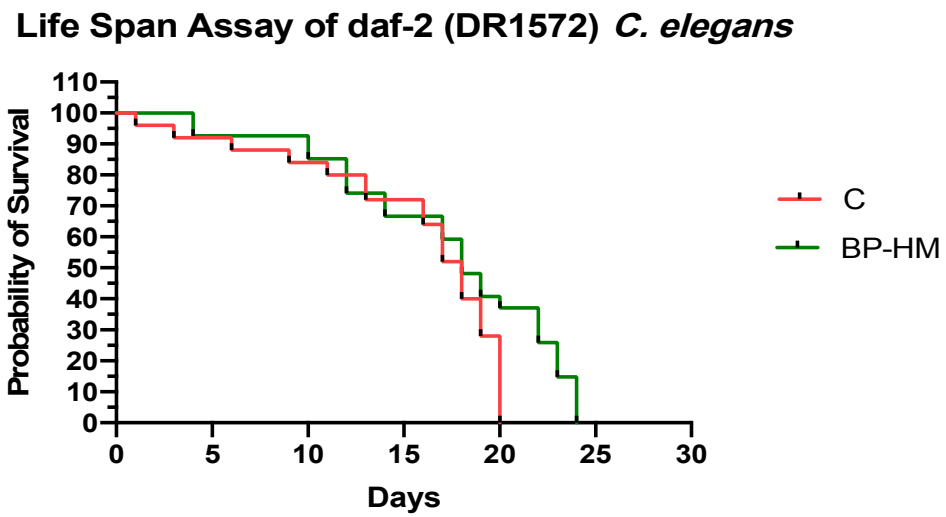
C–Control; BP-HM–*Bryophyllum pinnatum*

Figure 4 Lifespan assay of N2 (Wild type) *C. elegans*



C–Control; BP-HM–*Bryophyllumpinnatum*

Figure 5 Lifespan assay of daf-16 (TJ356) *C. elegans*



C–Control; BP-HM–*Bryophyllum pinnatum*

Figure 6 Lifespan assay of daf-2 (DR1572) *C. elegans*

Table 1 Lifespan assay of N2 (Wild type) *C. elegans*

Mean and Median for Survival Time of N2 (Wild type)								
Group	Mean[a]				Median			
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound			Lower Bound	Upper Bound
C	14.917	0.798	13.352	16.481	16.000	0.690	14.647	17.353
BP-HM	17.556	1.103	15.393	19.718	19.000	1.033	16.976	21.024
Overall	16.716	0.477	15.781	17.650	18.000	0.335	17.344	18.656

a - Estimation is limited to the largest survival time if it is censored

BP-HM treatment significantly extended the mean lifespan of N2 (wildtype) treated group ($p<0.033$) when compared to control (Fig.4; Table.1).

Table 2 Lifespan assay of daf-16 (TJ356) *C. elegans*

Means and Medians for Survival Time of Daf-16 (TJ356)							
Group	Mean[a]				Median		
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval
			Lower Bound	Upper Bound			Lower Bound
C	8.280	0.754	6.802	9.758	8.000	0.999	6.042
BP-HM	10.346	0.859	8.662	12.030	10.000	1.275	7.501
Overall	9.333	0.586	8.185	10.482	9.000	0.893	7.251

a - Estimation is limited to the largest survival time if it is censored

BP-HM treatment revealed significant enhancement in lifespan of daf-16 worms ($p<0.002$) compared to control worms (Fig. 5; Table 2).

Table 3 Lifespan assay of daf-2 (DR1572) *C. elegans*

Mean and Median for Survival Time of DAF-2 (DR1572)							
Group	Mean[a]				Median		
	Estimate	Std. Error	95% Confidence Interval		Estimate	Std. Error	95% Confidence Interval
			Lower Bound	Upper Bound			Lower Bound
C	11.360	0.945	9.508	13.212	12.000	1.489	9.081
BP-HM	12.179	1.226	9.776	14.581	12.000	0.655	10.717
Overall	11.792	0.781	10.262	13.323	12.000	1.031	9.979

a - Estimation is limited to the largest survival time if it is censored

Similar trend of significant increase in lifespan of daf-2 ($p<0.033$) was also noted compared to control worms (Fig. 6; Table 3).

DISCUSSION

Achieving longevity and a healthy life are prime quests of human beings.Globally, one of the major challenges at present is to delay the onset of aging and regulate age-associated diseases. A recent report suggests that a non-genetic, effective approach for anti-aging and health improvement is dietary intervention [35].

C. elegans and mammals have quite similar pattern of aging process and hence *C. elegans* is an important role model to study the aging process and age-related diseases and thus plays major role in discovery of anti-aging drugs. Guha *et al*. [36] have identified the modulation of daf-16 and osr-1 activity in *C. elegans* treated with cranberry.

One of the major causes of aging is reactive oxygen species (ROS) and many antioxidants have been reported to curb the ROS. Free radicals mediated oxidative damage can be controlled by antioxidants and therefore such antioxidants result into enhanced longevity and healthy aging process. Several researches have been undertaken to identify the potential anti-aging drugs. In the current study, we have assessed the efficacy of the extract of a traditionally used medicinal plant *Bryophyllum pinnatum*(BP) in regulating the aging and curtailing age-linked diseases.

Insulin/IGF-1signalling (IIS) pathway, TOR signalling pathway and gonad signalling pathway are considered as the plausible factors responsible for anti-oxidative stress and anti-aging [37].The longevity-regulating pathways in worms, including IIS, DR, TOR signalling, autophagy, ER stress response and mitochondrial respiration pathways have important roles in protein aggregation by modulating cellular proteostasis through cascades of signalling events that eventually control the transcriptional activities of relevant genes[38].

Insulin signalling pathway is the crucial pathway in controlling aging in *C. elegans* which is regulated by transmission of daf-2 insulin receptor through age-1/PI3K to the AKT-1/2 and then to daf-16 transcription factor and ultimately control metabolic processes and the lifespan [39]. Recently, Seo et al.[40] have reported that daf-2 mutants had comparatively longer lifespan and higher oxidative stress resistance. Further, age-1 mutants have also been reported to show an enhanced lifespan depending on the expression of daf-16 transcription factor [41].

Natural products could prove to be novel sources of potentanti-aging drugs. Several studies have documented such natural antioxidative compounds which possess anti-aging potential[42,43]. The results obtained in the present study revealed enhanced longevity and better healthspan in *C. elegans* treated with *Bryophyllum pinnatum* (BP-HM)(100µg) extract. Our study represented the beneficial effect of BP-HM treatment in terms of significant increase in body bend after 5 days ($p<0.033$) and 10 days ($p<0.002$) as well as significant enhancement in pharyngeal pumping after 5 days and 10 days ($p<0.001$) respectively. Body bend and pharyngal pumping are important hallmarks of aging in *C. elegans* and thus indicated the efficacy of BP in improving health span with increasing age.

In this study, we have evaluated the effect of BP-HM(100µg/ml) on two mutants, daf-2 and daf-16 which are regulating the IIS signalling pathway in *C. elegans*. The results obtained in this study revealed significant increase in lifespan of daf-16($p<0.002$) and daf-2 ($p<0.033$) mutants of *C. elegans* after BP-HM treatment. Furthermore, the BP-HM extract manifested a strong antioxidant property *in vitro*[44]and hence, antioxidant activity of BP-HM might also could be the probable factor for lifespan expansion in *C. elegans*. Generally, until the environmental conditions like stress and several ligands cause the stimulation and translocation of daf-16/FOXO from cytoplasm to nucleus, daf-16/FOXO remains inactive inside the cytosol. It has further been demonstrated by Murphy et al. [45] that the translocation of daf-16/FOXO triggers the expression of different genes which contribute to activation of the stress response. The results of the thermal stress assay also support the hypothesis that BP-HM extends lifespan of *C. elegans* by curbing free radicals and preventing their accumulation.

C. elegans and other mammalian species contain highly conserved IIS signalling components and pathway. Anti-aging and anti-stress effects of BP-HM might be due to the inhibition of such conserved mechanisms of aging. daf-16, a transcription factor of FOXO and its upstream components such as daf-2/IGF-1 receptor (IGFR) and age-1/PI3K play a central role in lifespan and stress related responses. In additionto this, results of the present study revealed significant enhancement of lifespan of mutants for daf-16 (TJ356) and daf-2 (DR1572).Our results also corroboratea previous studyby Sonani et al. [46] which stated that phycoerythrin (PE) mediated expression of daf-16, in daf-16::GFP transgenic animal showed non-significant difference in nuclear localization of the daf-16::GFP protein and hence, PE regulated lifespan effects are independent of the daf-2–age-1–daf-16 pathway.

Contrary to this, several reports suggest that hsf-1 is responsible for controlling stress induced gene expression which is an essential factor for life expansion. Recently, it has been demonstrated that hsf-1 activity is moderated by IIS pathway through the formation of multi-molecular complex containing conserved proteins DDL-1 and DDL-2 which causes cytoplasmic sequestration of hsf-1 [47,48]. Moreover,

the phycoerythrin (PE)-mediated effect on longevity was found to be hsf-1 dependent and IIS independent [46]. Similar results have also been observed in our study which showed BP-HM mediated increase in lifespan of the mutants of *C. elegans* which might be due to some parallel alternative activity along with the insulin signalling pathway.

Results of the present investigation revealed that hydromethanolic extract of BP has significantly reduced thermal stress compared to control group which indicates that BP has potential to enhance the thermal stress tolerance due to its antioxidant activity. Hence, BP-HM has revealed its efficacy to increase longevity and healthy aging in *C. elegans*. Thus, the effects demonstrated by BP-HM on longevity and aging in *C. elegans* proved the hypothesis that BP-HM is a potent antioxidant and possess beneficial effect on aging and age-associated diseases.

CONCLUSION

The findings of the present investigation reveal a novel role of *Bryophyllum pinnatum* hydromethanolic extract in lifespan extension and healthspan of *C. elegans*. Our results show that BP supplement not only enhance the lifespan but also promotes a healthy life by delaying aging effects like pharyngeal pumping and locomotion. BP-HM extract also enhances the stress tolerance capacity. Further, this study also demonstrates the probable mechanism of action for signalling pathway through which BP-HM treatment increases longevity and provides stress tolerance. Our result indicates that BP contains a parallel activity with components of conserved IIS pathway and acts even in the absence of factors for IIS pathway to prolong lifespan. However, results of thermal stress indicate that presence of hsf-1 is crucial in mediating anti-aging effects of BP-HM extract. Thus, results of the current investigation exhibit the beneficial role of BP-HM and shed light on the probable mechanism of anti-aging and stress resistance.

CONFLICT OF INTEREST

The authors have no conflicts of interest regarding this investigation**.**

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