Towards the development of phytoextract based healthy ageing cognitive booster formulation, explored through Caenorhabditis elegans model

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Received: 6 August 2022 / Accepted: 5 October 2022 / Published online: 10 November 2022
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Abstract
The positive effect of herbal supplements on aging and age-related disorders has led to the evolution of natural curatives for remedial neurodegenerative diseases in humans. The advancement in aging is exceedingly linked to oxidative stress. Enhanced oxidative stress interrupts health of humans in various ways, necessitating to find stress alleviating herbal resources. Currently, minimal scientifically validated health and cognitive booster resources are available. Therefore, we explored the impact of plant extracts in different combinations on oxidative stress, life span and cognition using the multicellular transgenic humanized C. elegans, and further validated the same in Mus musculus, besides testing their safety and toxicity. In our investigations, the final product—the HACBF (healthy ageing cognitive booster formulation) thus developed was found to reduce major aging biomarkers like lipofuscin, protein carbonyl, lipid levels and enhanced activity of antioxidant enzymes. Further confirmation was done using transgenic worms and RT-PCR. The cognitive boosting activities analyzed in C. elegans and M. musculus model system were found to be at par with donepezil and L-dopa, the two drugs which are commonly used to treat Parkinson’s and Alzheimer’s diseases. In the transgenic C. elegans model system, the HACBF exhibited reduced aggregation of misfolded disease proteins α-synuclein and increased the health of nicotinic acetylcholine receptor, levels of Acetylcholine and Dopamine contents respectively, the major neurotransmitters responsible for memory, language, learning behavior and movement. Molecular studies clearly indicate that HACBF upregulated major genes responsible for healthy aging and cognitive booster activities in C. elegans and as well as in M. musculus. As such, the present herbal product thus developed may be quite useful for healthy aging and cognitive boosting activities, and more so during this covid-19 pandemic.

Keywords Caenorhabditis elegans · Mus musculus · Cymbopogon khasianus · Ocimum tenuiflorum · Camellia sinensis · Phyto-extract · Longevity · Cognition · HACBF (healthy ageing cognitive booster formulation)
Graphical abstract

Introduction
With an exponential increase in elderly population worldwide, the incidences of age-related neurodegenerative diseases especially AD and PD are also growing very fast. These diseases cause progressive devastating cognitive, behavioral, memory and motor dysfunction. In recent years, there has been an incredible interest in research on medicinal plants to find an effective cure for neurodegenerative diseases. Maximum ROS is generated due to human body’s subjection to various kinds of pollutants and toxic materials. The latter contributes a lot to the mitochondrial dysfunctions and is often associated with misfolded proteins such as α-synuclein and amyloid-β [33, 34, 47, 50, 52, 53]. Since aging is the major contributing factor for the occurrence of various kinds of human diseases, it presents a societal burden on the younger generation and financial burden on health care system. In the USA, it has been estimated that reducing the commencement of age-related disorders by 2 years would save $ 7.1 trillion over the next fifty years [17]. During aging, the changes occurring in structure and function of body organs significantly reduces the metabolic rate, motor activity and cellular damage. For achieving healthy lifespan, the focal point is to discuss the biochemical and cellular changes happening during aging [10, 19, 32]. However, it is not feasible to observe age dependent changes in higher organisms and hence the multicellular organism C. elegans has proved to be one of the valuable organisms for developing healthy aging and cognitive booster resources for elderly as well as younger population [43, 45]. In view of the aforementioned factors about aging and cognition, it was planned to investigate the prospect of plant extracts of selected plant species for their antioxidant, stress tolerance, antiaging and cognitive booster activities, especially against Parkinson and Alzheimer’s by observing various indicative biomarkers in Caenorhabditis elegans.

Materials and methods
In the present investigation the major approach has been to study the prospect of various medicinal plant extracts (prepared as per AYUSH guidelines of the Government of
India) on healthy aging and cognition using multicellular model *C. elegans*. Further some experiments related to safety, toxicity, cognition and inflammation have also been performed involving *Mus musculus*. Beginning with initial screening of combination of twelve prospective plants reported in traditional literature for health promoting properties, we subsequently pinned down to test and examine the combinations of 3 plants viz. leaves of cv Suvarna of *Cymbopogon khasianus* (Hack.) Stapf ex Bor, herb of cv CIM-Agna of *Ocimum tenuiflorum* L. and leaves of *Camellia sinensis* (L.) Kuntze, in different ratios.

**Heavy metal analysis of plant extract**

For metal analysis, after overnight pre-digestion, acidified samples were digested in a microwave digester (Anton Paar Multiwave Pro oven) at 120 °C for 41 min. The samples were filtered. The metal in digested samples was determined by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Perkin Elmer Optima 5300 V).

**Maintenance and culture of *C. elegans* strains**

Various *C. elegans* strains were grown on nematode growth medium (NGM) and spotted with *E. coli* OP50 as a food source [9]. *E. coli* OP50 is an uracil auxotroph strain, used to limit the growth of bacteria on NGM. Nutrient food for bacteria i.e., minimal essential medium (MEM) was prepared by using standard protocol [49]. All the strains of *C. elegans* utilized in the current study were made available by Caenorhabditis Genetic Center (CGC), University of Minnesota (www.cgc.cbs.umn.edu). Sigma and Merck companies provided the chemicals used in the present study. The worm strains utilized in the present study were wild type Bristol N2, transgenic strain NL5901 (Punc-54::α-synuclein::YFP + unc-119), CL2006 (unc::54, Aβ1-42), CL4176 {dvIs27 [myo-3/Aβ 1–42 minigene + rol-6(su1006)]}, CF1553 (sod-3::gfp), CL2166 (gst-4::gfp).

**Embryo isolation to obtain synchronized population of *C. elegans***

Alkaline hypochlorite treatment method has been primarily used to produce age synchronized population of the worms for the different experiments [16] conducted in this study.

**Toxicity assay in *C. elegans***

Toxicity determination assays are imperative as only non-toxic extracts can be used for the various experiments, including aging and cognition. Acute toxicity assay was carried out using 1–5 mg/ml concentration in *C. elegans*. The worms were synchronized using the standard protocol [31]. The synchronized L1 worms were put into fresh NGM plates and the worms were grown upto L4 stage at 20 °C. Different concentrations (1–5 mg/ml) of HABCF were prepared in double distilled water and 20 worms were transferred to 24 well plates containing relevant concentration of HABCF in each of the test well. The assay was performed in triplicate. Touch provoked movement method was used to observe the survival of the worms [24]. The toxicity was analyzed using the formula (live worms/ total number of worms) × 100. SPSS software has been used to calculate the statistical significance using one-way ANOVA, followed by Scheffe and Dunnett tests.

**Toxicity assay in Swiss albino mice**

Acute and sub-acute oral toxicity of HABCF was carried out in Swiss albino mice for defining the safety limit in the rodent model for its further development. The experiment was carried out in accordance with the approved protocol and ethical guidelines of the institute.

**Acute oral toxicity**

Acute oral toxicity was carried out following the methodologies published earlier (11). HABCF was suspended in double distilled water (DDW) and was given orally at 5, 50, 300, 1000 and 2000 mg/kg body weight as a single oral dose. Control animals received only vehicle.

**Sub-acute oral toxicity**

In the sub-acute oral toxicity of HABCF, fractional doses of maximum tolerated doses from acute oral toxicity (2000 mg/kg) was considered and the animals were treated with the HABCF at 0.2, 2, 20 and 200 mg/kg once orally for 28 days following the methodologies previously published by us [11].

**Observational, hematological, biochemical and gross pathological study**

The animals were checked for mortality and morbidity at hourly interval on the day of administration of HABCF and a daily general examination of the animals was carried out.
for changes in skin, mucous membrane, eyes, occurrence of secretion and excretion and also responses like lachrymation, piloerection respiratory patterns etc. Further changes in gait, posture and response to handling were examined [5]. Body weights were recorded and blood samples were collected from all the animals on 7th day of the experiment in acute oral toxicity and were examined for serum biochemistry and hematology. Further, in the sub-acute oral toxicity experiment, animals were also examined for the observational changes as well as hematological and biochemical changes as stated in acute oral toxicity and blood samples were collected on 28th day of the experiment and analyzed for hematological and biochemical changes. The experimental animals were sacrificed at the end of experiment (7th day in acute toxicity and 28th day in sub acute toxicity) and vital organs were collected, and necropsied for any gross pathological changes. Weights of vital organs like liver, heart, kidney etc. were recorded [11]. Liver and Kidney samples were collected from animals of sub-acute oral toxicity and fixed in 10% buffered formalin and processed for histomorphological analysis [46].

Impact of HABCF treatment against carrageenan-induced paw edema inflammation in rats

In order to start an acute inflammation, 100 μl of saline containing 1% w/v carrageenan was injected into the sub-plantar injection of the left hind footpad of Charles Foster rats (4 rats/group). The same amount of vehicle was administered to the rats in the vehicle-treated group at the same time. A water Plethysmometer was used to calculate the paw volumes. Each left footpad’s volume was measured before to the carrageenan injection, and three hours later, the swelling of the footpads was measured. The HABCF (50,100, and 200 mg/kg) was given orally to the rats for three days in a row before the injection of carrageenan, and Diclofenac (15 mg/kg), a standard anti-inflammatory medicine, was given 30 min before the injection of carrageenan to examine the effect of the HACBF administered orally. After 3 h of carrageenan injection, the sub-plantar region of the left hind paw showed the highest inflammation. The percentage inhibition of edema was calculated as follows:

\[
\text{Percent inhibition of edema} = \left( \frac{V_o - V_t}{V_o} \right) \times 100
\]

where \( V_o \) = Volume (ml) of paw in vehicle treated rats (Carrageenan + Vehicle), \( V_t \) = Volume (ml) of paw in treatment group rats (Carrageenan + Treatment).

In vitro antioxidant assay

To make the stock solution test plant extracts and HABCF were suspended in sterilized DW (distilled water). The stock solution was diluted to prepare for required concentrations for antioxidant assays. The reagents used in the assay were prepared freshly before experiment. To study the various antioxidants the stable free radical, 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) (purple color, \( \lambda_{\text{max}} \) 515–517 nm) was used [55]. According to previous studies alteration in the absorbance of DPPH after adding test compound is used as an index of the antioxidant capacity of the compound. The antioxidant activity of the HABCF was quantified according to the previously known method by DPPH assay [1]. Briefly, 0.5 mM of DPPH (freshly prepared) and 0.1 M Tris buffer pH (7.4) were added to 100 μl of the sample (1.5 ml eppendorf tube) at various concentrations (1–10 mg/ml) of HABCF. The reaction combination was incubated in dark for 45 min at 37 °C temperature in an incubator followed by spectrophotometric quantification at 517 nm against control. The scavenging activity was computed using the following equation [57]

\[
\text{Inhibition\%} = \frac{A_c - A_t}{A_c} \times 100
\]

where \( A_c \) = absorbance of control; \( A_t \) = absorbance of the sample.

Stress resistance assays in *C. elegans*

To evaluate the effect of resource extract for oxidative stress assay in worms, juglone (5-Hydroxy-1, 4-naphthoquinone, Sigma-Aldrich), an intracellular ROS generator was applied. The age synchronized N2 wild type worms were raised from L4 to adult stage in presence/ absence of different doses of HABCF. The treated and untreated day 4 adult *C. elegans* was exposed to 24-well plates containing liquid NGM. Further, juglone (250 μM) was used in a total volume of 300 μl per well for inducing oxidative stress. The worms were placed in an incubator at 20 °C and scored for their viability after 24 h of continuous exposure [51].

Assessment of reactive oxygen species (ROS) by H\textsubscript{2}DCF-DA method in *C. elegans*

The established fluorescent H\textsubscript{2}DCF-DA (Molecular Probes, U.S.A) (2′,7′-dichlorodihydrofluorescein–diacetate) probe was utilized for determination of in vivo ROS level in worms. Following 72 h of different concentrations of formulation treated and control, day 4 wild-type worms were washed thrice using M9 buffer to wash off the extra *E. coli* OP50 and any residual extract. Then the washed worms were suspended in 1.5 ml micro-centrifuge tubes filled
with 300 μl of 0.1% PBST buffer (PBS with Tween 20). Approximately, 50 *C. elegans* were shifted into Corning® 96 Well Black Flat Bottom Polystyrene NBS™ Micro plate, and 1.5 μl of 10 mM H₂DCF-DA dye was dropped to each well before taking observation. Spectra Max M2 multimode micro plate reader, (Molecular Devices) was used to quantify the fluorescence at 485 nm excitation and 530 nm emission. The change in fluorescence was recorded for 120 min at 20 min intervals at 37 °C [48]. The assays were performed in triplicate. GraphPad Prism software was used to calculate the statistical significance using independent t-test.

**Lifespan assay in *C. elegans***

Exposure of same age L4 worms was done to varied concentrations of HABCF at 20 °C on NGM plates spotted with *E. coli*. 50 μM 5-Fluoro-2′deoxyuridine (FUdR) and was mixed to the NGM plates for inhibiting growth of the progeny. For avoiding contamination, the worms were moved to new OP50 seeded NGM plates every 3–4 days. The survival was assessed each day until the death of the last worm. The experiments were replicated thrice and the results showed as the mean life span per trial.

**Lipofuscin assay in *C. elegans***

The wild type N2 worms were raised from L4 larvae as in the lifespan assays and on 5th day of adulthood, fluorescent imaging was performed for lipofuscin and other GFP specific strains treated with an effective dose (4 mg/ml) of HABCF. The intestinal autofluorescence of lipofuscin and GFP fluorescence was measured in randomly selected worms (n = 30) from each set of experiments. Worms were mounted onto microscope slides coated with 3% agarose pads and 2% sodium azide was added to anesthetize for visualization of fluorescence. Images were captured with a fluorescence microscope (Leica, DMI3000); levels were quantified by determining the average pixel intensity in each worm using Image-J software (NIH).

**Estimation of protein carbonyl in *C. elegans***

For sample preparation age synchronized embryos were added to treatment plates and incubated at 22 °C for 48 h. After the 48-h incubation period, the worms were washed thrice using M9 buffer and sonicated in cell lysis buffer for 3 min at 30 percent amplitude with pulse on/off for 2 s. The sonicated worms were centrifuged for 7000 rpm for 7 min at 4 °C to remove cell debris. Supernatant was collected and stored at temperature -20 °C and protein estimation was done using Bradford method [8].

**Staining of worms with Nile red for the assessment of lipid level**

The effect of HABCF treatment on lipid levels of worms was measured using Nile red (a fluorescent dye used to stain intracellular lipid droplets) staining [6]. A 5 mg/ml stock solution of Nile red (9-diethylamino-5-benzoxazine) was prepared in acetone, further diluted with *E. coli* OP50 in a ratio of 1:250 and spotted onto NGM plates along with or without treatment. Thereafter, age synchronized L1 worms were transferred to treatment plates and incubated at 20 °C. After 72 h, the worms were washed off the plates using M9 buffer. Worms were anaesthetized using 100 mM sodium azide, mounted onto slides and were observed using rhodamine filter. The fluorescence intensity was calculated semi-quantitatively using Image J.

**GFP reporter assay in *C. elegans***

The age synchronized day 2 adult transgenic worms carrying inducible green fluorescence protein (GFP) specific strains were treated with an effective dose of HABCF (4 mg/ml). Reporter transgene viz sod-3 (CF1553), gst-4 (CL2166), were treated with HABCF and control in these two strains. The GFP fluorescence was captured using randomly selected 20–30 adult worms mounted on 3% agarose pads and anaesthetized by 2% sodium azide [7]. GFP expression was quantified at the site of its expression which varied from strain to strain. Images were captured using a fluorescence microscope (Leica, DMI3000) in GFP filter (with excitation 365 nm and emission 420 nm) at the 20 × objective [38]. Quantification of data was performed by using Image J software. The experiment was done thrice.

**Aldicarb assay in *C. elegans***

Aldicarb sensitivity assay is an indirect assay to check the relative effect on neurotransmission. Aldicarb is a carbamate insecticide which acts as an acetylcholinesterase inhibitor. Aldicarb assay was performed as per previously described protocol [26].

**Levamisole assay in *C. elegans***

Levamisole is an anti-helminthic compound which works as a nicotinic acetylcholine receptor agonist that causes continued stimulation of the parasitic worm muscles, leading to paralysis. Levamisole assay was done as per described method [36] with slight modifications.
The dopamine levels in worms were studied by an indirect repulsion assay through 1-nonanol. Worms raised from the L1 stage along with HABCF (4 mg/ml) treated/control worms were washed thrice at day 5 by using M9 buffer. The washed nematodes were exposed to 1-nonanol by placing a drop of 1-nonanol by using poking lashes near head region. The alteration in repulsion time was observed in both wild type and diseased worms and data was plotted against repulsion time ± SEM. The experiment was performed in three biological replicates.

Body bend assay

To investigate the health of motor neurons, body bend assay was performed. Treated with HABCF (4 mg/ml) and control hermaphrodite day 5 worms were used to study the neural behavior. Approximately 10 worms were transferred in a drop of M9 buffer on a glass slide and the body movement was observed (S-shaped curved movement represent 1 bend) for 20 s. The data of HABCF/Control was plotted against total bends per 20 s. The experiment was performed in three biological replicates.

Head thrash assay

For the examination of locomotion and movement alteration in worms, head thrash assay was performed. HABCF (4 mg/ml) treated and control worms were transferred (n = 10) on a glass side with 20 µl M9, observed and the to and fro motion in the head region of worms was recorded one by one for 20 s. The data was analyzed and compared for the movement of treated/control worms by using GraphPad Prism software. The experiment was repeated thrice independently.

α-Synuclein inhibition assay

This experiment was conducted to study the impact of HABCF on the aggregation of α-synuclein protein under a fluorescence microscope. For the quantification of α-syn aggregation levels, transgenic strain NL5901 (Punc-54::α-synuclein:: yellow fluorescence units (YFP); unc-119) was used by previously described method [21, 54]. Briefly, the synchronized eggs were transferred on different concentrations of HABCF (1, 2, 3, 4, 5 mg/ml) and control plates and incubated at 20 °C till the worms reached to adult day 5 stage. Randomly picked healthy day 5 adult hermaphrodite worms (n = 30) were placed on glass slides having 20 µl M9 along with 4 µl of 100 mM NaN3 and mounted under the cover slip. For the observation of α-syn aggregation, images were captured under fluorescence microscope with excitation at 488 nm and emission at 530 nm at 20×. The fluorescence intensity was calculated semi-quantitatively using Image J and represented in terms of normalized values of Corrected Total Cell Fluorescence (CTCF) [CTCF = Integrated Density – (Area of selected cell×Mean fluorescence of background readings)].

RNA isolation

Total RNA was extracted from adult worms using RNAzol reagent (Molecular Research Centre) according to the manufacturer’s protocol.

cDNA synthesis

cDNA synthesis was carried out from 1 µg of total C. elegans RNA in a 96 well thermal cycler using High-Capacity cDNA synthesis Kit (Thermo Scientific) according to manufacturer’s protocol. cDNA samples were quantified using nanodrop spectrophotometer and stored at -80 °C.

Quantitative real time PCR

qRT-PCR studies were done using SYBR Green (Puregene, Genetics Asia, catalogue no. Pgk022) technology. Briefly, 10 µl total reaction mixture was prepared by mixing cDNA template (125 ng), RNAase free water, forward and reverse primer and SYBR green dye. gpd-1 was used as internal control. Applied Biosystems 79,000 HT was used to perform the RT-PCR studies using the program of pre incubation cycle of 50 °C for 2 min and 95 °C for 10 min followed by 35 amplification cycles for 15 s and 58 °C for 30 s and final extension at 72 °C for 20 s. Relative expressions were calculated by 2-ΔΔCT method. Primers were procured from Integrated DNA technologies.

Statistical analysis

Statistical analysis and graphical representation of data were carried out using Graph Pad Prism Version 5. Analysis of variance and independent t-test was used to calculate statistical significance wherever applicable. For survival assays long rank analysis were performed using Kaplan–Meier survival analysis. The level of statistical significance was ascribed at \( p < 0.05 \).

Results and discussion

Qualitative analysis of plant extracts

The analysis of extract was done for examining the occurrence of active molecules and purity of plant extracts. Blank and certified reference material were included in
each batch of samples for quality control. Diverse class of secondary metabolites viz. caffeic acid, rutin, and rosmarinic acid in *Ocimum tenuiflorum* cv Agna; citral, gallic acid, quercetin, and syringic acids in *C khasianus* cv Suvarna; and catechins epigallocatechin gallate, epigallocatechin, epicatechingallate, epicatechin, catechin) and caffeine in *C. sinensis* are reported in the ingredient herbs of the present decoction formulations. The content of key phytochemicals viz. caffeic acid (7.20 mg/100 g), rosmarinic acid (30.22 mg/100 g), quercetin (0.25 mg/100 g), epicatechin (21.33 mg/100 g), catechin (1.70 mg/100 g) were quantified in the decoction formulations using external standard HPLC method (Fig. 1).

**Heavy metal analysis of plant extract**

The concentrations of heavy metals (mg/kg dry weight) were found as follows: Al 0.5, Ca 0.2, Cd 0.027, Cu 4.125, Fe 0.7, Mg 0.775, Mn 1.0, Zn 0.15, which are below the permissible limit. The concentrations of Cr, Co, Pb and Ni were not detected in HABCF. The concentrations of all other metals in the HABCF samples were within accepted limits set for herbal extract according to international regulatory bodies (Fig. 2).

**Genotoxicity analysis of plant extract**

During the experimentation no genotoxicity has been detected in the HABCF up to 10 mg/ml concentration (full data is Supplementary Table 1).

**HACBF is devoid of toxic effects at lower doses**

The HACBF at 1, 2, 3, 4 mg/ml was found non-toxic whereas at 5 mg/ml showed significant toxicity (Fig. 3). Therefore 1, 2, 3, 4 mg/ml were selected as experimental concentrations for further studies. *C.elegans* were exposed to 0.05% DMSO that served as a vehicle control. The experiment was independently replicated thrice.

No observable toxicity was recorded throughout the experimental period in all the groups of animals up to the tested dose levels of 2000 mg/kg of HACBF including control in acute oral toxicity and up to 200 mg/kg in sub acute oral toxicity. Haematology and serum biochemistry showed non-significant changes in all the
haematological and biochemical parameters like haemoglobin level, total RBC, WBC count, differential leucocyte counts, ALP, SGPT, SGOT, total cholesterol, triglycerides, creatinine, bilirubin, albumin and serum protein levels (Supplementary Table 2 and Fig. 4a) except significant increase in serum albumin level in animals treated with HACBF at 1000 and 2000 mg/kg. Similarly, animals on gross pathological study showed no significant changes in any of the organ weight pertaining to their absolute and relative weight (Fig. 4b, c) in acute oral toxicity. Similarly, the lyophilized HACBF in sub-acute oral toxicity at 0.2, 2, 20 and 200 mg/kg once daily for 28 days did not produce any observational changes, morbidity or mortality during the experimental period. Haematological, biochemical and gross pathological studies also showed nonsignificant changes among all the doses studied (Supplementary Table 3 and Fig. 4d–f). Liver and kidney tissues from the animals of all groups in the sub-acute oral toxicity experiment were saved for histopathological studies. The sections of liver and kidney from all the groups including control showed no observable changes and are presented in Supplementary Fig. 1. The present acute and sub-acute oral toxicity study in Swiss albino mice suggests that HACBF is well tolerated by the experimental mice up to 2000 mg/kg in acute oral toxicity and up to 200 mg/kg body weight in sub-acute oral toxicity when dosed daily for 28 days.
Anti-inflammatory profile of HACBF against carrageenan-induced paw edema inflammation in rats

Oral treatment of HACBF (200 mg/kg) has shown significant inhibition of carrageenan-induced paw edema inflammation in rats when compared to vehicle-treated rats (Fig. 5). The persistent rise in inflammatory marker concentration in the blood is a characteristic of aging, which has become a major public health problem with a large socioeconomic component globally. Chronic low-grade inflammation is regarded to be a major contributor to many age-related diseases.

**HACBF possesses in vitro free radical scavenging activity**

Aggregation of free radicals is associated with many health problems. Reactive oxygen species (ROS) play a key part in various biological manifestations [10, 14]. Antioxidants are the defense machinery of a cellular system to protect them by scavenging the ROS. Plant-derived active molecules are rich antioxidants that are able to scavenge the free radicals [32, 45]. The antioxidant potential of HACBF was examined by colorimetric method using DPPH as a

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**Fig. 4** Effect of lyophilized extract powder as a single acute oral dose at 5, 50, 300, 1000 and 2000 mg/kg body weight in Swiss albino mice. a differential leucocytes counts, b absolute organ weight, c relative organ weight.; Effect of lyophilized extract powder at 0.2, 2, 20 and 200 mg/kg body weight once orally for 28 days in Swiss albino mice. d Differential leucocytes count, e absolute organ weight, f relative organ weight (Mean ± SE, n = 6)

**Fig. 5** Anti-Inflammatory profile of HACBF against carrageenan-induced acute inflammation in rats

**Anti-inflammatory profile of HACBF against carrageenan-induced paw edema inflammation in rats**

Oral treatment of HACBF (200 mg/kg) has shown...
colouring reagent. Different concentrations of HACBF ranging from 1 to 10 mg/ml were selected for the assay. The results exhibited a decrease in absorbance of DPPH in a dose dependent manner with the increasing concentration of HACBF (Fig. 6). This result suggested that HACBF possesses potent free radical scavenging effect on DPPH. Therefore, the results revealed that HACBF possess a significant antioxidant potential.

**Assessment of reactive oxygen species (ROS) by H$_2$DCF-DA and juglone assay**

ROS is generally produced by various pollutants especially heavy metal ions, radiation, UV etc. During studies it was found that ROS play a major role in the intracellular signaling process [28, 35, 53]. The increased oxidative stress level with raise in intracellular ROS result in cellular damage.

Fig. 6 In vitro antioxidant activity of HACBF

Fig. 7 Lifespan studies in C. elegans. a DCF-DA test in wild-type C. elegans. b Pre-treatment with different doses of HACBF exerts oxidative stress reduction in C. elegans. c Mean lifespan of C. elegans affected with HACBF d Survival curves of animals control and treated with different doses (HACBF treated worms at1mg/ml, 2 mg/ml, 4 mg/ml) of HACBF. e 4 mg/ml HACBF impacted average lifespan by 27%, f HACBF significantly reduced the protein carbonyl level as compared to control.
which has a significant part in the pathology of several elderly diseases [58]. The additional retention of ROS inside the cells can be related to the toxicity in wild-type worms during thermal threat. Therefore, to evaluate the correlation between HACBF mediated enhanced survival and intracellular ROS level on the wild-type C. elegans at the 2nd day of lifespan, the intracellular ROS level was quantified employing DCF-DA method using live worms. The pre-treatment with different doses of HACBF reduced in vivo ROS level (Fig. 7a), indicating HACBF might postpone ROS production during the aging process and subsequently a decline in intracellular ROS could lead to extension in lifespan. Maximum reduction of ROS was observed at 4 mg/ml concentration. Similarly, maximum survivality was observed in juglone assay at different doses of HACBF (Fig. 7d). Present studies indicate that HACBF brought tolerance in C. elegans against oxidative and this might have resulted from its ROS scavenging activity.

HACBF prominently increased the longevity

According to previous reports, activation of stress response in cells and tissues is mandatory for survival of multicellular life forms. In this group of stress associated responses, stress resistance is related to various types of interferences that enhance life expectancy [29]. In the present experimentation, it was observed that HACBF treatment with different doses enhances life span in C. elegans and maximum enhancement was observed at 4 mg/ml followed by other doses. The longevity enhancement capability of HACBF in wild type N2 was investigated. The wild type N2 population was given treatment of various doses (0, 1, 2, and 4 mg/ml) of HACBF at the early embryonic stage on NGM plates. Further, we noticed a marked surge in mean lifespan of HACBF treated worms at 1 mg/ml (16.30 ± 0.42, \( p \leq 0.01 \)), 2 mg/ml (16.72 ± 0.51, \( p \leq 0.001 \)), and 4 mg/ml (19.06 ± 0.57, \( p \leq 0.001 \)), as compared with control (14.95 ± 0.36) (Fig. 7c–e). The utmost addition in average lifespan of 27% was displayed by 4 mg/ml concentration (\( p < 0.001 \), Fig. 7c, e). For the first time the present study reveals the competence of HACBF to modify the normal lifespan of wild-type C. elegans (Bristol N2). In the present experiment it was noticed that reductions in protein carbonyl, lipofuscin and lipid levels are directly related to the longevity of worms.

HACBF increases mitochondrial viability and protein carbonyl content in C. elegans

Aging is a progressive multifactorial biological process and mitochondria play a central role in it. The mitochondrial dysfunction in aggregation with altered mitochondrial dynamics drives aging. The low levels of ROS trigger a stress response and biogenesis pathways that increase the mitochondrial dynamics which is protective and prevent aging [23]. However, the increased free radicals can break down the cellular components like DNA, RNA, protein or lipids, which may also contribute to aging. The wild-type worms were treated with HACBF (4 mg/ml) for 2 days and then mitochondrial health was quantified using a MitoTracker. The viability of mitochondria was found significantly increased in HACBF treated worms (4 mg/ml), compared to control (Fig. 8a–c). The accumulation of protein carbonyl and lipofuscin contents are the result of oxidative and cellular damage and it increases with age in C. elegans [2]. The results showed significant reduction in protein carbonyl content in the HACBF treated (4 mg/ml) wild-type worms, compared to control (Fig. 7f). These findings suggest that dietary supplementation of HACBF notably attenuate the oxidative stress in worms by the augmentation of mitochondrial viability, and decreasing the levels of protein carbonyl content.

The amount of endogenous lipofuscin is an important biomarker of aging in multicellular organisms. This auto fluorescent protein accumulates with the passage of time in cells and tissues with a declined turnover. This auto
fluorescent age pigment fluoresces yellow to red wavelength when excited with UV or blue light [18]. It is generated during aging process and its contents are reduced through usage of phytomolecules [13]. Therefore, the effect of the most effective dose of HACBF (4 mg/ml) was evaluated by observing pre-treated HACBF day 4 worms to examine the lipofuscin levels in the intestines. The HACBF supplementation was able to reduce intestinal lipofuscin accumulation in worms by 34% (p < 0.01**) in comparison to untreated control worms where a high level of lipofuscin build-up was observed (Fig. 9d–f). These results suggest HACBF extends lifespan by reducing oxidative stress level and stress-mediated macromolecular damage. The decline in age pigment lipofuscin is consistent with DR like effect mediated by HACBF treatment. The decline in intestinal lipofuscin autofluorescent suggests a reduction in oxidative stress and delay in aging in C. elegans. The result indicates that aging progression is directly proportional to lipofuscin accumulation. HACBF delayed aging process may be due to its inherent antioxidative property.

HACBF decreases lipid levels in C. elegans

Fat accumulation is a biomarker of aging and is associated with higher ROS levels [27, 37, 40, 41]. The fat storage is also found to alter with the energy expenditure and diet [30]. Here, we used Nile red staining methods to probable the effect of HACBF on the fat accumulation in wild type-N2 worms. The progression in age is associated with higher accumulation of triglycerides which is correlated with age related diseases like obesity, cardiovascular diseases and cancer [4, 20, 30]. The C. elegans fats are majorly stored as triglycerides and it is found to deplete in case of DR [3]. The HACBF treated worms were found to have lower fat levels significantly by 54% (Fig. 9a–c). This result indicates that HACBF can be a useful remedy against increased lipid peroxidation, which is the major pathological hallmark behind age-associated neurodegenerative disorders.

Effect of HACBF on expression of SOD-3∷GFP and GST-4∷GFP in transgenic strain of C. elegans

The cellular signaling pathways are associated with lifespan and cellular stress levels [13, 39, 42, 56]. The cellular antioxidant defence system counteracts this process by detoxifying ROS [15]. Superoxide dismutase (SOD-3), is vital for oxidative stress resistance in worms [12, 22] and binding domain of DAF-16 is located in a transcriptional promoter region of SOD-3, and expression of SOD-3 depends on DAF-16 activity [25]. Therefore, the effect of HACBF exposure on expression of antioxidant enzyme encoding stress response gene SOD-3 and GST-4 was evaluated in transgenic strains CF1553 (SOD-3∷GFP) and CL2166 (GST-4∷GFP). In the present experiment 4 mg/ml HACBF displayed higher SOD-3∷GFP and GST-4∷GFP expression as compared to control (Fig. 10a–f). The results indicated that the HACBF at 4 mg/ml may considerably enhance SOD-3∷GFP expression by 67% in transgenic strain CF1553 of

Fig. 9 Effect of HACBF on fat accumulation and relative lipofuscin levels. The age synchronized wildtype worms were grown till their adulthood with or without HACBF treatment (4 mg/ml). a Microphotograph untreated control worms, b microphotograph of day 2 HACBF (4 mg/ml) treated worms. c HACBF (4 mg/ml) supplementation reduced the fat content. d Relative lipofuscin content in untreated control worms, e Lipofuscin content in HACBF (4 mg/ml) treated worms. f HACBF (4 mg/ml) supplementation reduced lipofuscin level in treated worms with respect to untreated control worms.
C. elegans (Fig. 10a–c). In addition, the effect of HACBF on GST-4 expression was studied because it is a major phase II detoxification enzyme, which is regulated by SKN-1 in response to oxidative stress. Plant extract (4 mg/ml) boosted the expression of GST-4::GFP significantly by 96% over the control (Fig. 10d–f). Antioxidant genes viz. sod-3, gst-4 genes were also upregulated in treated worms as compared to untreated control wild-type N2 worms (Fig. 13a). Such genes are directly controlled by DAF-16, which behave as stress-sensitive reporters to predict longevity in C. elegans [44]. It is suggested that up-regulation of SOD-3 and GST-4 and decline in oxidative stress level could be responsible for HACBF mediated life span extension and stress tolerance in worms. These results are consistent with previous studies where the decline in oxidative stress level and elevated level of antioxidant gene promoted mean lifespan in an organism [32, 25].

**Impact of HACBF on synaptic acetylcholine availability**

To study the effect of HACBF on synaptic ACh availability, aldicarb assay was performed with different doses i.e., 1 mg/ml, 2 mg/ml, 3 mg/ml, 4 mg/ml and 5 mg/ml. In the present study it was observed that increase in percentage of worms paralyzed was found to be dose dependent. Treatment with 1 mg/ml HACBF was 54.33 ± 4.41 (p ≤ 0.001) followed by 2 mg/ml HACBF 67.33 ± 2.84 (p ≤ 0.001), 3 mg/ml HACBF 87.81 ± 2.08 (p ≤ 0.001), 4 mg/ml HACBF 89.45 ± 1.85 (p ≤ 0.001), and 5 mg/ml HACBF 50.33 ± 1.4 (p ≤ 0.001) as compared to that of control 47.33 ± 1.33. The results suggested that HACBF increased synaptic Ach content (Fig. 11a).

**Surge in the activity of nicotinic acetylcholine receptor through HACBF**

In addition to synaptic Ach level enhancement, the involvement of nicotinic acetylcholine receptor (nAChR) in HACBF mediated elevation of cholinergic transmission, using levamisole assay was also ascertained. nAChR responsiveness is proved by the percentage of paralyzed worms at a time point. In comparison to the control (45.15 ± 0.57), increased percentage of paralyzed worms was noticed upon treatment with 4 mg/ml HACBF (73.33 ± 3.28, p ≤ 0.01) showing the useful effects of HACBF on nAChR (Fig. 11b).
Effect of HACBF on genes related to acetylcholine cascade in C. elegans and mice

qPCR experiment was carried out to examine the impact of different genes on ACh synthesis and nAChR activities. The genes selected for transcript examination were related to ACh synthesis; cha-1 (choline acetyl transferase), degradation; ace-1 and ace-2 (genes coding for AChE), transport; unc-17 (acetylcholine transporter) and cho-1 (high affinity choline transporter), and receptor; unc-29 (non-alpha subunit of nAChR) and unc-38 (alpha subunit of nAChR), and unc-50 (regulator of nicotinic acetylcholine receptor). In comparison to the control, at 4 mg/ml HACBF[CO7], a significant upregulated expression of cho-1 (2.82 ± 0.15, p ≤ 0.001), unc-17 (1.98 ± 0.23, p ≤ 0.001), and unc-29 (1.81 ± 0.13, p ≤ 0.001) and downregulated expression of ace-1 (0.60 ± 0.20, p ≤ 0.05), and ace-2 (0.63 ± 0.10, p ≤ 0.05) was found (Fig. 11c). The rest of the genes were devoid of any significant alteration (11c). Moreover, the study was further validated by up regulated expression of

HACBF declines the α-syn aggregation in transgenic worms

In the present experiment, a marked reduction at day 5 in α-syn levels of HACBF treated transgenic C. elegans at 1 mg/ml HACBF (18.57 ± 1.27, p ≤ 0.001), 2 mg/ml HACBF (15.47 ± 0.84, p ≤ 0.001), 3 mg/ml HACBF (12.72 ± 0.85, p ≤ 0.001), 4 mg/ml HACBF (11.43 ± 0.21, p ≤ 0.001), and 5 mg/ml HACBF (16.84 ± 1.4, p ≤ 0.001), was seen as compared with control (25.62 ± 0.73) (Fig. 12a, b). The maximum reduction of α-syn level (55%) was observed at 4 mg/ml HACBF treatment.
HACBF alters expression of stress responsive and longevity promoting genes in *C. elegans*

During experimentation, HACBF treatment overexpress the mRNA level of insulin signaling pathway genes *daf-2* (11.16-fold) and *daf-16* (11.64-fold) (Fig. 13c). Here, a significant upregulation of *skn-1* (6.97-fold), *gst-4* (1.73-fold) and *gst-7* (2.02-fold) genes which are directly related to aging process was observed. Additionally, an increase in mRNA transcript levels of *pha-4* (2.55-fold), and its downstream target genes *sod-3* (1.91-fold), and *sod-4* (1.73-fold) was seen (Fig. 13a, c). Further the change in the mRNA expression level of genes *bec-1* (14.36-fold) and *lgg-1* (11.33-fold) confirmed the role of HACBF in autophagy-mediated by DR (Fig. 13c). Besides, we observed a significant change in the expressions of stress responsive genes *ctl-2* (2.15-fold) and *jnk-1* (3.35-fold) which shows that HACBF also requires antioxidant defense system for the longevity promotion. Moreover, the study was further validated by upregulated expression of antioxidant and longevity promoting genes in male and female mice respectively *ctl-1* (5.57-fold and 1.84-fold), *sod-2* (12.66-fold and 4.16-fold), *sod-3* (5.65-fold and 2.33-fold), and *sod-4* (3.60-fold and 1.65-fold), (Fig. 13b).

HACBF impact on dopamine

During the present experimentation the usefulness of HACBF on dopamine was investigated in wild type N2 *C. elegans* and reported higher repulsion time (1.33±0.04) in wild type control worms as compared to treated worms (0.77±0.03,..
Effect of HACBF on head thrash and body bends

Various activities in worms, especially head thrash and body bends show the motor neuron health and dopamine level in the worm. Usefulness of HACBF was noticed by observing the head thrash and body bend in *C. elegans*. In the result it was documented that HACBF treated N2 worms showed greater number (Fig. 12e) of head thrash ($40.17 \pm 0.87$, $p = 0.02$) compared to untreated control worms ($35.83 \pm 1.55$). Subsequently, wild type treated worms ($14.67 \pm 1.45$, $p < 0.001$) showed significant improvement in body bends ($8.33 \pm 0.88$) (Fig. 12d).

Functionality of HACBF on neuromodulator genes expression

It is well established that α-syn aggregation play a major role in onset of PD [33, 43] and many genes are also responsible for α-syn suppression in *C. elegans*. That is why the qPCR studies of PD was performed to identify the mechanism behind the HACBF modulation of α-syn, lipid, dopamine levels and mitochondrial functioning through *ubc-12, lagr-1, ymel-1, pink-1*, and *pdr-1*. It was found that HACBF[C07] showed a significant useful effect in fold change of mRNA expression of *ubc-12* ($1.96 \pm 0.06$, $p < 0.05$), *pdr-1* ($2.08 \pm 0.02$, $p < 0.01$), *lagr-1* ($2.98 \pm 0.34$, $p < 0.001$), *pink-1* ($1.68 \pm 0.04$, $p < 0.01$), and *ymel-1* ($12.93 \pm 0.80$, $p < 0.001$) in HACBF treated worms (Fig. 12f). Moreover, the study was further validated by upregulated expression of neuromodulatory genes in male and female mice respectively *ubc-12* (2.52-fold and 4.67-fold) and *pink-1* (1.61 fold and 2.75 fold), (Fig. 12g).

Conclusion

The present study is the first of its kind that demonstrates the effect of HACBF on ageing and cognition in *C. elegans* model system. The healthy ageing activities of medicinal plant extracts can be attributed for stress resistance, lifespan extension and cognitive boosting activities. Our results suggest that HACBF altogether alleviate oxidative stress,
promote anti-aging activities and boost cognition in *Caenorhabditis elegans*. The traditional Indian system of ayurvedic medicine utilizes a mixture of herbs for treating various ailments. Thus, such research work opens new avenues for the development of anti-aging and cognitive booster herbal products as an herbal therapy for delaying aging and age-related disorders. The formulations thus developed could be conveniently implemented in the form of decoctions/tea.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s13237-022-00407-1.

**Acknowledgements** The authors are also thankful to *Caenorhabditis elegans* Genetics Center (CGC), University of Minnesota, MN, USA, funded by the NIH National Center for Research Resources (NCRR) for the sharing of the *C. elegans* strains. Senior author is highly grateful to CSIR-HRDG for granting CSIR Emeritus Scientist Scheme.

**Author contributions** RP conceived, and designed the research study. RP wrote the research manuscript with analyzed input from all authors. All authors conducted different experiments and analyzed the results as provided to RP.

**Funding** Rakesh Pandey and Mashu Trivedi is receiving financial support from HRDG Emeritus Scientist Scheme 21 (1099)/20/EMR-II, New Delhi, India.

**Declarations**

**Conflict of interest** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Research involving human participants and/or animals** This article does not contain any studies with human participants performed by any of the authors.

**Informed consent** All authors consented to publish.

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