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Original Article

COMPREHENSIVE MOLECULAR STUDY REVEALS THE POTENTIAL ROLE OF CHEBULINIC ACID AND BOERAVINONE B TO ESTABLISH REDOX HOMEOSTASIS IN METABOLICALLY STRESSED CELLS

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ABSTRACT

Objective: Our objective was to assess the multi-dimensional protective mechanism of Chebulinic acid (CA) and Boeravinone B (BB) against MG-induced metabolic stress and cytotoxicity.

Methods: In this study, we have studied CA-and BB-mediated changes in molecular markers of highly dynamic mechanisms of mitochondrial disintegration, nuclear damage and cell death pathways associated with early ageing in MG-exposed *Saccharomyces cerevisiae* cells using biochemical assays, qRT-PCR and flow cytometry.

Results: We found that CA and BB interventions during MG-induced stress in *S. cerevisiae* reduce the rate of extracellular nitrite production, protein carbonyl content, lipid peroxidation and in addition, photo components positively modulate the expression patterns of genes involved in different cell death pathways. Furthermore, CA and BB treatments to MG-stressed cells reduced the number of cells in late apoptosis by 13.4% and 28.3%, respectively. On top of that, CA and BB supplementation during MG-stress restored mitochondrial membrane potential ($\Delta\Psi m$) by 63.0% and 62.5%, respectively.

Conclusion: Based on the results of this study, it seems CA and BB phytotherapy protect against MG-induced cytotoxicity through their natural antioxidant properties by establishing redox homeostasis; thus, CA and BB defend the cell components from oxidative damage of different biomolecules and organelles, ultimately increase longevity.

Keywords: Redox homeostasis, Stress signaling, Mitochondrial membrane potential, Mitochondrial damage control, Apoptosis

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INTRODUCTION

Since the beginning of the 19th century, the elderly population worldwide has faced major socioeconomic and health-related challenges [1]. To overcome these challenges, detailed knowledge of the fundamental biochemical mechanisms involved in cell death and aging is required for adopting interdisciplinary intervention approaches. The end of the 20th century is denoted as an "end of an epoch" and is characterized by the rise of scientific hopefulness and therapeutic involvement to combat oxidative stress-related lifestyle disorders involving the research for life span extension [1]. It is reported that heavy metals, ultraviolet (UV) radiation, herbicides, air pollutants, xenobiotic and other exogenous factors can induce the substantial generation of reactive oxygen species (ROS). ROS are usually generated by electron escape from the electron transport chain during aerobic respiration (oxidative metabolism) in the inner mitochondrial membrane. Elevated levels of ROS induce oxidative stress, a condition that is broadly specified as inequality in pro-oxidant and antioxidant levels [2].

Constantly increasing environmental pollution is a major cause of elevated oxidative stress and related pathophysiology. The extensively used aquatic pollutant malachite green (MG) is a coloring agent in major burlap, leather, silk, wool, paper, and woven fabric manufacturing industries; it is also utilized as a food additive anthelmintic and disinfectant [3]. MG accumulated in eukaryotes induces oxidative stress, intercalates with DNA and leads to DNA damage, lipid peroxidation, protein aggregation, mutagenesis, chromosomal fractures, tumor promoter activity, cytotoxicity and has the ability to induce cell transformation, which results in adverse health effects [4, 5]. Prolonged accumulation of MG increases its toxicity which causes random damage to cellular components such as proteins, nucleic acids and lipids; further resulting in cell death and lowered quality of life (or aging) [6]. Aging is universally defined as a progressive failure in organismic functioning over the years, linked with an increased threat of illness, disease and death. In light of a growing body of research on aging worldwide, it has become evident that aging is a universal set of multiple complex processes that may occur at different rates among different species and involves multiple interconnected molecular mechanisms [7]. In humans, longevity is affected by heredity along with various man-made factors such as lifestyle, pollution, nutrition, childhood stress and socioeconomic status [8]. If aging is to be determined by an accumulation of free radicals and reactive oxygen species (ROS) and damage induced by them, then the main challenge is to identify the organelles and cellular components that are most likely to participate in the primary mechanisms of highly networked pathways involved in cellular damage and repair. It is also imperative to understand the roles and mechanisms of action that these cellular components play during redox homeostasis. Mitochondria are energy stations and most vital organelles for appropriate cellular functions. Irregularity and alteration in mechanisms that control proper mitochondrial operation and morphology maintenance are detrimental and can impair the cellular homeostasis, which further leads to cell death [9].

Genetic and molecular studies on model organisms have revealed major pathways that are developmentally conserved and control the progression of programmed cell death and lifespan within the species. During the evolutionary history of living cells, these pathways are likely to evolve as an adaptation to various intrinsic and extrinsic stimuli. However, organisms have evolved a huge battery of antioxidant defense systems to combat the noxious characteristics of oxygen, mainly because of their tendency to produce reactive oxygen species (ROS) [10]. Elimination of ROS is important to suppress alterations in metabolism; genetically open

model systems in yeast have been heavily employed in research pursuits and serve as an excellent model to understand how eukaryotes fight against oxidative stress and other adverse effects [11]. *S. cerevisiae* has evolved antioxidant defense systems to overcome oxidative stress, including the transcription factors *Msn2/4, Skn7, Yap1* and their respective genes. Genes involved in apoptosis, necrosis, pexophagy, mitophagy and autophagy pathways are also well evolved [12].

Previous studies have revealed that caloric restriction and pharmacological treatments are central regulators of the conserved cell death pathways in experimental organisms such as *S. cerevisiae* and mice. Recently, polyherbal extract and multi-molecule formulations are being used for the preparation of dispersible tablets to treat kidney disorders [13]. The use of plant-derived secondary metabolites can expand endurance in heterotrophic organisms by means of developmentally retained mechanisms, in which structurally diverse phyto-components regulate an intricate network of signals that harmonize a diverse group of immunomodulatory and longevity-determining cellular mechanisms [14]. Formerly, herbal intervention in stressed *S. cerevisiae* cells has been shown to be effective in reducing oxidative stress, restoring normal growth phases, and securing cells from nuclear damage. It was later proved that chebulinic acid (CA) and boeravinone B (BB) also act in the rejuvenation of stressed *S. cerevisiae* cells by lowering the biological markers of programmed cell death (PCD) and necrosis [15]. In the present article, we report molecular insights into highly dynamic processes that interconnect redox homeostasis to mitochondrial homeostasis, nuclear metabolism, and conserved signaling processes, which control longevity during CA and BB interventions and extend the lifespan in stressed *S. cerevisiae* cells by either direct or indirect modulation of various genes involved in cellular homeostasis mechanisms.

MATERIALS AND METHODS

Chemicals

MG, dextrose anhydrous (GRM077), peptone type-III bacteriological (RM7709), yeast extract powder (RM668) and agar powder (GRM026) were acquired from Hi-Media Laboratory Products (India). Total RNA isolation kit and SYBR Green qPCR Master Mix were procured from GeNei™, India. Annexin-V-PI staining kits and Rhodamine 123 were obtained from Sigma Aldrich, India. Phyto-molecules, chebulinic acid and boeravinone B were obtained from Natural Remedies Pvt Ltd (India). The primers used for gene expression analysis were purchased from Eurofins Genomics (India). All other substances and dissolvents used in the experiments were of analytical grade and the highest purity available.

Culture growth conditions and experimental setup

The yeast strain *S. cerevisiae* (NCIM-3594) was purchased from the NCIM Research Center, Pune (India). The cell culture was maintained on 3% agar slants containing yeast extract (1%), peptone (1%) and dextrose (5%). Cells used for the current study were consistently grown on minutely amended culture media consisting of yeast extract (0.5%), peptone (0.5%) and dextrose (2%). For the entire study, five experimental sets were designed as a) a control set with untreated *S. cerevisiae* cells; b) MG set with MG (1.0 µM)-exposed cells; c) (MG+vit-C) set with MG-exposed cells treated with 5 µg/ml vit-C; d) (MG+CA) set with MG-exposed cells treated with 5 µg/ml CA; and e) (MG+BB) set with MG-exposed cells treated with 3 µg/ml BB. Cells used in the experimental setup were allowed to grow at 35±2 °C in modified liquid media with continuous shaking at 120–150 rpm.

Nitrite estimation by griess method

MG-induced nitric oxide (NO^{2-}) generation in *S. cerevisiae* was estimated using the Griess method [16]. *S. cerevisiae* cells treated with either MG, MG+CA, MG+BB along with untreated cells (control) were centrifuged at 3000g independently and the cell-free supernatant was used for analysis. To estimate the amount of nitrite generated, 150 µl of sample supernatant was mixed independently with 150 µl of Griess reagent and incubated for 10 min in the dark. OD was measured at 540 nm using a spectrophotometer (Eppendorf, Hamburg, Germany).

Estimation of total protein carbonyl content by the DNPH method

Total cellular protein of all four samples (control, MG, MG+CA and MG+BB) was isolated using a previously described protocol with slight modifications. One milliliter of cells from four sets was taken and centrifuged at 3000 g and the supernatant obtained was discarded. The pellet retained after centrifugation was washed with PBS twice and cells were re-suspended in 1.5 ml of ice-cold phosphate buffer (50 mmol) at pH 7.4; further, cells were sonicated independently by maintaining the probe amplitude at 60. Three strokes of sonication each of 45 s with a 2 min delay after each stroke were allowed to take place at 4 °C. The homogenized cells were then spun for 5 min at 3000 g and the supernatant obtained was used as a source of protein. The total protein content of the four samples was estimated as per Lowry's method [17, 18].

The 2,4-dinitrophenylhydrazine (DNPH) assay was performed in triplicate and as mentioned in the Product Information Bulletin of Protein Carbonyl Content Assay Kit (MAK094-Sigma Aldrich India), with slight modifications. The persistent DNP-hydrazone adducts formed in the reaction mixture of the four samples was estimated by measuring the absorption at 375 nm [19].

Anti-lipid peroxidation assay

The anti-lipid peroxidation (ALPO) potential after CA and BB treatment was ascertained using the thiobarbituric acid (TBA) assay method [20]. Cells from all four sets were suspended in ice-cold 1.5 ml of Tris–HCl buffer at pH 7.4, followed by independent sonication by maintaining the probe amplitude at 60. Three strokes of sonication each of 10 s with a 60 s delay after each stroke were allowed to take place at 4 °C. The homogenized cells were then spun for 5 min at 3500 g, and the supernatant obtained was used as a source of cellular lipids. The reaction mixture contained 200 µl of sample (5 mg/ml), 500 µl of acetic acid (20 %), 200 µl of SDS (10 %) and 500 µl of TBA (0.8 %). The reaction mixture was incubated for 1 h in a boiling water bath and then cooled. After cooling, 1.5 ml of n-butanol: pyridine (15:5) were added and spun for 5 min at 8000 g [21]. The upper layer of the organic solvent was isolated carefully and OD was measured at 532 nm using a spectrophotometer. The percent anti-lipid peroxidation potential of CA and BB was calculated as follows:

$$\% \text{ ALPO} = \frac{(\text{OD of MG Induced} - \text{OD of treatment sample})}{(\text{OD of MG induced} - \text{OD of control})} \times 100$$

mRNA isolation, cDNA preparation, and quantitative real-time PCR (qRT-PCR) assay

Total RNA isolation was performed using an isolation kit (Genei, India). Total RNA was quantified using a NanoDrop Biospectrophotometer (Eppendorf, USA). Of the total RNA isolated, 50 ng of RNA were used for cDNA synthesis using the protocol and chemicals provided in the cDNA Synthesis Mix Kit (Sigma-Aldrich, India).

For quantitative real-time PCR, SYBR Green and related chemical components were purchased from QIAGEN, India. Of the synthesized cDNA, 50 ng of cDNA templates of all four respective sets with 15 µM of both forward and reverse primers of each gene were used. Gene-specific alterations in the expression patterns of all genes were studied using a StepOnePlus RT-PCR System (Applied Biosystems, AB). Differences in cycle threshold (ΔCT) values were evaluated by deducting the cycle threshold value of the *ACT* gene from the cycle threshold values of all the respective genes analyzed. Gene expression results were analyzed and further refined using the automatic mode of quantification provided in StepOnePlus Software (Applied Biosystems), and the results were confirmed by the delta-delta Ct method ($2^{-\Delta\Delta Ct}$). The primer pairs used in the present investigation are listed in table 1.

Table 1: List of the primer pairs with the nucleotide sequences used for the qRT-PCR gene expression study

Primer name	Primer sequence
Msn2_F	TCACCATTTCCCACAGCA
Msn2_R	TCCTTCGTAACCCCAGCA
Msn4_F	TTGGCGACTTCTGGTGTT
MSn4_R	TGTTTGGGCTTACCGTTG
Skn7_F	CAACCCAGCAACAACAAC
Skn7_R	CGTCATCTTCCACCAACA
Fis1_F	CACCGCTACCATACAGTCA
Fis1_R	GACGACAACACCCTTGAGT
Dnm1_F	TGGGGTTTGTTGGTGTAG
Dnm1_R	GCTCTTGTTCGGTTTGAG
Nuc1_F	CCACCTACCCAGAAACCTA
Nuc1_R	CGAGGACCCAATAAGGAT
Ste20_F	GCCACTACCACCAATACCTC
Ste20_R	CGTCTTTCCCTTTCCTCTCT
TatD_F	GCTCCGTGGTGTGAGATTA
TatD_R	TCCTTGACTTCCGATACGAC
Mca1_F	ATGGCTCCTCCACCTAACCA
Mca1_R	ATTGTGCCTTTGCCTGTTCC
Ubc5_F	TTCCTCCAAGCGTATTGC
Ubc5_R	AGCGTAGGGTGAGTCTGAAG
Slt2_F	CTGACGAACCTGTGTGTAGTG
Slt2_R	GAAGGCTGCTGTTGCTGT
Pep4_F	ACTTCGCTGAGGCTACCA
Pep4_R	GGGACCACCTTATCAACAGA
Atg8_F	TTCCTGCTGACCTTACCG
Atg8_R	CCGTCCTTATCCTTGTGTTC
Atg12_F	GGAACGGCAATGGAAAGA
Atg12_R	TGTCACTCGCCAAACCAA

Flow cytometric analysis

To assess the phases (early apoptosis, late apoptosis and necrosis) of PCD, *S. cerevisiae* cells from four sets were rinsed twice with PBS and the cell wall was disintegrated using zymolyase for 30 min (at 30 °C) [22]. Post-protoplast formation cells were collected, rinsed with binding buffer provided by the kit and stained using Annexin-V and PI containing labeling solution. The labeling solution was prepared by mixing Annexin-V labeling reagent (20 µl) in 960 µl of binding buffer and then 20 µl of PI solution was added. Cells from all sets were centrifuged at 3000 g and resuspended in 250 µl of labeling solution. Cells were then incubated in the dark for 15 min at ambient temperature and diluted with binding buffer (750 µl). Cell acquisition and assessment of stained samples were carried out using a CytoFLEX Flow Cytometer (Beckman Coulter Life Sciences, USA). For excitation, a 488 nm laser was used and the fluorescence signal was detected using FIT-C (515 nm) and PE (>560 nm) filters for Annexin-V and PI, respectively.

Assessment of mitochondrial membrane integrity and potential (ΔΨm) was carried out using 50 nM Rhodamine 123 (Rh123) dye according to with a previously described protocol with minor variations [23]. For this study, cells from all the sets were rinsed twice and suspended in PBS; 1 µl of Rh123 dye (from a 50 mmol stock solution prepared in ethanol) was mixed with 1 ml of cells suspended in PBS. This mixture was then incubated for 10–15 min in the dark at 37 °C. Cell acquisition and assessment of stained (by Rh123) samples were performed using a CytoFLEX flow cytometer (Beckman Coulter Life Sciences, USA). For excitation, a 488 nm laser was used, and the fluorescence signal of Rh 123 was detected using an FIT-C filter. Final data analysis and processing of both experiments were performed using the trial version of FlowJo Software (FlowJo LLC, USA).

Statistical analysis

All experiments involved in the study were conducted three times in parallel and the data obtained are presented as mean±SD. Statistical interpretation of the data obtained was performed using Microsoft Excel. Experimental data were examined and analyzed by the two-way ANOVA method followed by the Tukey's multiple comparison test. A p-value less than 0.05 ($p<0.05$) was considered statistically significant. The coefficient of variance for all experiments was measured as previously reported [24].

RESULTS AND DISCUSSION

It is well understood that MG induces a free radical holocaust process resulting in high levels of ROS and disturbance in redox homeostasis in different cellular compartments of *S. cerevisiae* [5,15]. High levels of ROS trigger oxidative damage to biomolecules such as proteins, lipids and nucleic acids, along with alterations in normal metabolic functioning of vital cell organelles such as mitochondria, peroxisomes and nuclei [25]. Changes in the overall redox status of cells and collapsed mitochondrial functioning are linked to the initiation of cell death pathways in *S. cerevisiae* [9]. Alterations in redox status and metabolic functioning of cells may occur due to a primary antioxidant defense system against oxidative imbalance that is impaired, lowered or rendered inaccessible, which prompts the initiation of cell death pathways, in which extrinsic antioxidant treatments may provide defense against oxidative damage in stressed cells [14]. In the present study, the lifespan-extending potential of the phyto-components CA and BB by altered gene expression in *S. cerevisiae* was evaluated in terms of lowering reactive nitrogen species, protein carbonylation, lipid peroxidation, mitochondrial damage and cell death rate.

CA and BB treatments reduce the rate of extracellular nitrite production

In addition to ROS, enhanced generation of RNS (Reactive Nitrogen Species) leads to severe alterations in cellular redox homeostasis resulting in post-translational damage to cellular and mitochondrial proteins, which further disturb normal cellular metabolisms and induce severe chronic disease [26]. In the present study, we analyzed the production of NO^{2-} after MG accumulation and the role of phytocomponents in neutralizing elevated concentrations of NO^{2-}. We observed that the extracellular levels of NO^{2-} were increased by 36.7 nM/ml after MG accumulation; NO^{2-} levels were reduced by 12.7 nM/ml (34.60 %) and 29.0 nM/ml (79.02 %) after CA and BB treatments, respectively (fig. 1a). This indicates that CA and BB served as RNS scavengers and helped to restore the elevated NO^{2-} concentration to near-normal levels, which improved the deteriorated cellular homeostasis process. Earlier reports have stated that the detrimental effects of increased extracellular nitrite concentration in blood plasma are linked to obesity, cardiovascular diseases and aging in Old Order Amish people [27]. The lowered concentration of extracellular nitrite and re-establishment of cellular homeostasis after CA and BB treatments in stressed *S. cerevisiae* cells demonstrates the potential role of natural antioxidants in therapy, which can be correlated with higher organisms.

CA and BB treatments decrease total protein carbonyl content

MG-induced free radicals catalyze the attachment of carbonyl groups to proteins resulting in the formation of stable protein carbonyls. Thus, a long-term accumulation of these protein carbonyls can be used as a hallmark of oxidative injury to cellular proteins in *S. cerevisiae* cells, which can be further correlated with MG-induced cytotoxicity to cellular proteins in higher organisms [28]. These stable protein carbonyls were quantified using DNPH at 375 nm. In our study, a 2.80 nmoles/mg increase in total carbonyl content of MG-exposed cells indicated elevated levels of oxidative protein damage as compared to the control. On the contrary, total carbonyl content was reduced by 1.55 nmoles/mg (55.36 %) and 2.57 nmoles/mg (91.78 %) post-CA and-BB treatments, respectively (fig. 1b). In a recent report, researchers have described several intrinsic and extrinsic factors involved in the chronological decline of antioxidant potential combined with elevated levels of ROS (such as protein carbonyl species) from cellular oxidative metabolism in aging skin cells [29]. In addition, protein carbonylation is described as an oxidative damage biomarker that is strongly linked to programmed cell death and associated diseases [4]. Herein, we report reduced protein carbonyls by CA and BB treatments in stressed *S. cerevisiae* cells; thus, CA and BB could be of medicinal use to conquer protein carbonylation and associated metabolic dysfunction during PCD process of higher organisms.

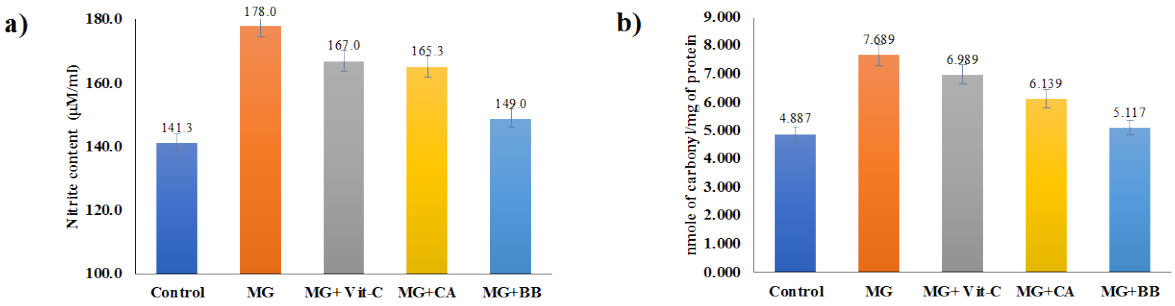

Fig. 1: Biochemical assays for a) nitrite estimation by the griess method b) estimation of total protein carbonyl content by the DNPH method. Gene expression data are presented as the mean±SD of the three corresponding experimental sets (n=3). CV and p values of the treatment sets substantially differed from those of the MG set (*p≤0.03; CV≤0.05)

CA and BB act as anti-lipid peroxidation agents

MG-induced oxidative stress, which can cause major oxidative injury to cellular lipids, results in the formation of lipid peroxides, further leading to *S. cerevisiae* cell death and can be correlated with higher organisms [30]. Lipid peroxides were quantified using TBARs at 532 nm. The anti-lipid peroxidation potential of CA and BB was determined by calculating the reduction in TBARs in stress-induced cells after CA and BB treatments. Assuming control with 100% of ALPO activity and MG-stressed cells of 0% ALPO activity, ALPO activity in CA-and BB-treated cells is measured to be 76.54% and 99.38%, respectively. Herein, we conclude that MG-exposed cells demonstrate zero ALPO activity, while ALPO activity shifted to near-normal levels after treatment with CA and BB. Previous studies have reported that structural damage to lipids results in the formation of lipid peroxides. Lipid peroxides and their derivatives can act as secondary messengers of oxidative stress, can provoke PCD and aging-related disorders [31], which can be treated using natural antioxidants such as CA and BB.

Quantitative real-time PCR (qRT-PCR) assay

CA-and BB-mediated alterations in transcription factors involved in the primary defense mechanism

As the study revolves around redox homeostasis and programmed cell death, the genes involved in redox homeostasis and ROS scavenging mechanism were targeted; of these genes, the transcription factors *Msn2*/4 and *Skn7* were further studied. During non-stress conditions, Msn2p and Msn4p were determined to be located in the cytosol. When stressed, Msn2/4p is hyper-phosphorylated, re-localized to the nucleus, and exhibited periodic nucleo-cytoplasmic transport that corresponded to stress levels (fig. 2a). Nuclear transport of Msn2p has been proven to be exportin Msn5p-dependent and is governed by PKA [32]. When *Msn2* expression in MG-challenged cells was studied, the expression levels were found to be elevated by 1.58-fold compared to controls; meanwhile, with CA and BB treatments, expression levels were observed to be elevated by 1.05-and 0.48-fold, respectively, which is comparable to normal levels (fig. 5a). Similarly, the expression levels of *Msn4* were enhanced by 1.3-fold in MG-exposed cells but were reduced to 0.41-and 0.34-fold after CA and BB treatments, respectively, when compared to controls (fig. 5b). The product of another transcription factor, *Yap1* (Yap1P) regulates the expression of antioxidant genes such as TRR1, TRX2, GSH1 and GLR1. Skn7p also regulates many of the genes regulated by Yap1p, indicating the coordinated action of Yap1p and Skn7p against different stresses (fig. 2b) [33]. During MG stress, 5.18-fold higher expression of *Skn7* was observed, indicating a higher requirement of antioxidant defense proteins regulated by *Skn7*. Furthermore, due to the ROS scavenging action of phyto-components, expression levels of *Skn7* in CA-and BB-treated cells were found to be 0.57-and 0.30-fold, respectively, compared to controls (fig. 5c). Thereby, during MG-induced stress, higher expression of *Msn2*/4 and *Skn7* indicated the increased requirement of primary antioxidant defense proteins regulated by these transcription factors. Potentially due to the protective effects of natural antioxidants, near-normal expression levels of these transcription factors were observed in CA-and BB-treated cells.

Previous reports suggest that supplementation of tea polyphenols showed radio protective potential in hematopoietic system restoration, transition of serum inflammatory cytokines and improved antioxidant capability in mice exposed to radiation, demonstrating that the radio protective role of tea polyphenols may be partly due to their free radical scavenging activity which lowers the activation of antioxidant enzymes (superoxide dismutase) involved in primary defense [34]. In our findings, the protective role of CA and BB in the primary antioxidant defense system was observed at the molecular level, which coincides with previous data.

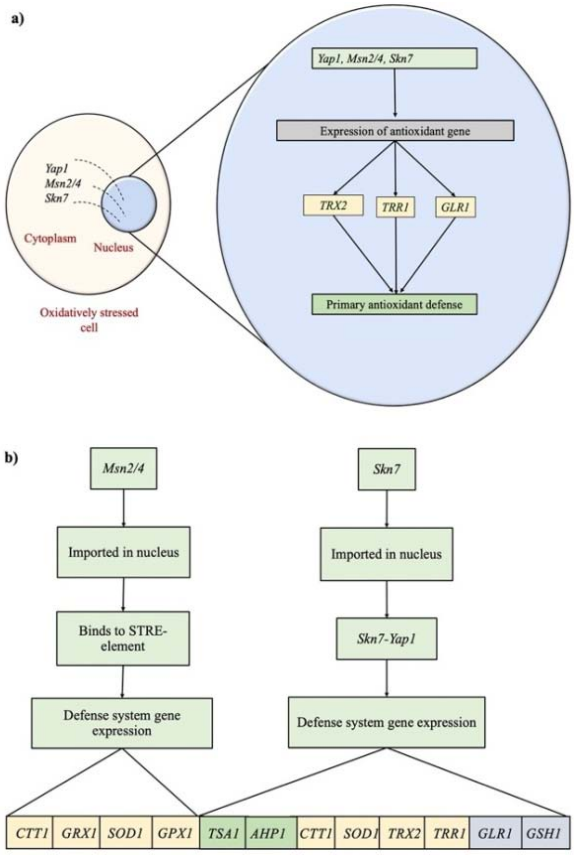

Fig. 2: a) Action mechanism of transcription factors involved in primary defense mechanism during stress condition b) Genes involved in primary defense mechanism during stress condition regulated by *Msn2, Msn4* and *Skn7* transcription factors

Protective role of CA and BB to retain mitochondrial redox homeostasis

Previous reports have concluded systematic reduction in expression of certain mitochondrial functional genes (molecular markers) as the potential cause of aging in skeletal muscles of mice and may serve as useful biomarkers for future diagnosis and treatment in aging [35]. Considering this, we have studied genes involved in mitochondrial fission and membrane potential maintenance. Mitochondrial morphology and membrane potential are maintained by normal expression of genes (*Fis1*, *Dnm1*, and *Mdv1*) in mitochondrial fusion and fission, mitochondria-mediated apoptosis and mitophagy; their overexpression was observed in cells under oxidative stress (fig. 3a) [36]. Compared to controls, 1.38-and 1.98-fold higher expression of *Fis1* and *Dnm1*, respectively, were observed in MG-treated cells, suggesting that mitochondria could not sustain a higher concentration of ROS due to the collapse of the mitochondrial membrane potential, thus leading to Cyt-C release (a marker of mitochondria-mediated apoptosis) and apoptosis or progression into mitophagy (fig. 5d and e). However, due to the protective role of external antioxidants in the maintenance of mitochondrial redox homeostasis, expression of *Fis1* was 0.26-and 0.32-fold, respectively, in CA and BB supplementation; *Dnm1* expression levels were also reduced to 0.67-and 0.53-fold in CA and BB treatments, respectively (fig. 5d and e).

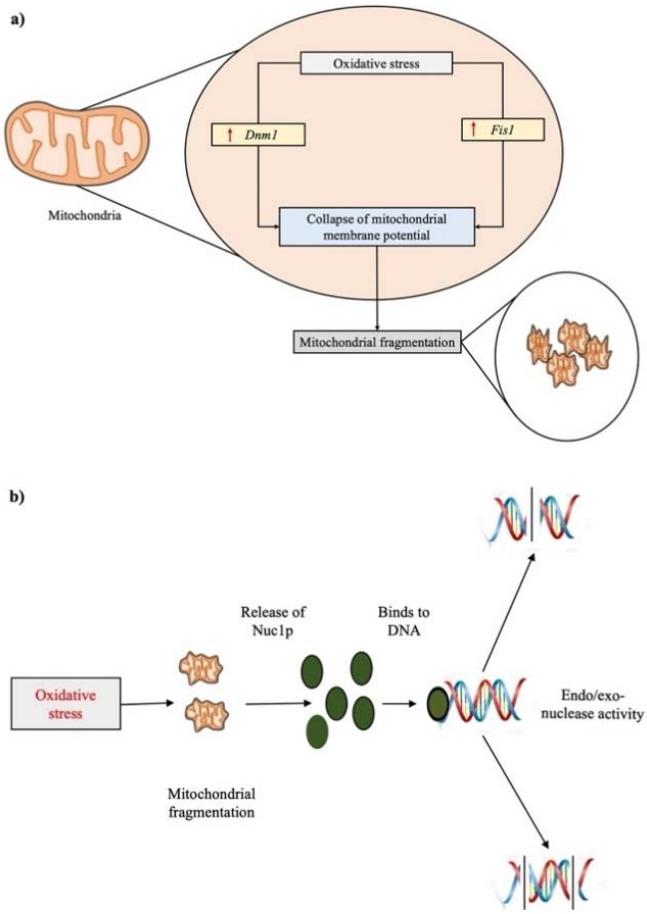

Fig. 3: a) *Fis1 and Dnm1* mediated mitochondrial fragmentation during oxidative stress b) Action mechanism of *Nuc1p* mediated DNA damage in stressed cells

CA and BB provide protection against nuclear damage in stressed cells

Eight apoptotic nucleases have already been reported and alterations in the expression of the major mitochondrial nuclease Nuc1p (Nuclease1) were studied in this work. In MG-challenged cells, a 2.57-fold increase in *Nuc1* expression was observed, which indicated higher RNAse and DNAase that display endo/exo-nuclease activities associated with genomic DNA fragmentation during programmed nuclear destruction (fig. 3b). In contrast, down regulation of Nuc1 (0.61-and 0.39-fold) was observed in CA and BB treatments, respectively; this normalization in expression is suggestive of either lowered or absent DNA fragmentation in phytocomponent-treated cells (fig. 5f). Another apoptotic nuclease, namely *TatD* (analogous to the *Caenorhabditis elegans* apoptotic nucleases) was investigated for its expression, which is conserved across the kingdom [37]. In MG-treated cells, a 4.59-fold elevation in the expression of *TatD* was demonstrated, thus suggesting higher Mg^{2+} metal-dependent endo-/exonuclease activity, which can lead to the initiation of apoptosis and cell death pathways. CA and BB treatments reduced the expression of *TatD* to 0.85-and 0.74-fold, respectively, compared to the control, resulting in the normal nuclear metabolism of cells (fig. 5g).

It has been reported that Ste20p activity is associated with H2B phosphorylation. Ste20p is translocated to the nucleus and immediately phosphorylates the serine 10 of histone 2B, resulting in chromatin condensation during hydrogen peroxide-mediated PCD in *S. cerevisiae* cells, which is similar to mammalian cells undergoing apoptosis induced by hydrogen peroxide (fig. 4a) [38]. In the present study, the expression levels of *Ste20* were observed to be 1.54-fold higher than that in controls in MG-exposed cells, whereas CA and BB treatments demonstrated 0.55-and 0.30-fold expression of this gene, respectively. This represents an elevated chance of chromatin condensation leading to apoptotic cell death in MG-exposed cells than in CA-or BB-treated cells (fig. 5h). This demonstrates that phytotherapy can have potential applications against DNA damage and nucleotide alteration.

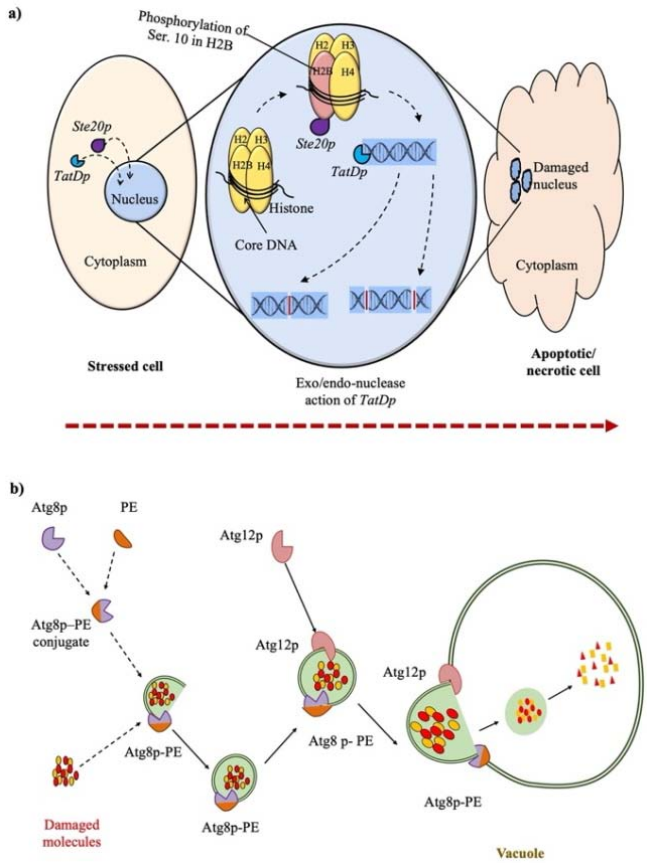

Fig. 4: a) Action mechanism of *TatD* and Ste*20* mediated nuclear damage in stressed cells b) Action mechanism Cytoplasm-to-vacuole targeting selective autophagy pathway regulated by *Atg8p* and *Atg12p* during oxidative stress

Positive regulation of protein homeostasis by CA and BB

Effects of MG exposure along with the defensive effects of CA and BB treatment on the genes involved in the protein homeostasis of MG-stressed cells were investigated. The central component in protein homeostasis during the cellular stress response is ubiquitin-conjugating enzymes as they degrade short-lived, unfamiliar or excess proteins. Of these, UBC5, which encodes a ubiquitin-conjugating enzyme (Ubc5p) that links ubiquitin (Ubi4p) to lysine residues of target proteins, was studied; it also plays a role in the polyubiquitination of proteins [39]. The present study revealed that MG stress causes abnormalities in proteins leading to defective homeostasis. A 1.67-fold increase in Ubc5 expression in MG-exposed cells suggested an elevated requirement of ubiquitination machinery to degrade abnormal and misfolded proteins, while CA and BB treatment shifted *Ubc5* expression to 0.72-and 0.50-fold, respectively, compared to the control condition. This confirmed normal protein homeostasis as a result of ROS scavenging by supplementation with external antioxidants (fig. 5i).

Similar to *Ubc* genes, caspases are major players involved in protein homeostasis. Yeast caspase *Mca1* is a Ca^{2+}-dependent cysteine protease that plays a crucial role in protein quality control as it is recruited to JUNQ and IPOD during proteostatic stress by counteracting the accumulation of insoluble protein aggregates and misfolded and unfolded proteins [40]. As a consequence of MG accumulation and its toxicity, MG-treated cells showed 1.18-fold higher expression of *Mca1*, indicating elevated proteostatic stress and a higher chance of cell death pathway progression in stressed cells. In fact, CA and BB treatments shifted the expression of *Mca1* by 0.47-and 0.26-fold, respectively, relative to controls which indicates a potential reduction in MG-induced cytotoxicity and maintenance of normal proteostasis by natural antioxidants (fig. 5j).,

Furthermore, the *Pep4* gene involved in mitophagy, nucleoporin degradation and membrane protein damage was studied. This gene encodes proteinase-A (vacuolar aspartyl protease), which is closely related to mammalian aspartyl proteases such as cathepsin D, pepsin, chymosin and renin. Pep4p is synthesized as a zymogen that is self-activated and subsequently further initiates the maturation and activation of other vacuolar hydrolases [41]. Under elevated oxidative stress, Pep4p migrates out of the vacuole into the cytoplasm and mediates mitochondrial and nucleoporin degradation by cleaving the bond between two adjacent hydrophobic residues [42]. In the present study, a 1.88-fold higher expression of *Pep4* in MG-stressed cells was found, which caused damage to membrane proteins of mitochondria, nuclei and other cell organelles but CA and BB treatments lowered the expression levels by 0.59-and 0.56-fold, respectively, compared to that in controls indicating lowered activation of peptidase ultimately lowers damage and results in normal homeostasis in natural antioxidant-treated cells (fig. 5k).

Another key player is *Slt2* which encodes serine/threonine MAP kinase. The resulting enzyme is involved in the regulation of cell cycle progression, spindle assembly and maintenance of cell wall integrity. Dual phosphorylation of threonine and tyrosine residues leads to *Slt2* activation as an adaptive negative feedback loop response of cells during oxidative damage, affecting the maintenance of the morphology of cellular components and the cell wall [43]. Reports also suggest the role of *Slt2* in macro autophagy, such as mitophagy (mitochondrial degradation) and pexophagy (peroxisome degradation). *Slt2* helps in the critical cargo-packing step of macro-autophagy; thus, the recruitment of mitochondria and peroxisomes to the phagophore assembly site is carried out by *Slt2* [44]. In this study, a 1.67-fold higher expression of *Slt2* in MG-stressed cells than in controls was associated with damaged cellular morphology reported earlier, compared to that in CA-and BB-treated cells, which showed a clear shift in expression levels to 0.66-and 0.41-fold, respectively, relative to controls. Thus, this finding reveals the protective effect of natural antioxidants in maintaining normal morphology during stress (fig. 5l).

Our study coincides with previous work that suggests supplementation with iodine ions (can be applied within a 0.1–10 μM range by oral supplementation in humans) can lower damage to proteins and cellular components by modulating oxidative protein damage caused by the inflammation-associated heme enzyme myeloperoxidase [45].

CA and BB positively alter expression of genes involved in autophagy

Genes involved in the highly conserved multistep pathway of autophagy were analyzed and alterations in the expression of *Atg8* and *Atg12* were observed. These genes are components of the ubiquitin-like systems of the Cytoplasm-to-vacuole targeting pathway (Cvt pathway) and are involved in the intracellular degradation of cellular material [46]. Atg8p is conjugated with phospholipid phosphatidylethanolamine (PE) to form an Atg8p-PE conjugate which further plays a role in membrane fusion and phagophore expansion during autophagosome formation. Atg12p is another ubiquitin-like modifier involved in the Cvt pathway and auto-phagocytosis. Auto-phagosomes formed by conjugation systems (Atg8 and Atg12) are fused with vacuoles. Autophagic bodies are then discharged in vacuoles that are further degraded and their components are dissolute for re-utilization in biosynthesis (fig. 4b) [47]. In the present study, the stressed cells showed a 1.42-fold increase in *Atg8* expression, while in CA and BB treatments gene expression levels were reduced to 0.63-and 0.50-fold, respectively, compared to that in control cells (fig. 5m). Similarly, the expression level of *Atg12* in stressed cells was 2.18-fold higher than that in controls, whereas CA and BB treatments reduced expression to 0.66- and 0.51-fold, respectively (fig. 5n). In MG-exposed cells, elevated levels of genes involved in autophagy suggest greater damage to biomolecules, organelles and other cellular components. In addition, as a response to this damage, cells proceeded with the cell survival mechanism of autophagy which degrades damaged cell components. Defensive properties of phyto-components against oxidative stress in stressed *S. cerevisiae* cells were clearly evident as the overexpression of genes associated with autophagy activation was not observed, thereby suggesting normal morphology and functioning of cellular components after CA and BB treatments.

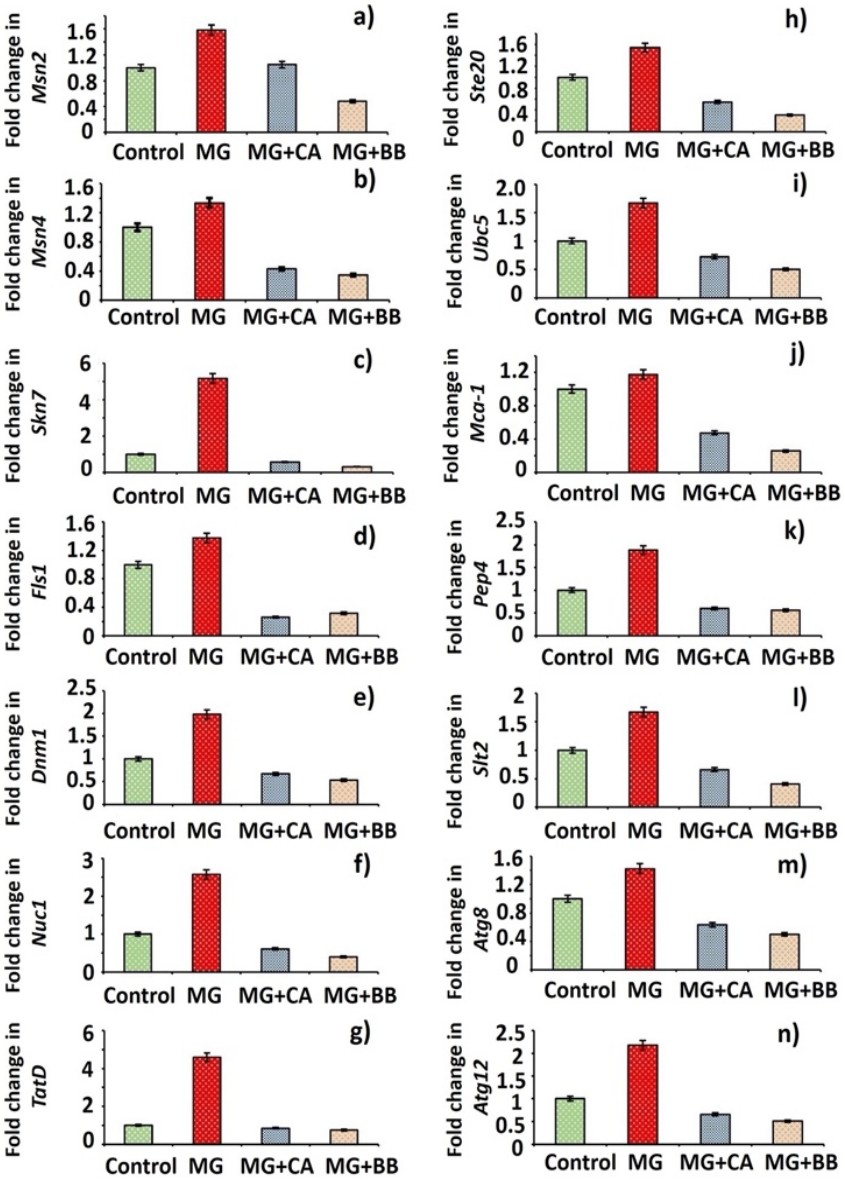

Fig. 5: Modification in the expression patterns of a) *Msn2* b) *Msn4* c) *Skn7* d) *Fis1* e) *Dnm1* f) *Nuc1* g) *TatD* h) *Ste20* i) *Ubc5* j) *Mca1* k) *Pep4* l) *Slt2* m) *Atg8* n) *Atg12* genes in comparison with those of control *S. cerevisiae* cells, during MG exposure and in CA (5 μg/ml) and BB (3 μg/ml) treatments. Gene expression data are presented as the mean±SD of the three corresponding experimental sets (n=3). CV and p values of the treatment sets substantially differed from those of the MG set (*p≤0.03; CV≤0.05)

Table 2: Comparison of differentially expressed genes between different experiment sets control, MG, MG+CA and MG+BB)

Gene name	Treatments			
	Control (DE)	MG (DE)	MG+CA (DE)	MG+BB (DE)
Msn2	0	+0.582	+0.049	-0.517
Msn4	0	+0.334	-0.572	-0.655
Skn7	0	+4.178	-0.429	-0.69
Fis1	0	+0.376	-0.741	-0.685
Dnm1	0	+0.978	-0.327	-0.464
Nuc1	0	+1.574	-0.391	-0.602
Ste20	0	+0.545	-0.452	-0.696
TatD	0	+3.593	-0.152	-0.256
Ubc5	0	+0.669	-0.275	-0.498
Mca1	0	+0.176	-0.526	-0.742
Pep4	0	+0.844	-0.401	-0.443
Slt2	0	+0.671	-0.339	-0.593
Atg8	0	+0.425	-0.367	-0.501
Atg12	0	+1.179	-0.344	-0.491

*DE: Differential expression

Antioxidant defense by CA and BB restores the expression of genes involved in evolutionarily conserved pathways of cell death

Accumulation of a MG-induced free radical holocaust results in a tremendous generation of ROS and causes damage to various cellular organelles. As a response to ROS generation, the primary defense mechanism is activated; when primary defense fails to scavenge ROS, vital organelles are damaged in the stress-challenged cell. This initiates secondary defense mechanisms and related pathways, among which disturbed mitochondrial homeostasis leads to collapse in ΔΨm. Key genes involved in mitochondrial fragmentation (*Fis1* and *Dnm1*) were found to be overexpressed during MG-induced stress and near-normal levels could be observed in CA and BB treatments. Furthermore, mitochondrial fragmentation leads to the release of cytochrome-C (a marker of apoptosis). Cytochrome C initiates a cascade of caspases involved in protein degradation. In addition to caspases, ubiquitin conjugates play a crucial role in protein homeostasis. Genes involved in protein degradation mechanisms such as *Mca1*, *Ubc5*, *Slt2* and *Pep4* were found to be overexpressed during MG stress conditions; however, in response to CA and BB treatments, their expression was regulated to near-normal levels. Mitochondrial disintegration also releases mitochondrial nuclease (*Nuc1*), which is translocated to the nucleus and proceeds with DNA endo/exonuclease activity. Expression levels of *Nuc1* and other genes involved in nuclear damage (*TatD* and *Ste20*) were observed to be elevated in stressed cells, whereas in CA and BB treatments, they were down regulated to normal levels. Similarly, genes involved in autophagy, *Atg8* and *Atg12* were overexpressed in stress conditions, while their expression was significantly reduced to normal levels after CA and BB treatments. A cumulative picture of various genes involved in altered stress responses in the form of a hypothetical pathway is shown in (fig. 7).

Our findings are in accord with earlier experimental and epidemiological data, suggesting herbal products to be potential remedies to regain life expectancy of an organism against metabolic syndrome and PCD. Supplementation with dietary polyphenols— such as resveratrol, coumarins, phenolic acids, and curcumin, and which are regarded as anti-aging natural compounds can recuperate aging-related cellular injuries by diminishing excessive ROS generation and positively altering oxidative impairment, inflammation, cellular senescence and aging components [48]. In line with these findings, CA and BB alter evolutionarily conserved cell death mechanisms at the molecular level and ultimately enhance cell survival in stressed *S. cerevisiae* cells.

Flow cytometry analysis

CA and BB help maintain mitochondrial membrane potential and morphology during stress

In the present study, flow cytometry was used for the assessment of mitochondrial function, as expressed by the ΔΨm, since intrinsic cell death pathways are triggered via mitochondrial malfunction. It was observed that 100% of cells showed normal ΔΨm in the control set, while MG exposure showed collapsed ΔΨm in 64.5% of cells and normal ΔΨm in 35.5% of cells; however, CA and BB-treated cells restored ΔΨm in 98.5% and 98.0% of cells, respectively (fig. 6a). This implicates that the accumulation of MG and associated free radicals in cells show collapsed ΔΨm due to excessive ROS generation which can be correlated with disturbed mitochondrial function in MG-exposed cells. All these observations seem to correlate with the expression patterns of *Dnm1* and *Fis1,* which are the genes involved in mitochondrial fission; therefore, it could be confirmed that ROS-mediated mitochondrial fission initiates the cascade of apoptosis and necrosis by releasing Cyt-C and Nuclease1 (fig. 3a). Earlier studies have showed the protective effects of fucoxanthin supplementation restored structural integrity of mitochondria in case of kidney damage caused by the Cadmium-exposed mice model [49]. In accordance with earlier reports, in our investigation stressed cells attained normal morphology and mitochondria functioning after CA and BB treatments.

CA and BB alter cellular redox homeostasis, thus reducing death in stressed cells

Furthermore, apoptosis and necrosis in all four treatment sets using Annexin-V and PI staining were carried out. Compared to the control, in response to MG exposure, 9.18% of cells were in the early apoptotic phase, while 55.3% of cells were in the late apoptotic phase. CA treatment maintained 10.3% of cells in early apoptosis yet minimized late apoptosis to only 39.9%; similarly, superior results were observed with BB treatment, which reduced the early apoptosis percentage to 7.96% and the late apoptosis percentage to 27.0% in stressed cells (fig. 6b). Upon assessment of flow-cytometry results with gene expression studies, corroborating findings were observed in the qRT-PCR gene expression study of key genes involved in apoptosis and necrosis pathways (fig. 7). In light of these observations, it became evident that the external antioxidants CA and BB scavenge excessive ROS, which modulates redox homeostasis during stress to increase longevity by modulating the molecular mechanisms involved in the cell death pathways of stressed *S. cerevisiae*.

Previously, in addition to demonstrating antioxidant capability, Coenzyme-Q10 showed an anti-apoptotic effect directly by inhibiting PTP opening, which inhibits mitochondrial membrane depolarization, mitophagy, Cyt-c release and Nuc1p release; ultimately inhibits caspase9 activation, which is involved in the apoptosis pathway [50]. Natural antioxidant resveratrol supplementation to H_2O_2-stressed retinal ganglion cells provided protection against H2O2-induced apoptosis by suppressing MAPK cascade, therefore, shows potential to prevent glaucoma [51]. In accordance with previous data, CA and BB are potent antioxidants that play a role in primary antioxidant defense against cell death pathways such as mitophagy, apoptosis, necrosis and autophagy, which ultimately alters the evolutionarily conserved cell death network at the molecular level.

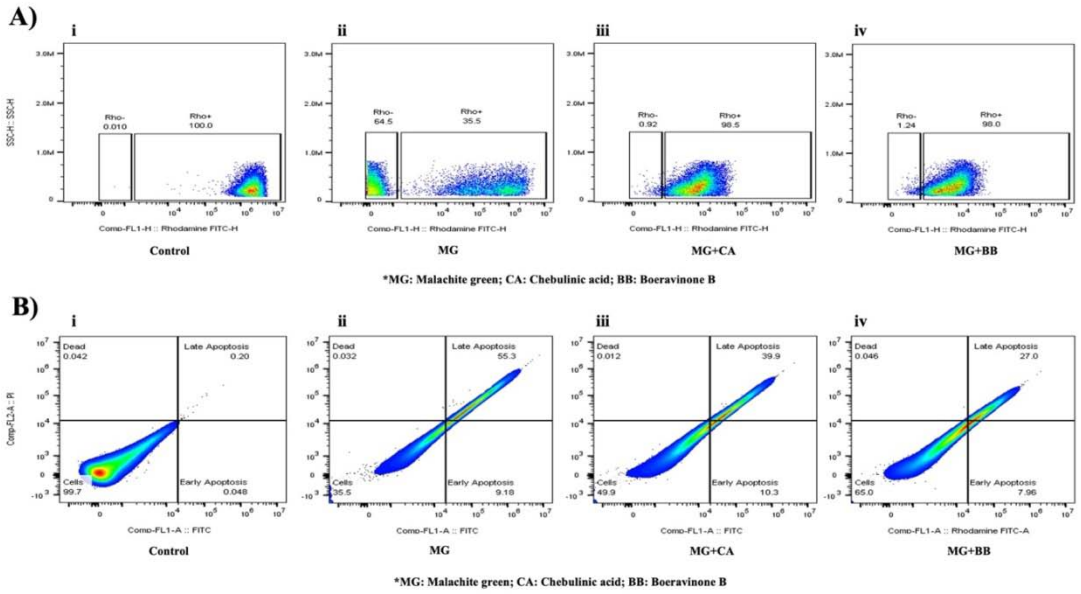

Fig. 6: Flow-cytometry data of a) Rhodamine 123 staining for the assessment of mitochondrial functioning and b) Annexin-V and PI staining for the assessment of programmed cell death pathways of MG-exposed *S. cerevisiae* cells in comparison with CA (5 µg/ml) and BB (3 µg/ml) treatments

CONCLUSION

The findings of this study provide new insights into the pharmacological treatments and phyto-therapies against metabolic stress in *S. cerevisiae* which can be correlated with higher organisms. The natural antioxidants CA and BB provide protection against MG-induced free radical holocaust and oxidative stress by scavenging them into the cytosol. Protective effects of these phyto-molecules can also be observed in the context of mitochondrial redox homeostasis, morphology and function, as they suppress the expression of proteins involved in mitochondrial disintegration. Mitochondrial disintegration is a major player in intrinsic apoptosis pathways, as mitochondrial Nuclease1 and other cytosolic nucleases play a role in nuclear damage; expression levels of these nucleases are downregulated by these antioxidant interventions. After the mitochondrial breakdown, cytochrome-c release is important in caspase activation, which plays a role in protein breakdown and initiates the apoptosis cascade. Moreover, we observed that caspases were downregulated during phyto-component treatments. In addition, cytosolic cytochrome c oxidase activity indicates the presence of pro-apoptotic mitochondrial membrane fragments and proteins in the cytoplasm. Furthermore, membrane proteases, such as *Pep4* were downregulated during CA and BB treatments and such enzymes play a role in the degradation of nucleoporins and other membrane proteins and are involved in cell death pathways. CA and BB interventions proved beneficial in maintaining nuclear integrity by suppressing involved nucleases. Gene expression levels of *Atg8* and *Atg12*, which are involved in the cytoplasm to vacuole targeting (Cvt) pathway of apoptosis, were also downregulated during CA and BB treatments, revealing reduced damage to biomolecules. Altogether, the natural antioxidants CA and BB participate in enhancing cell survival and lifespan expansion during oxidative stress conditions.

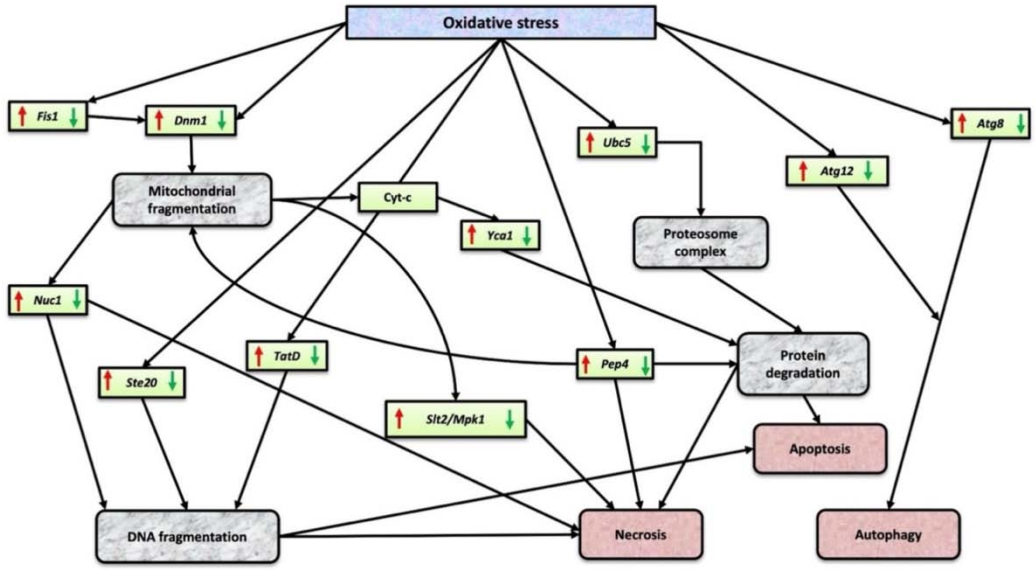

Fig. 7: A schematic representation of known or proposed steps in the programmed cell death network, leading to up-regulation (↑) of certain genes on MG-induced oxidative stress and down-regulation (↓) during CA (5 µg/ml) and BB (3 µg/ml) treatments in *Saccharomyces cerevisiae*

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AUTHORS CONTRIBUTIONS

Harshad Bote: Conceptualization, Methodology, Software, Formal analysis, Investigation, Visualization, Writing an original draft, Writing-review and editing.

Samidha Kakade: Methodology, Formal analysis, Investigation, Visualization, Writing-review and editing.

Shivtej Biradar: Methodology, Formal analysis, Visualization, Writing-review and editing. Rahul Khandare: Writing-review and editing.

Pankaj K. Pawar: Conceptualization, Funding acquisition, Resources, Investigation, Writing-review and editing.

CONFLICT OF INTERESTS

We confirm that there are no known conflicts of interest associated with this publication.

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