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RESEARCH ARTICLE

Mitigation of oxidative stress in yeast cells by *Bacopa monnieri* methanolic extract

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Abstract

The objective of the present study was to assess the influence of the methanolic leaf extract of *Bacopa monnieri* on the extent of cell death in yeast cells due to induced oxidative stress. The yeast (*Saccharomyces cerevisiae*) cells were employed and hydrogen peroxide (H_2O_2) was the oxidant used to cause oxidative stress. The cells were exposed to oxidative stress and by examining distinctive apoptotic processes, the extracts' ability to mitigate this stress was evaluated. The various cytotoxicity assays 2-(4,4-dimethyl-2-tetrazoyl)-2,5-diphenyl-2,4-tetrazolium salt (MTT) and sulforhodamine B (SRB) and changes related to apoptosis viz., morphological and nuclear changes by various staining techniques (Giemsa, propidium iodide [PI], ethidium bromide [EtBr] and 4',6-diamidino-2-phenylindole [DAPI]) and DNA damage (diphenylamine method) were assessed in *S. cerevisiae* cells with/without leaf extract and oxidant (hydrogen peroxide), statistically analyzed and $P<0.05$ was considered significant. The outcomes made it abundantly evident that H_2O_2 caused a sharp increase in the number of *S. cerevisiae* cells going through apoptotic cell death. The amount of apoptosis was not increased by *Bacopa monnieri* leaf extract alone and the apoptotic cells were significantly reduced when the plant extract was co-administered with H_2O_2. Therefore, it is clear that the methanolic extract of *Bacopa monnieri* leaves has been shown to protect *Saccharomyces cerevisiae* cells from stress-induced oxidative damage.

Keywords: apoptotic events; *Bacopa monnieri;* H_2O_2; oxidative stress; *Saccharomyces cerevisiae*

Introduction

Oxygen is a necessary component of aerobic living. Still, it can significantly impact our well-being under specific conditions by generating Reactive Oxygen Species (ROS), including free and non-free radical species. Oxidative stress is typically defined as a state wherein cells are damaged due to increased generation of ROS, free radicals and oxidant-related processes (1). Various cellular systems respond to endogenous and external stimuli by producing hydroxyl, hydrogen peroxide and superoxide radicals, which indicate the degree of oxidative stress in a cell (2). Many cellular macromolecules are harmed by oxidative stress from free radicals, including hydrogen peroxide, superoxide anions, hydroxyl, nitric oxide and peroxynitrite. They consist of proteins, lipids and DNA molecules. Diabetes mellitus, carcinogenesis, myocardial infarction, atherosclerosis, anaemia, inflammation, asthma, neurological disorders and arthritis are just a few of the illnesses that might arise from this damage (3).

Antioxidants help neutralize the damaging effects of excessive ROS. They are essential for maintaining optimal health and supplementing with natural antioxidants is often recommended for conditions like cancer, cardiovascular diseases and other health disorders. In the treatment of several metabolic diseases associated with free radicals, including diabetes, cancer, atherosclerosis and neurodegeneration, antioxidant therapy has become more important (4). Research is now primarily focused on finding plant-based crude medications with antioxidant properties. Accordingly, evaluating the antioxidant activity of the potential herb *Bacopa monnieri* was the main goal of the current investigation. Brahmi, scientifically known as *Bacopa monnieri* (L.) Pennell, is included in the Scrophulariaceae family. The banks of rivers and lakes serve as the natural habitat for *Bacopa monnieri*. It has been ranked second on the priority list of the most important medicinal plants due to its medical significance, commercial value and potential for advanced exploration and improvement (5). It possesses

anti-inflammatory, bronchovasodilatory, anti-ulcer, hepatoprotective and anti-helicopylori qualities (6, 7). *Bacopa* protects oxidative damage through decreased protein carbonyl levels in cytosol and mitochondria in all brain regions (8). Evidence suggests that *Bacopa* exhibits inhibitory activity in preventing lipid peroxidation in the prefrontal cortex, striatum and hippocampus of rats (9). In the presence of free radicals, the primary defence mechanism is the antioxidant superoxide dismutase, which rises in response to oxidative stress. A study found that *Bacopa* treatment in diabetic rats reduced superoxide dismutase activity to normal levels, indicating a balance between oxidant and antioxidant species (10). Oxidative stress significantly depleted reduced glutathione levels in diabetic rats compared to non-diabetic rats (11). *Bacopa* has been shown to enhance reduced glutathione levels (12). *Saccharomyces cerevisiae* (yeast) is a valuable model organism because it is a primitive eukaryote and non-pathogenic fungus. It shares notable molecular and organelle -level similarities with mammalian cells (13). Given the broad-spectrum pharmacological properties, this study aimed to evaluate the antioxidant efficacy of *Bacopa monnieri*. The *in vitro* antioxidant potential of the methanolic leaf extracts was assessed in response to H_2O_2-induced oxidative stress using *Saccharomyces cerevisiae*. *Saccharomyces cerevisiae* (yeast) is a valuable model organism because it is a primitive eukaryote and non-pathogenic fungus. It shares notable molecular and organelle-level similarities with mammalian cells.

Materials and Methods

The leaves of *B. monnieri* were gathered from plants grown in pots within the Avinashilingam University campus, Coimbatore, situated at 11°01′11″N latitude and 76°56′58″E longitude. They were carefully rinsed with running tap water to remove any grime or soil elements, then smoothly blotted amongst sheets of tissue paper to eliminate every excess drop of water. The plant was recognized with the aid of Flora of the Presidency of Madras. After that, 1 g of *B. monnieri* leaves was blended with 10 mL of methanol. The extract was dried at 60 °C under light protection after being centrifuged for 5 min at 2000 rpm. Dimethyl Sulfoxide (DMSO) was utilized to dissolve the residue after it had been weighed to obtain the desired concentration (20 mg/20 µL).

1000 mL of distilled water with a pH of 6.5, yeast extract (10 g), peptone (20 g) and dextrose (20 g) constituted the yeast extract peptone dextrose (YPD) media. After aliquoting, it was autoclaved to ensure sterilization. After cooling, the aliquots were kept at room temperature until needed and often examined for contamination. On the penultimate day of each test, the media was inoculated with the yeast cells and the flask was then incubated for the whole night at 30 °C in a temperature-controlled orbital shaker. Cells in the exponential growth phase are most suitable for all *in vitro* tests. Centrifugation was done for 15 min at 1000 g to separate the media. Two saline washes were performed on the cells gathered in the pellet. After that, saline was used to resuspend the pellet. Aliquots comprising 10^6 cells (as determined by Neubauer ruling) remained treated for 60 min at 30 °C in the presence and absence of 200 µM H_2O_2 and 20 mg of *B. monnieris'* methanolic leaf extract. While the viability of suspended cells was assessed after incubation, a smear of the treated cells was prepared and used for several staining methods.

The level of cytotoxicity in the oxidant-induced cells, both with and without the leaf extract, was measured using the 2-(4,4-dimethyl-2-tetrazoyl)-2,5-diphenyl-2,4-tetrazolium salt (MTT) dye reduction assay (14). The MTT is reduced by living cells to form its formazan derivative. The amount of formazan produced serves as an indicator of the count of viable cells. Subsequently, after dissolving the formazan in an appropriate solvent, the survival of the cells is quantified with the help of a microtitre plate reader. In this assay, the cells (100 µL) were treated with 50 µL of MTT and incubated at 37° C for 3 hr. Following incubation, 200 µL of Phosphate Buffered Saline was added to each sample and the liquid was gently removed. Next, 200 µL of acid-propanol was introduced and the samples were kept in the dark overnight. The absorbance was then recorded at 650 nm using a microtiter plate reader (Anthos 2020, Austria). The optical density of the control cells was set as 100 % viability and the cell viability percentages for the other treatment groups were determined accordingly.

The sulphorhodamine B (SRB) test was used to examine the degree of cell survival subjected to stress caused by reactive oxygen species with and without leaf extracts (15). Sulphorhodamine B is a vibrant pink aminoxanthene dye containing two sulfonic groups. In mildly acidic conditions, SRB attaches to essential amino acids in proteins of cells fixed with trichloroacetic acid (TCA), offering a profound indicator of intracellular protein content that correlates directly with cell survival rate. The SRB assay is a highly delicate method for measuring cytotoxic effects caused by drugs and quantifying clonogenicity. It is particularly well-suited for high -throughput automated drug screening. In this assay, a 350 µL of 40 % TCA (ice-cold) was added to the treated cells and maintained at 4 °C for 60 min. The cells were washed with 200 µL of cold PBS (five times). After removing the buffer, 350 µL of SRB dye was introduced into each well and left at room temperature for 30 min. Surplus dye was eliminated by rinsing the cells 4 times using 1 % acetic acid (350 µL). Next, 350 µL of 10 mM Tris, a solubilizing agent, was added to each well to dissolve the protein-bound dye and the plate was gently shaken for 20 min. Tris solution from each well was transferred to a new 96-well plate and the absorbance was measured using a microtiter plate reader (Anthos 2020, Austria) at 492 nm. Cell existence was determined as a percentage of absorbance relative to the untreated cells.

Shrinkage, chromatin and cytoplasm condensation, cell separation from neighbouring cells, nucleus disintegration and membrane blebbing are the hallmark morphological characteristics of apoptotic cells. Giemsa staining was used to observe the morphological variations in the cells both with and without the leaf sample and/or oxidizing agent (16). The cells were made static, stained for 10 min using Giemsa and examined below a phase-contrast microscope (Nikon, Japan), following the method described (17). An aliquot (10 µL) of Giemsa stain (diluted) was added to the slide and spread by placing a cover slip over it. Then, the

cells were examined for morphological variations under a phase contrast inverted microscope (Nikon, Japan) at 400X magnification. The following formula was used to calculate the apoptotic ratio in Equation 1:

$$\text{Apoptotic ratio} = \frac{\text{Number of apoptotic cells}}{\text{Number of normal cells}} \quad \text{(Eqn. 1)}$$

Changes in the early stage of apoptosis are marked by chromatin condensation around the nuclear membrane. These nuclear alterations were detected in the cells, with and without leaf extracts and/or the oxidizing agent, using propidium iodide (PI), ethidium bromide (EtBr) and 4′,6-diamidino-2-phenylindole (DAPI) staining.

The nuclear variations in the apoptotic cells were detected using PI staining, as outlined by (18). Propidium iodide is a fluorescent dye that binds to nucleic acids by intercalating between their bases, allowing it to stain the nuclear changes associated with apoptotic cells. The treated cells were rinsed with PBS to eliminate residual medium and serum. Subsequently, they were permeabilized using a 50 µL mixture of acetone and methanol in the ratio of 1:1 at -20 °C for 10 min. Afterwards, a 10 µL aliquot of PI was added and evenly spread by placing a coverslip on top, followed by incubation at 37 °C for 30 min in the dark. Apoptotic cells with fragmented nuclei were observed with the green filter of a fluorescence microscope (Nikon, Japan) at 400X magnification. The apoptotic ratio was determined using the previously described formula.

The standard method was used, albeit somewhat modified (EtBr staining), to identify the nuclear alterations in cells that underwent programmed cell death (19). Ethidium bromide binds to nucleic acids, allowing the visualization of nuclear changes in apoptotic cells. A 10 µL drop of ethidium bromide was applied to the treated cells and evenly distributed by placing a coverslip. The slides were then incubated at room temperature for 5 min. Apoptotic cells exhibiting chromatin condensation and nuclear fragmentation were observed using a fluorescence microscope (Nikon, Japan) equipped with a G-2A filter at 400X magnification. The apoptotic ratio was then calculated. as described previously.

The cells that underwent apoptosis were detected using the DAPI staining technique (20). DAPI binds to double-stranded DNA, forming fluorescent complexes that enable nuclear visualization. In dying cells, DAPI staining reveals intensely stained, fragmented nuclei and condensed chromatin. Additionally, nuclear apoptotic bodies and chromatin margination can be observed after DAPI staining. After treatment with the oxidizing agent, with or without the plant extract, the cells were fixed with 50 µL of 3 % paraformaldehyde for 10 min at room temperature. They were then permeabilized with 50 µL of 0.2 % Triton X-100 for 10 min at room temperature and stained with 10 µL of DAPI for 3 min. A coverslip was placed over the cells to ensure uniform stain distribution. The apoptotic ratio was assessed by counting cells with condensed chromatin and fragmented nuclei under an inverted fluorescence microscope (Moticam, Hong Kong) using a DAPI filter at 400X magnification. The apoptotic ratio was calculated as previously described.

All parameters examined throughout the study were statistically analyzed using SigmaStat (Version 3.1) software. Statistical significance was determined using one-way analysis of variance (ANOVA), with a P-value of <0.05 considered significant.

Results and Discussion

Cell and tissue damage involves the production of ROS and reactive nitrogen species (RNS), which subsequently results in alterations in lipids, DNA and proteins, ultimately resulting in cellular dysfunction and cell death. The involvement of free radicals in cell death mechanisms, particularly in apoptosis induction, has garnered increasing attention in cancer therapy research (21).

In this study, the consequence of *B. monnieri* leaf extract on oxidative stress-instigated apoptosis was examined in *S. cerevisiae* cells, with H_2O_2 as the oxidant. The results obtained are presented below.

MTT and SRB tests were used to determine the degree of survival of *S. cerevisiae* cells exposed to oxidative stress both with and without *B. monnieri* leaf extract. The results of quantifying the viability percentage are shown in Figs. 1 and 2, respectively. The results demonstrated that exposure to H_2O_2 (200 µM) caused a significant increase in the number of cells enduring apoptosis. The leaf extract of *B. monnieri* (20 mg) alone failed to induce a noticeable rise in apoptosis. However, when treated with H_2O_2, the methanolic extract significantly reduced the number of apoptotic cells. This suggests that the leaf extract protects against oxidative stress-induced cell death in yeast cells.

MTT is regarded as a trustworthy test for assessing the degree of cell survival. The number of viable cells drastically decreased after being exposed to H_2O_2. The impact of H_2O_2 was successfully counteracted by administering *B. monnieri* leaf extract (methanol), which returned the number of viable cells to almost the control levels. Both the MTT assay and the SRB assay showed the same pattern. The survival of yeast cells exposed to H_2O_2-induced oxidative damage was markedly enhanced by the methanolic extract of *Curcuma amada* Roxb. leaves and rhizomes (22). MTT and SRB experiments exhibited that the extract (methanolic) of *Euphorbia antiquorum* latex enhanced cytotoxicity in yeast cells in a dose-dependent means (23). The ethanolic extract of *Pleurotus ostreatus* inhibited HL-60 leukaemia cell proliferation dose-dependently (24). *Piper sarmentosum* methanolic extract exhibited anticarcinogenic effects in HepG2 cells (25). A cytotoxic effect was demonstrated by the ethanolic extract of *Celastrus orbiculatus* on human melanoma A375-S2 and human cervical carcinoma HeLa cell lines (26). Hydrogen peroxide-induced apoptosis in PC-12 cells was significantly reduced by *Bambusae caulis in Liquamen* (27).

The results of the SRB assay in this study followed the same trend as the MTT assay. A dose-dependent decrease in cell viability was observed in primary rat hepatic stellate cells

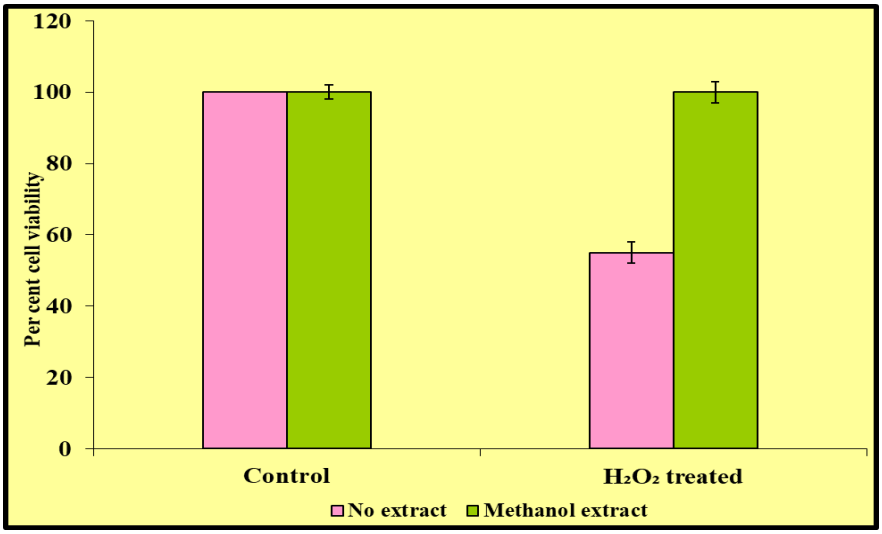

Fig. 1. Impact of *B. monnieri* leaf extract on *S. cerevisiae* cell viability under oxidative stress as assessed by the MTT test. The values of the negative (untreated) control group were fixed as 100 % viability and the percentage of viabilities in the other groups was calculated relative to this. Percent viability in: Control - 100 %; Methanol extract - 100 %. In H_2O_2 treated: 55 %; H_2O_2 + methanol extract treated - 98 %.

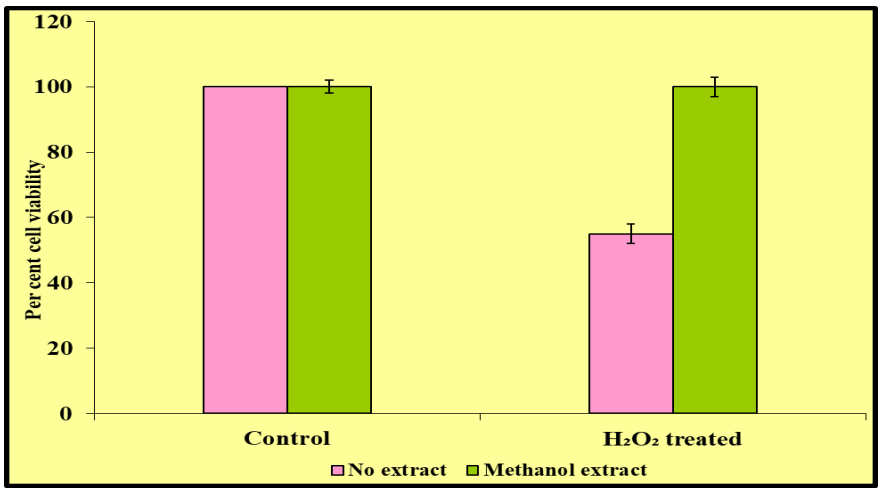

Fig. 2. The SRB test assessed the Impact of B. monnieri leaf extract on *S. cerevisiae* cell viability under oxidative stress. The values of the negative (untreated) control group were fixed as 100% viability and the percentage of viabilities in the other groups was calculated relative to this. Percent viability in: Control - 100 %; Methanol extract - 100 %. In H_2O_2 treated: 54 %; H_2O_2 + methanol extract treated -99 %.

and the human hepatic stellate cell line LX-2 treated with Withaferin A, as determined by the SRB assay (28). Similarly, treatment with *Cistus incanus L.* and *Cistus monspeliensis L.* extracts significantly reduced prostate cell line viability (29). Additionally, an anthocyanin extract from blueberries and an anthocyanin-pyruvic acid adduct extract significantly inhibited cell proliferation in two breast cancer cell lines (MDA -MB-231 and MCF7), as assessed by the SRB assay (30).

The morphological hallmarks of apoptosis include membrane blebbing, cell shrinkage, the formation of membrane-bound apoptotic bodies and chromatin condensation. This study effectively mitigated oxidative stress-induced cell death in yeast cells by *Bacopa monnieri* leaf extract. Using phase contrast microscopy, these alterations were seen and measured in the yeast cells exposed to oxidative stress both with and without the leaf extracts. Each experimental groups' number of cells exhibiting apoptotic morphological alterations was tallied; the findings are shown in Table 1. The number of cells going through apoptosis increased sharply after being exposed to H_2O_2. The amount of apoptosis was not increased by the plant extract alone. The amount of apoptotic cells was significantly reduced when the plant extract was given in conjunction with H_2O_2. It investigated how the plant extract *Rhinacanthus nasutus* affected the apoptosis caused by oxidative stress in *S. cerevisiae* cells (31). Morphological changes in L02 cells, detected through Giemsa staining, indicated that *Polygonum multiflorum* might induce apoptosis in these cells (32). Clitocine, a natural bioactive compound derived from the mushroom *Leucopaxillus*

Table 1. Impact of *B. monnieri* leaf extract on morphological alterations in oxidatively stressed *S. cerevisiae* cells (Giemsa staining)

Sample	No. of apoptotic cells / 100 cells		Apoptotic ratio	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	4 ± 2	85 ± 2[a]	0.04	5.66
Methanol extract	2 ± 2	24 ± 1[abc]	0.02	0.32

The values are mean ± SD of triplicates. a - Statistically significant (P<0.05) compared to untreated control; b - Statistically substantial (P<0.05) compared to oxidant treated group; c - Statistically substantial (P<0.05) compared to plant extract treated group

giganteus, triggered cell death, as evidenced by morphological alterations (33). Similarly, julibroside J8, isolated from *Albizia julibrisin*, inhibited the growth of BGC-823, BEL-7402 and HeLa cell lines (34). Additionally, oridonin, a diterpenoid extracted from medicinal herbs, exhibited effective anticancer action with minimal adversative effects on U937 cells (35).

Cell apoptosis is indicated by nuclear alterations such as atomic fragmentation and marginalization. The distinctive nuclear changes related to apoptosis were enumerated using PI, EtBr and DAPI staining. The study results indicated an increase in the number of apoptotic cells in yeast exposed to oxidative stress. However, administering *Bacopa monnieri* leaf extract did not exhibit cytotoxic effects on yeast cells. Propidium iodide (PI), a chemical too big to penetrate living, breathing cells, becomes more permeable to cells undergoing apoptosis. As a result, the degree of apoptosis in the cells is gauged by PI staining (36). Table 2 shows the number of yeast cells that show apoptosis-associated PI staining in the presence or absence of H_2O_2 and/or *B. monnieri* leaf extract. A significant proportion of yeast cells were permeable to PI after exposure to the oxidant, suggesting oxidation-induced apoptosis. The leaves of *B. monnieri* extract (methanolic) significantly reduced the number of cells going through H_2O_2-induced apoptosis.

An ethanolic extract of *Serenoa repens* fruit caused cell death (apoptosis) in several cancer cell types, including prostate (MDA MB231), breast, kidney (Caki-1), bladder (J82), colon (HCT 116) and lung (A549) cells, as shown using PI staining (37). Similarly, methanolic and methylene dichloride extracts from the above-ground parts of *Larrea divariculata* showed toxic effects on MCF-7 breast cancer cells (38). The alterations in the nucleus during apoptosis may be seen using the intercalating substance ethidium bromide (39). Ethidium bromide EtBr labelling was used to measure the induction of apoptosis in *S. cerevisiae* cells both with and without *B. monnieri* leaf extract. Table 3 displays the values that were obtained. It is evident from the tabulated figures that oxidative stress increased the number of cells going through apoptosis significantly (P<0.05). The co-administration of the *B. monnieri* leaf methanolic extract successfully reversed this cytotoxic damage. The ethanolic extract of *Curcuma aromatica* was found to cause cell death (apoptosis) and stop the formation of new blood vessels (angiogenesis) in Ehrlich Ascites Tumor cells, as shown by EtBr staining (40). Similarly, staining with acridine orange and ethidium bromide (AO/EB) exposed that 2',4'-dihydroxychalcone, a key compound in *Herba oxytropis*, caused apoptosis-mediated cell death in human gastric cancer MGC-803 cells (41).

Nuclear changes that occur during apoptosis can be observed by fluorescent staining using DAPI. Table 4 displays *S. cerevisiae* exposed to H_2O_2 and/or *B. monnieri* extract. Hydrogen peroxide-induced a considerable (P<0.05) fraction of the cells to commit to apoptosis, as shown by their increased permeability to DAPI, as may be inferred from the data. This oxidant-induced cell demise was effectively countered by the methanolic extract of *B. monnieri* leaves. By reducing apoptotic events as measured by EtBr, PI and DAPI staining, the methanolic extract of *Zea mays* leaves demonstrated the highest level of protection for yeast cells exposed to oxidative stress (42). In human liver cancer (Hep 3B) cells, the ethanolic extract of *Euchresta formosana* root caused cell death through apoptosis, as confirmed by DAPI staining (43). Similarly, DAPI staining showed that extracts of *Astrodaucus persicus* lowered the growth of human breast cancer cells (44). Additionally, *Phyllanthus niruri* protected liver cells (hepatocytes) from apoptosis caused by tertiary butyl hydroperoxide, helping to maintain normal cell function, as shown by DAPI staining (45). The DAPI staining results from our studies show that the methanolic extract of *Bacopa monnieri* leaves reduced the number of apoptotic (dying) cells in normal cells.

B. monnieri leaf extract inhibited H_2O_2-induced apoptosis in yeast cells. This antiapoptotic effect is likely due to its antioxidant properties. Along with the adaptive response of yeast cells, treatment with plant extracts, which are rich sources of antioxidants, appears to enhance the levels of antioxidant enzymes in stressed cells, playing a crucial role in cellular defence against oxidative stress. The increased antioxidant enzyme levels contribute to improved yeast cell survival under H_2O_2 exposure. Our preliminary research findings indicate that *B. monnieri* leaves protect lipids and DNA from H_2O_2-induced oxidative stress (46).

Table 2. Impact of *B. monnieri* leaf extract on nuclear alterations in oxidatively stressed *S. cerevisiae* cells (PI staining)

Sample	No. of apoptotic cells / 100 cells		Apoptotic ratio	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	7 ± 2	81 ± 3[a]	0.08	4.26
Methanolic extract	2 ± 2[a]	30 ± 3[abc]	0.02	0.43

The values are mean ± SD of triplicates. a - Statistically significant (P<0.05) compared to untreated control; b - Statistically substantial (P<0.05) compared to oxidant treated group; c - Statistically significant (P<0.05) compared to plant extract treated group

Table 3. Impact of *B. monnieri* leaf extract nuclear alterations in oxidatively stressed *S. cerevisiae* cells (EtBr staining)

Sample	No. of apoptotic cells / 100 cells		Apoptotic ratio	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	7 ± 3	86 ± 2[a]	0.08	6.14
Methanol extract	6 ± 2[a]	10 ± 2[bc]	0.06	0.11

Table 4. Impact of *B. monnieri* leaf extract on nuclear alterations in oxidatively stressed *S. cerevisiae* cells (DAPI staining)

Sample	No. of apoptotic cells / 100 cells		Apoptotic ratio	
	Control	H_2O_2 treated	Control	H_2O_2 treated
No extract	8 ± 2	76 ± 3[a]	0.09	3.17
Methanol extract	6 ± 2	14 ± 3[abc]	0.06	0.16

The values are mean ± SD of triplicates. a - Statistically significant (P<0.05) compared to untreated control; b - Statistically substantial (P<0.05) compared to oxidant treated group; c - Statistically significant (P<0.05) compared to plant extract treated group

Conclusion

In conclusion, this study investigated the cellular events of apoptosis in yeast cells treated with plant extract using a model system in which H_2O_2 induced oxidative stress. The research was conducted with ethical considerations to minimize animal suffering. The findings confirm that *B. monnieri* leaf extract is a potent antiapoptotic agent, effectively reducing oxidative stress caused by the highly reactive H_2O_2. However, *in vivo* studies need to be carried out to confirm the results.

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Authors' contributions

All the authors have contributed equally to data collection, analysis, writing the original manuscript draft, editing and reviewing.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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