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# Induction of apoptosis in *Saccharomyces cerevisiae* cells by Heliamine, an alkaloid from cactus *Backebergia militaris*

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## ABSTRACT

***Heliamine, an alkaloid from Backebergia militaris, was found to induce apoptosis in Saccharomyces cerevisiae cells. Heliamine induced apoptosis was preceded by increase in ROS in cells and antioxidant treatment could inhibit apoptosis. Pathway details of heliamine induced apoptosis was studied by using mutants of apoptosis inducing factor (aif1), metacaspase (yca1) and AIF over expressing strains. Heliamine treated aif 1 cells showed more growth than wild type cells and yca1 cells, indicating role of AIF in heliamine induced apoptosis. In a confirmatory experiment, the growth of similarly treated, AIF overexpressing strains was found to be reduced, ascertaining AIF induced apoptosis. Moreover, other known effects of AIF, namely induction of chromatin condensation and DNA fragmentation were also observed in agarose gel electrophoresis and DAPI staining respectively. These results confirm that heliamine induces apoptosis in S. cerevisiae cells by metacaspase independent, AIF mediated pathway.***

**Keywords: Heliamine; *Saccharomyces cerevisiae*; Reactive Oxygen Species; apoptosis; metacaspase 1.**

## INTRODUCTION

According to World Health Organization (WHO), cancer is the leading cause of death in the world after cardiovascular and respiratory diseases [1], although in current pandemic respiratory diseases predominate. Cancer is caused by mutation in one or more cell cycle regulatory genes, which results in uninhibited growth of cells resulting into tumor formation. Normal cells when mutated, initiate cell death by apoptotic pathway, however cancerous cells fail to induce apoptotic pathway. Many successful chemotherapeutic agents as well as radiation therapies employed for the treatment of various types of cancers have long been observed to induce apoptosis in transformed cells, indicating that the treatments that increases the rate of apoptosis, could be useful for the treatment of cancer [2]. Therefore, apoptosis is widely studied in cancerous as well as chemotherapy-treated as well as untreated tissues to get insight of pathogenesis of cancer. Apoptosis induced cell death is extremely useful in anti-cancerous therapy [2, 3].

Existing treatments of chemotherapy and radiotherapies have multiple long term toxic effects as they not only cause death of cancerous cells but also of normal ones. There are numerous cytotoxic compounds extracted from plants

known to induce cell death by apoptotic pathway [2]. The advantage of such natural compounds is that they have little or no effect on the normal cells. Although these plant-derived compounds are extremely useful, very few of them are being used in medicine due to their lower efficacy and lack of details of their mode of action.

*S. cerevisiae* is used as a model to study apoptosis since more than two decades as they offer several advantages like culturing yeast cells is methodologically simple, also yeast is a eukaryotic cell with known genome sequence [4]. *S. cerevisiae* cells used in the present studies exhibit typical markers of apoptosis and physiologically quite similar to multicellular organisms and many yeast orthologues (genes that share common ancestors) of mammalian yeast proteins have been identified [4].

Various chemicals, heavy metals and pharmacological agents have been found to induce apoptosis in yeast cells by one of the two types of pathways, one is metacaspase dependent and another is metacaspase independent pathway. Apoptosis inducing factor 1 (AIF1) induced apoptosis is metacaspase independent pathway.

In the present study, we have investigated the effective concentration and mechanism of anti-proliferative action of one of such plant derived cytotoxic alkaloid, Heliamine (6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline). Heliamine is an alkaloid, isolated from a tree-like cactus *Backebergia militaris*. Previous study of this compound on rat sarcoma 45 cells revealed that, it has anti-cancerous effects [5]. Our aim in the present studies is to understand the mechanism of cell death induced by heliamine. For this, we have studied its effect on ROS levels and role of AIF, metacaspase in inducing apoptosis and tried to develop an insight into its mechanism of action in a model organism *S. cerevisiae*.

**Materials and Methods**

Heliamine was procured from Sigma-aldrich and dissolved in water.

**Yeast strains and Growth conditions**

*S. cerevisiae* wild type BY4741 (*MATa, his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*) was procured from MTCC, Chandigarh, India. Mutant strains of *S. cerevisiae* Δ*yca1* (MATa; ura3-52; leu2-3,112; his3-Δ1;YOR197w::kanMX4) and Δ*aif1* (MATa, his3Δ1; leu2Δ0; met15Δ0; ura3Δ0;YNR074C::kanMX4) were previously obtained as a gratis. All the cells were grown at 30°C in YPD medium containing 1% Bacto-yeast extract, 2% Bacto-peptone and 2% Dextrose [6]. Solid medium was prepared by addition of 2% agar. For each experiment, the cells were grown up to the number of 1.5 x $10^6$ at 30 °C and incubated at the same condition for 24 hours.

**Viability assays**

Viability assays were carried out using trypan blue exclusion assay. Live cells, having intact cell membrane inhibited the passage of trypan blue

dye, were seen colorless whereas dead cells, which lost the cell membrane, were stained in blue color. The exponentially growing cells in YPD broth were treated with heliamine at different concentration ranging from 1mg/ml to 10mg/ml in 30ml cell culture media and incubated for 24 hours at 30˚C in rotary shaker (150 rpm). After the treatment, cells were harvested by centrifuging at 10,000 rpm for 5 min and washed thrice with PBS. Percentage viability was calculated from three independent experiments of trypan blue staining [7]. At least 300 cells were counted in each treatment which are compared with control one.

**Acridine orange (AO) / Ethidium bromide (EtBr) staining**

The co-staining of AO/EtBr is extremely useful for detection of apoptotic or necrotic cells. Acridine orange stains both live and dead cells whereas ethidium bromide stains only dead cells. The staining can evaluate the morphology of nucleus. Apoptotic cells have fragmented or condensed nuclei whereas necrotic cells resemble to viable cells in nuclear morphology. Live and early apoptotic cells appear in green color. Late apoptotic and necrotic cells appear in orange color [8]. The nucleus morphology was also evaluated using DAPI staining.

For detection of apoptosis, 24 hours heliamine treated as well untreated *S. cerevisiae* cells were evaluated using Acridine orange (AO; Sigma-Aldrich) / Ethidium bromide (EtBr; Sigma-Aldrich) staining [8]. Briefly, treated and untreated cells were harvested by centrifugation to pellet down the cells. Cells were suspended in 1 X PBS. 2µl of AO/EtBr solution (containing equal volume of 100µg/ ml AO and 100 µg/ml EtBr) was added in 20 µl of cell culture. Dye and cells were mixed properly and 10µl mixture was taken immediately for observation in fluorescence microscope. Around 300 cells were examined under fluorescence microscope using fluorescence filter at 100X objective (Olympus Bx41, Japan).

**DAPI staining**

For the detection of chromatin changes, the chromatin specific dye 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI; Himedia, India) was used [9]. In brief, for DAPI staining cells were harvested after the treatment for 24 hours. Then cells were washed with PBS (pH 7.4), incubated with 1 µg/ mL DAPI in PBS for 10 min at room temperature in dark. Cells were rinsed thrice with PBS and examined the change in chromatin structure under fluorescence microscope using fluorescence filter at 100X objective (Olympus Bx41, Japan).

**DNA fragmentation study**

DNA of 24 hours treated and untreated *S. cerevisiae* cells were isolated and electrophoresed on 0.8% agarose gel as described by Patel *et al* [10]. Briefly, treated and untreated

*S. cerevisiae* cells were harvested by centrifugation at 8000rpm for 5min. Supernatant was discarded and cell pellet was washed twice with PBS to remove extra impurity. Cells were resuspended in 0.5ml of distilled water in 2ml of micro-centrifuge tube. 0.2mL of lysis buffer (2% Triton X-100, 1% Sodium dodecyl sulphate, 100mM NaCl, 10 mM Tris HCl (pH-8.0), 1 mM EDTA (pH-8.0) and 0.2mL of Phenol:chloroform:isoamyl alcohol (25:24:1) were added in to cell suspension. To this acid-washed glass beads were added and vortexed for 5 min with intermittent cooling to break around 90% of the cells. DNA was separated from cell debris through by centrifugation for 5min at room temperature. Upper aqueous layer was transferred in separate tube and added with $1/10^{th}$ volume of 3M sodium acetate (pH-5.2) and 2.5 volume of 100% ethanol (chilled) to precipitate the DNA. This was spun using microfuge for 5 minutes, pellet was washed with 70% chilled alcohol, air dried and resuspended in 20-30 µl of Tris-EDTA buffer (pH-7.5). DNA was run on agarose gel electrophoresis using 0.8% agarose and Tris - acetate – EDTA buffer (pH -8.2). Image of electrophoresed agarose gel (pre-stained with ethidium bromide) was captured using ChemiDoc XRS+ (Bio Rad) instrument and analyzed by image lab software.

**ROS detection**

Accumulation of intracellular reactive oxygen species (ROS) were evaluated using ROS sensitive fluorescence probe 2', 7'-dichlorofluorescein diacetate (DCFDA; Sigma-Aldrich) as described earlier [11]. Exponentially growing cells were treated with heliamine (5mg/ml) for a period of 3 hours. After the treatment cells were harvested at 8000 rpm for 10 minutes and rinsed with PBS for thrice. Pellets were resuspended in PBS (0.1M, pH 7.4) and incubated with 10 µM DCFDA for 30 minutes in dark at 30°C. Stained cells were observed under fluorescence microscope (Olympus Bx41, Japan) using fluorescence filter at 40X objective.

**Mutant assay**

*S. cerevisiae* BY4741 wild-type, Δ*yca1* and Δ*aif1* cells were grown for overnight in YPD liquid media. When the cell-count reached to 1.5 x $10^6$ cells/ml, it was treated with heliamine at 5mg/ml concentration for a period of 24 hours at 30°C. Untreated cells were also grown for same period of time and condition. After treatment period, cells were diluted using fresh YPD medium at different dilution i.e. $10^{-1}$, $10^{-2}$, $10^{-3}$ and $10^{-4}$. The 10µl of diluted cells suspension was spread on YPD agar plates to grow the survival colonies for 3 days at 30°C. The survival colonies of treated cells were compare with untreated one.

**Statistics**

Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparisons test and two-way ANOVA followed by Bonferroni's multiple comparisons test. The

results represented as the mean ± SD using Graph Pad Prism 6.0 software, San Diego, USA.

## RESULTS

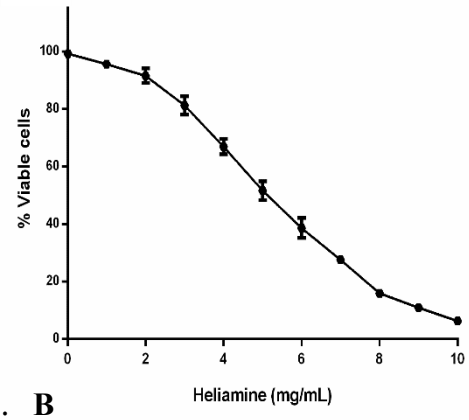
### Heliamine inhibits the growth of *S. cerevisiae* cells

The effect of heliamine on growth of BY4741 wild-type *S. cerevisiae* cells was evaluated by treating with different concentration of heliamine for a period of 24 hours. A significant reduction in cell viability was noticed in heliamine treated cells in a dose dependent manner compared with control, which was treated with the same volume of extra pure water (Figure 1A). At higher concentration (20mg/mL), number of viable cells found was not significant. The IC50 concentration found from this study was 5mg/mL, which was used for further studies.

### Heliamine induces the apoptosis

Whether the heliamine induced death in *S. cerevisiae* cells was by apoptosis or necrosis was confirmed using AO /EtBr dual as well as by DAPI staining. Heliamine was observed to induce apoptosis at 5mg/mL ($IC_{50}$ concentration) (Figure 2). Compared to the control, significant number of early and late apoptotic cells were seen, whereas live cell count was decreased due to heliamine treatment (Table 1).

1. **A**




1. **B**

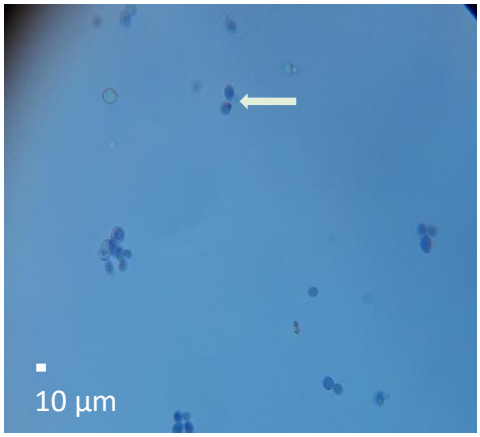



**Figure 1** Heliamine inhibits the growth of *S. cerevisiae* cells. Wild type (BY4741) *S. cerevisiae* cells grown in YPD broth till 1.5 x $10^6$ cells/mL cell density were treated with different concentrations of heliamine for 24 hours, cells treated with same volume of extrapure water was taken as negative control. **A.** The plot of % cell viability versus heliamine treatment was presented using trypan blue dye exclusion assay. **B.** Representative picture of trypan blue dye exclusion assay where the dead cells were stained with trypan blue, arrow indicates the dead cells.

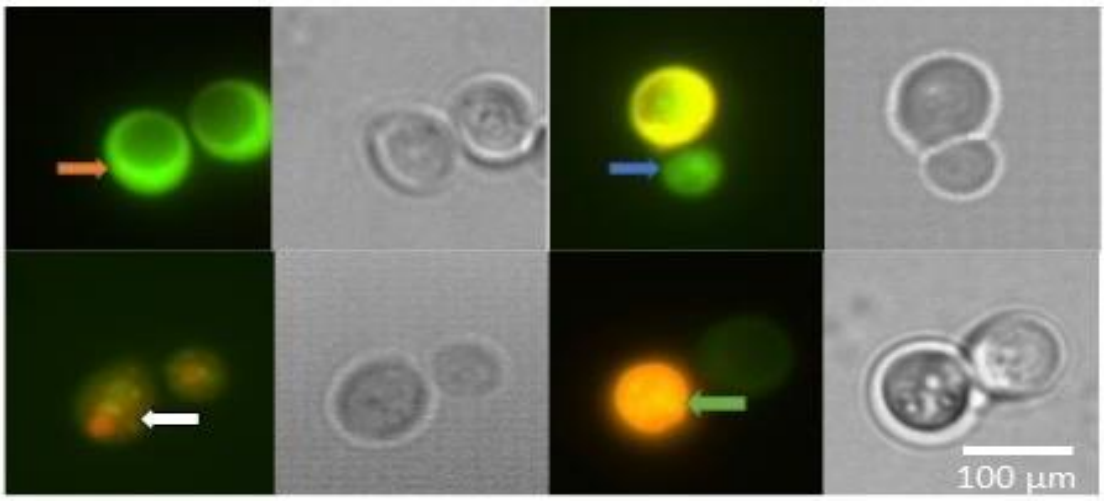
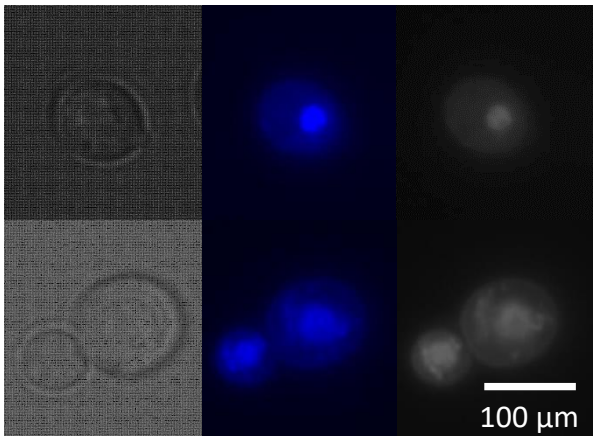



**Figure 2** Acridine Orange / Ethidium bromide staining of *S. cerevisiae* cells. Cells treated with heliamine showing early apoptosis (blue arrow) as well late apoptosis (white arrow) due to condensed chromatin, whereas live cells seen in uniformly green color (red arrow). The necrotic cells were seen in orange color (green arrow).

| Treatment | Live cells (in %) | Early apoptotic cells (in %) | Late apoptotic cells (in %) | Necrotic cells (in %) |
|---|---|---|---|---|
| Control | 92 ± 3 | 1.6 ± 0.5 | 3.7± 1.7 | 5 ± 1 |
| Heliamine 5mg/ml | 45 ± 4.5 | 19 ± 5 | 51.57± 7.8 | 8.7 ± 3 |

**Table 1** Around three hundred cells were examined from each sample for apoptosis evaluation under the fluorescent microscope at 100X objective. Each value represents the mean ± SD of triplicate determinations from three independent experiments.

3. **A**




**3. B**

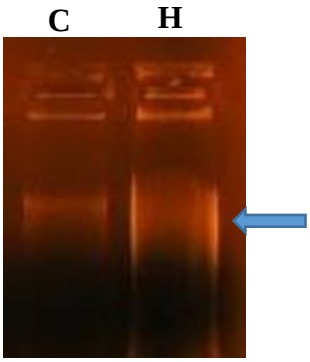



**Figure 3A** DAPI staining of *S. cerevisiae* cells. Cells treated with Heliamine showing condensed chromatine (lower side) compare to live cells (upper side). **3B**. Effect of heliamine on DNA integrity. Arrow indicates the degradation of DNA due to heliamine treatment. C, Control; H, Heliamine treated.

DNA condensation was also evaluated using DAPI staining. Heliamine induced the chromatin condensation at the concentration of 5mg/mL (Figure 3A). Compared to the control around 50% more condensed chromatin appeared in *S. cerevisiae* cells (Table 2).

| Treatment | Chromatin condensation in yeast cells (DAPI staining) |
|---|---|
| Control | 8.79 ± 1.53 % |
| Heliamine 5mg/ml | 48.56 ± 6.3 % |

**Table 2** Around three hundred cells were examined from each sample for apoptosis evaluation under the fluorescent microscope at 100X objective. Each value represents the mean ± SD of triplicate determinations from three independent experiments.

**Heliamine induces the ROS**

Accumulation of intracellular reactive oxygen species is one of the major biochemical responses during apoptosis. The fluorescence probe DCFDA was used to detect ROS. DCFDA is converted into DCF by cellular esterase. DCF after reacting with ROS gives green fluorescence [12]. Heliamine induced more ROS generation compared to control in *S. cerevisiae* cells (Figure 4A). Around 3.5 times more ROS was produced in heliamine treated cells compared to control. Heliamine induced ROS level was decreased due to co-treatment with NAC, which suggests that NAC could control the ROS levels in *S. cerevisiae* cells.

**Heliamine induces the apoptosis inducing factor 1 (*aif1*) mediated apoptosis.**

Whether the induction of apoptosis by Heliamine in *S. cerevisiae* cells involved caspase dependent or caspase independent pathway was studied using metacaspase 1 (Δ*yca1*) and apoptosis inducing factor 1 (Δ*aif1*) mutant strains. Heliamine treated and untreated wild type as well as mutant cells were grown on solid media and colonies were counted. The survival of heliamine treated *aif1* mutant cells was increased compared to wild type and Δ*yca1* cells whereas aif1 overexpressed cells exhibited more susceptibility towards heliamine treatment (Figure 5). As lack of AIF resulted in increased growth but its overexpression prompted in lesser growth, this clearly indicated that apoptosis inducing factor 1 has a potent role in heliamine mediated apoptosis.

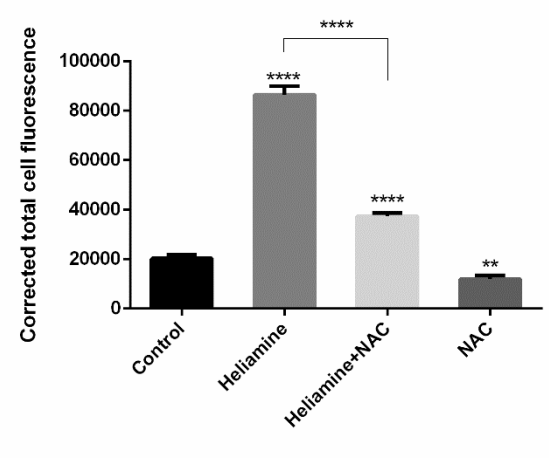



**Fig 4 B** The ROS induced by various treatments were quantified in the form of corrected total cell fluorescence using image J software.

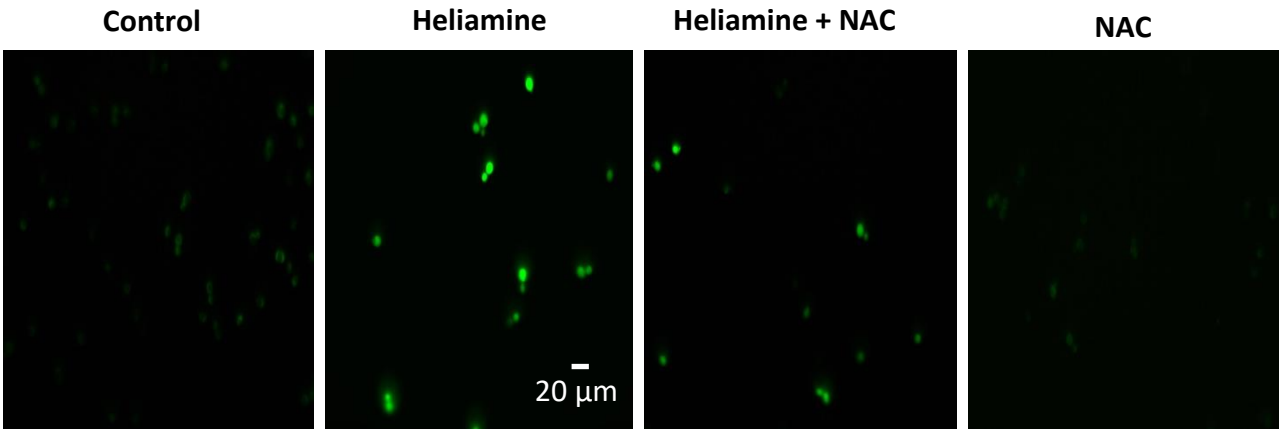



**Figure. 4 A**. ROS levels in *S. cerevisiae* cells treated differently were detected using DCF DA probe. Heliamine treatment significantly increased the ROS levels and co-treatment with NAC controlled it.

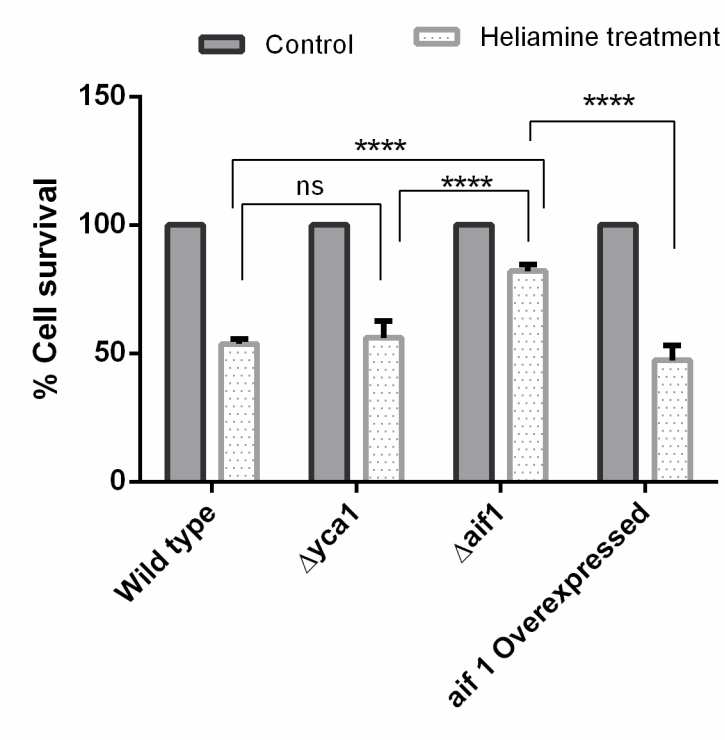



**Figure 5. Heliamine induces apoptosis by activation of apoptosis inducing factor 1 in *S. cerevisiae* cells.** Wild-type, metacaspase mutant (Δycal), apoptosis inducing factor 1 mutant (Δaif1) and apoptosis inducing factor 1 overexpressed of *S. cerevisiae* cells were treated with heliamine (5mg/ml) for 24 hours. After three days of treatment the number of cells survived were counted on YPD agar plats.

## DISCUSSION

Heliamine exhibited dose dependent effect on *S. cerevisiae* cells. At lower concentration it did not inhibit the growth of *S. cerevisiae* cells, however with increasing concentration, it affected the viability significantly. The effective concentration range of heliamine was found to be from 1mg/mL to 10 mg/mL and its IC50 was found to be 5mg/mL (Figure 1). It is useful to find the IC50 concentration of effective compound as it directs its usefulness for medicinal usage. The IC50 concentration of heliamine found in the present studies is quite high as many effective anti-cancerous metabolites reported in the literature have effective concentrations below 1 mg. However, heliamine might be useful for simultaneous treatments with chemotherapeutic agents. Many compounds like citral (3,7-dimethyl-2,6-octadienal), curcumin ((1E,6E)-1,7-bis (4-hydroxy- 3-methoxyphenyl) -1,6-heptadiene-3,5-dione) used in the combination treatments have been found to reduce toxicity and increase the efficacy [10, 13]. Although, Heliamine is reported to have anticancer activity on rat sarcoma 45 cells [5], its IC50 concentration and mechanism of action has not been reported.

Heliamine was found to induce programmed cell death in *S. cerevisiae* cells, as confirmed by AO/EtBr staining. The common characteristics of cells undergoing apoptosis like chromatin condensation and DNA fragmentation were observed in DAPI staining and agarose gel electrophoresis results (Figures 3 A, 3 B). Many compounds at higher concentrations show undesirable effects, for example hydrogen peroxide induces apoptosis at lower concentrations but becomes necrotic at higher concentrations, but heliamine was found to induce apoptosis even at 5 mg/mL concentration.

ROS is the central molecule for apoptosis induction and regulation [5, 11]. ROS was found to be increased in heliamine treated cells and simultaneous treatment by NAC (anti-oxidant) could control the ROS levels as well as the anti-proliferative activity of heliamine (Figures 4 A, 4 B). Mechanism for induction of apoptosis by heliamine was further investigated using apoptosis pathway mutants of *S. cerevisiae*. The metacaspase mutants of *S. cerevisiae* cells were expected to grow in heliamine treated cells, as they lacked the metacaspase enzyme, however such cells showed almost similar growth as compared to heliamine treated wild type cells, which implied that metacaspase might not have any role in the heliamine induced apoptosis. The other mutant strains, *aif 1* mutants, on the contrary showed more growth in heliamine treated cells in spite of lack of apoptosis inducing factor (AIF) (Figure 5). This result clearly indicated that AIF might be required to induce apoptosis in heliamine treated *S. cerevisiae* cells. To confirm this, *aif1* overexpressing strains were employed, which upon heliamine treatment, could not grow well and confirmed induction of apoptosis in the presence of *aif1*. AIF1 is a

mitochondrial flavoprotein in the intermembrane space, important for cellular homeostasis. Upon induction of apoptosis, it gets translocated to nucleus, where it leads to chromatin condensation and DNA fragmentation [14, 15]. DNA fragmentation in yeast cells was confirmed by agarose gel electrophoresis and chromatin condensation was observed by DAPI staining. These results confirm that heliamine induces apoptosis in *S. cerevisiae* cells by caspase independent, AIF mediated pathway.

## CONCLUSION

Heliamine was found to have anti-proliferative effect as it induces apoptosis in *S. cerevisiae* cells and its IC50 was found to be 5 mg/mL. The metacaspase mutant strain of *S. cerevisiae* was observed to be sensitive to heliamine treatment but *aif1* mutant was found to be resistant. The reduced growth in metacaspase mutant and increased growth in *aif1* mutant cells in heliamine treated cells, in comparison to similarly treated wild type strain, indicated that AIF might be involved inducing the apoptosis. The *aif1* overexpressing cells exhibited more susceptibility (reduced growth) in heliamine treated cells, which confirmed the role of AIF in heliamine mediated apoptosis. Based on these results it was concluded that heliamine induces ROS mediated, apoptosis inducing factor 1 dependent apoptosis in *S. cerevisiae* cells. The results found could be useful in assessing the role of heliamine in combinatorial chemotherapy treatment.

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