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Polyherbal decoction modulates redox homeostasis during Malachite green induced metabolic stress in *Saccharomyces cerevisiae*

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Malachite green (MG) is aquatic pollutant that induces oxidative stress when comes in contact with the living organisms. In *Saccharomyces cerevisiae*, MG produces intracellular reactive oxygen species (ROS) and these ROS disturb redox homeostasis and cellular functions leading to early cell death. Exogenous supply of natural antioxidants containing polyherbal decoction may play a crucial role in re-establishment of redox homeostasis by ensuring the cell survival. Exposure of MG to *Saccharomyces cerevisiae* resulted in a significant decrease (97.8%) in colony forming units (CFU). An Ayurvedic polyherbal formulation *'Vayasthapana Rasayana'* (VR) which contains natural antioxidants from plants *viz. Terminalia chebula, Clitoria ternatea, Boerhaavia diffusa, Centella asiatica, Phyllanthus emblica, Asparagus racemossus and Tinospora cordifolia* at 1.0 mg/mL concentration could arrest the oxidative stress during MG exposure. Levels of ROS elevated up to 67.3% on MG exposure; while VR supplementation reduced it by 54.7%. MG induced cellular apoptosis in 38% and necrosis in 27% cells, while VR augmentation reduced it to 8%. Activities of antioxidant enzymes like catalase, superoxide dismutase and glutathione peroxidase in MG exposed cells were induced by 408, 144 and 140%, respectively, whereas, VR supplementation lowered the expressions to 102, 57 and 111%, respectively. Induction in caspases 3/7 activity was also found to be reduced by 65.39% after VR augmentation. Similarly, VR modulated activities of oxido-reductases like lignin peroxidase, laccase, NADH-DCIP reductase and MG reductase. VR supplementation also maintained the MG utilization potential of *S. cerevisiae* up to 20th exposure cycle which was otherwise arrested to 8th cycle. The treatment also decreased the ROS accumulation and nuclear damage, restoring the cell viability up to 94% and retained normal growth dynamics. Thus, VR supplementation could significantly decrease oxidative stress, enhance cell viability and ultimately protect the dying *S. cerevisiae* cells during MG exposure.

Keywords: Anti-aging, Antioxidants, Apoptosis, Aquatic pollutantion, Ayurvedic, Herbal, Oxidative stress, Redox homeostasis, *Vayasthapana Rasayana*

Synthetic textile dye and a common antimicrobial agent Malachite Green (MG) is regularly used as colouring agent, food additive, disinfectant and anthelmintic[1]. In aquaculture industries, MG is used as a potent antifungal agent[2]. According FAO and WHO; MG is classified as category C-III polutant[3]. MG is reported as cytotoxic, genotoxic and oxidative stress inducer on the kidney and gill cell lines of *Channa striata* fish[4]. MG was responsible for inducing free radical formation in cultured mammalian cells and is also regarded as a potent tumor promoter and is known to induce malignancy[5]. The mechanism of anti-Candida (*Candida albicans*) action of MG has been reported because of the increased generation of ROS, labile iron deprivation and cell necrosis[6].

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MG, in contact with the living cells, exerts oxidative stress by disturbing balance between the levels of oxidants and antioxidants in favor of oxidants. These oxidants are a group of free radicals mainly reactive oxygen species (ROS). Elevated levels of ROS can also be observed in certain physiological conditions leading to lipid peroxidation and oxidative DNA damage. Intracellular ROS mainly comprise $O_2^{\bullet-}$, $NO^{\bullet-}$, oxidizing radicals like $^{\bullet}OH$, $RO^{\bullet}$, $ROO^{\bullet}$, $^1O_2$, etc.[7]. ROS react with biomolecules such as lipids, proteins and DNA and generate a number of lipid, sugar, amino acids and thiyl as secondary radicals[8]. Interactions between ROS and membrane lipids also lead to lipid peroxidation forming lipid hydroperoxide (LOOH). ROS can react with proteins causing changes in their tertiary structure *viz*. protein-protein cross linkages, fragmentation and proteolytic degradation. In addition, ROS can interact with DNA leading to mutation in DNA, strand breakage, DNA-protein cross-linkage and damage the DNA repair system[9].

Effect of oxidative stress can be monitored using model organisms like yeasts. The yeast system has been used to study the relationship between different kinds of stress like heat stress, oxidative stress by different oxidants like H_2O_2, paraquat, diamide, osmotic stress and salt tension[10-13]. Furfural induced oxidative stress has been reported to show accumulation of ROS with disintegration of nuclear chromatin, vacuole membranes, mitochondrial and actin cytoskeleton in *Saccharomyces cerevisiae*[14]. Effect of menadione induced oxidative stress on transcriptional, post transcriptional, translational and post translational processes were studied in *S. cerevisiae* to elucidate the regulation of gene expression of antioxidant enzymes[15]. Acrolien, chromate and arsenic induced oxidative stress in *S. cerevisiae* and *Escherichia coli* K-12 was responsible for cell death, physiological changes, caspase and mitochondria mediated apoptosis[16-18]. Such stress may lead to inhibition of growth because of arrested replication due to nuclear damage, lipid peroxidation and aggregation of proteins.

In this work, oxidative stress exerted by MG on *S. cerevisiae* was hypothesized to be relieved using natural antioxidants from a decoction of polyherbal ayurvedic formulation popularly known as '*Vayasthapana Rasayan'* (VR). The literal meaning of VR is 'anti-aging formulation'. Several reports showing the application of natural antioxidants against aging and oxidative stress are available. Traditionally known important herbs such as *Terminalia chebula*, *Clitoria ternatea*, *Boerhaavia diffusa*, *Centella asiatica*, *Phyllanthus emblica*, *Asparagus racemossus*, and *Tinospora cordifolia* were used to prepare this formulation[19]. VR has potential to scavenge free radical and anti-aging property. Additionally, VR contains pharmacological properties *viz.* hepatoprotective, immunomodulation, antianxiety, antistress, antidepressant, adaptogenic, etc. which attenuates the aging process[19]. The benefits of VR showed brilliance, long life, excellent memory, pleasant colour, great strength of body and sense organs. It also offers fine strength and delay of the aging process[19]. In the present study, *S. cerevisiae* was used as a model organism to study protective effect of VR against MG induced oxidative stress, caspases mediated apoptosis and necrosis.

Materials and Methods

Chemicals

Annexin-V-FLUOS Staining Kit (Roche), caspase-Glo® 3/7 assay kit (Promega), MG and ABTS (2, 2-azino-bis-3-ethyl benzothiazoline-6-sulphonic acid) were obtained from Hi media Laboratory, India. Superoxide dismutase (SOD) assay kit (Cat. No. 19160), glutathione peroxidase (GPx) assay kit (Cat. No. CGP1), catalase (CAT) assay kit (Cat. No. CAT100), 4', 6-diamidino-2-phenylindole (DAPI) (Cat. No. D9542), and 2'7'-dichlorofluorescin (H_2DCF) (Cat. No. D6883) were procured from Sigma Aldrich, USA. Rest of the chemicals and solvents used were of analytical grade.

Culture strain and maintenance

Baker's yeast *S. cerevisiae* used in the study was obtained from National Collection of Industrial Microorganisms (NCIM-3594). Culture was routinely maintained on 5% dextrose, 1% peptone, 1% yeast extract and 3% agar (w/v). Cells used for present experimentation were always grown on slightly modified medium containing 2% dextrose, 0.1% peptone, 0.1% yeast extract (w/v). The cells required for studies were harvested from the medium inoculated with 10% (v/v) inoculum at room temperature (30±2°C) for 24 h by centrifugation at 7000 rpm for 7 min at 4°C.

Plant material

Entire plant of *Boerhaavia diffusa*, *Clitoria ternatea*, *Phyllanthus emblica*, unripe fruits of *Terminalia chebula*, leaves of *Centella asiatica*, mature roots of *Asparagus racemossus* and mature stem of *Tinospora cordifolia* were procured from M/s Green Pharmacy, Pune, India. The plant material was finely powdered using domestic grinder and used further for extraction.

VR decoction preparation

Standard VR Formulation was prepared by mixing the fine powders of all plants in equal proportion. Three grams of powdered sample of each plant was added in 100 mL ethanol and kept for shaking at 150 rpm, 30°C for 24 h[20]. The extracts were filtered through Whatman filter paper No. 1 and centrifuged at 10000 rpm, at 10°C for 10 min to obtain a clear supernatant. After determining the extraction yield, concentration of the extract was adjusted in a way to obtain 1 mg/mL working concentration and stored in amber colored bottle at 4°C till further use[19].

MG utilization Pattern by *S. cerevisiae*

Two grams of *S. cerevisiae* cells were transferred to 100 mL medium containing MG alone (100 mg/L), similarly, extracts were augmented to flask, independently and MG augmented with VR extract separately (1.0 mg/mL) which was kept at 150 rpm

and room temperature (30±2°C). A biotic control at the static condition was kept at the same temperature. Similarly, abiotic controls with dye and devoid of yeast cells were also kept at shaking as well as static conditions. The utilization of MG was observed at different time intervals. For this, an aliquot (3 mL) of the medium was withdrawn, centrifuged to separate the cell mass and optical density of the supernatant was measured at 620 nm. The percentage utilization was calculated using the following equation.

$$\% \text{ MG Utilization} = \frac{(\text{Initial absorbance} - \text{observed absorbance})}{\text{Initial absorbance}} \times 100$$

Cell viability studies

After MG utilization, the cell of *S. cerevisiae* was investigated for their viability. One milliliter of culture media containing cells from the utilization experiment were serially diluted (10^{-9}) in 0.85% saline solution and spread on Petri plates with a slightly modified culture medium containing 2% dextrose, 0.1% peptone, 0.1% yeast extract and 3% agar (w/v). These cultures were incubated at room temperature (30±2°C) for 48 h and colony forming units (CFUs) were counted by using colony counter.

Effect of exposure of MG and augmentation of VR on the growth of *S. cerevisiae*

Growth curve of *S. cerevisiae* exposed to MG alone and MG in combination with VR along with control was studied in order to understand growth pattern of *S. cerevisiae*. For this, a loop full culture of *S. cerevisiae* was inoculated in growth medium mentioned earlier which was fortified with MG alone and MG in combination with 1.0 mL of VR (1 mg/mL). Growth of cells was monitored by measuring absorbance at 620 nm at every 1 h interval for 72 h.

Determination of intracellular ROS

Intracellular ROS levels were determined using 2'7'-dichlorofluorescin (H_2DCF) on fluorescence spectrophotometer[21]. The dye H_2DCF penetrates plasma membrane and reacts with ROS present inside the cell. Non-fluorescent H_2DCF on oxidation by ROS shows fluorescence which is determined spectrofluorometrically. For this analysis, cells were harvested during and after MG utilization at three different time intervals (0, 1 and 2 h), respectively by centrifugation in 2 mL aliquots. Harvested cells were washed with 2 mL of 50 mM phosphate buffer (pH 7.4) thrice and re-suspended in same buffer. The cell suspension was pre-incubated at 28°C for 10 min. To this suspension, 1 mM ethanolic stock solution of H_2DCF was added to achieve 10 µM final concentration of the dye and incubated at 28°C for 20 min. After incubation, cell suspension was centrifuged and supernatant was measured for fluorescence (excitation at 488 nm and emission at 520 nm for each sample) using fluorescence spectrophotometer 8300 (Agilent Technologies, USA).

Enzymatic analyses

Two grams of *S. cerevisiae* cells of control, MG exposed and cells exposed to MG augmented with VR were independently homogenized in chilled 1.0 mL of 50 mM phosphate buffer (pH 7.4) using a homogenizer and then sonicated with output at 60 amp, giving three strokes each of 30s at 2 min intervals at 4°C. The homogenate thus obtained was then centrifuged. The supernatant was used as source of laccase, lignin peroxidase, NADH-DCIP reductase, MG reductase, glutathione peroxidase, superoxide dismutase and catalase.

Activities of laccase, lignin peroxidase, NADH-DCIP reductase and MG reductase were measured as per the methods described by Jadhav and Govindwar[22]. During utilization of MG; the cells of *S. cerevisiae* undergo oxidative stress and hence expression levels of glutathione peroxidase, superoxide dismutase and catalase were measured as a function of exposure to MG alone and cells exposed to MG augmented with VR. The enzyme activity was measured strictly as per the manufacturer's protocol. All enzyme assays were performed at 27°C and were run in triplicates. The protein content of all the samples was determined as per Lowry's method[23].

Caspase 3/7 activity during MG exposure along with VR supplementation

Caspase 3/7 activities in *S. cerevisiae* were measured using a Caspase-Glo® 3/7 Assay kit (Promega). The Caspase-Glo® 3/7 Assay is a luminescent assay that measures caspase 3 and 7 activities in purified enzyme preparations or cultures of adherent or suspension cells. Proluminescent caspase-3/7 substrate with tetrapeptide sequence (DEVD) is cleaved to release aminoluciferin, a substrate of luciferase leading to generation of light. Addition of the single Caspase-Glo® 3/7 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate and generation of a "glow-type" luminescent signal (Caspase-Glo® 3/7 Assay kit Promega).

A 100 µL of *S. cerevisiae* (10^6) cells collected after MG exposed and MG with VR augmentation were

washed thrice with the sterile distilled water and re-suspended in 100 µL of 50 mM phosphate buffer (pH 7.4) independently at time intervals of 0, 1 and 2 h. Re-suspended cells were homogenized and then sonicated at 60 amp, giving three strokes each of 30 s with 2 min intervals at 4°C. The homogenate thus obtained was further centrifuged and used for monitoring the caspases activity. The 1:1 proportion of Caspase-Glo® 3/7 Reagent solution was independently added in to the above supernatants and incubated for 2 h at room temperature. The luminescence of each sample was measured in using Fluorescence/Luminescence Spectrophotometer 8300 (Agilent Technologies, USA).

Fluorescence microscopic analysis

Fluorescence microscopic analysis was carried out on Zeiss Axio-scope A.1 trinocular phase contrast microscope with fluorescent attachment. The analysis of intracellular oxidation and nuclear chromatin damage was studied using fluorescent H_2DCF, DAPI and Annexin V which were specific for intracellular oxidation, nuclear chromatin and identification of pre apoptotic cells. The cells were collected during and after MG utilization at 3 time intervals (0, 1 and 2 h), respectively, washed thrice with the sterile distilled water and re-suspended in 0.1 mL of 50 mM phosphate buffer (pH 7.4). To this, 1 mM ethanolic stock solution of H_2DCF was added to achieve 10 µM final concentration of the dye and incubated at 30°C for 2 h. Cells were again centrifuged after incubation, washed and re-suspended freshly in 50 mM phosphate buffer (pH 7.4). The cells were observed using FITC-Spectrum Green (Chroma3101) comprising 25 mm diameter filters, excitation filter (D 480/30), beam splitter (440 DCLP) and emission filter (D 535/40) and images were taken. For observing organization of nuclear chromatin, the cells were harvested during and after MG utilization at 3 time intervals (0, 1 and 2 h), respectively, washed with sterile distilled water, re-suspended in 10 µL of sterile distilled water and fixed by addition of formaldehyde (1%). The fixed cells were stained with 1.0 µL of 20 mg/mL of 4',6-diamidino-2-phenylindole (DAPI)[14] and observed under DAPI filter (Chroma 3100) comprising 25 mm diameter filters, excitation filter (D 350/50), beam splitter (400 DCLP), emission filter (D 460/50) and images were taken.

The analysis of apoptosis and necrosis during the exposure of MG alone and augmentation with VR was carried out using annexin-V-fluos staining kit (Roche). The analysis of phosphatidylserine on the outer leaflet of apoptotic cell-membranes was performed using annexin-V-fluorescein and propidium iodide (PI) for the differentiation from necrotic cells and its labeling with cell surface marker for characterization. Preparation of diluted annexin-V-fluos labeling solution was carried out taking pre-diluted 20 µL annexin-V-fluos labeling reagent in 1 mL of incubation buffer and adding 20 µL PI solutions. The cells (10^6) were collected after MG utilization, washed with phosphate buffer saline (PBS) and centrifuged at 3000 rpm for 5 min. The cell pellet was re-suspended in 100 µL of diluted annexin-V-fluos labeling solution and incubated for15 min at 25°C. The cells were observed using FITC-Spectrum Green (Chroma3101) comprising 25 mm diameter filters, excitation filter (D 480/30), beam splitter (440 DCLP), emission filter (D 535/40) and images were taken.

Application of VR during repetitive MG utilization cycles

Role of augmentation with VR in increasing the utilization efficacy of *S. cerevisiae* was investigated. For this, 2 g cells of *S. cerevisiae* were collected by centrifugation at 7000 rpm for 7 min under cold conditions and transferred to 100 mL broth containing MG (100 mg/L), and three independent sets containing MG augmented with VR (1.0 mg/ mL) and kept at room temperature (30±2°C) with shaking at 150 rpm. The utilization of MG was measured after every 2 h and cells were separated by centrifugation. The removed media was used for the study of MG utilization by measuring the absorbance at 620 nm. This process was considered as one cycle of MG utilization. Further, fresh 100 mL growth medium containing MG and VR was added in same cell mass and incubated as described earlier. Twenty cycles were similarly performed and the MG utilization percentages for all the treatments noted.

Statistical analysis

Data were analyzed by two-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test. A value of P <0.05 was considered statistically significant.

Results

Reversal of MG induced ROS accumulation, nuclear disorganization and apoptosis after VR supplementation

Intracellular ROS generation was estimated using H_2DCF and subsequent fluorescence measurement. Increase in fluorescence is directly proportional to the magnitude of ROS present. In present study, the MG

exposed cells showed 17.5 a.u. of fluorescence while the control cells ROS gave 2.6 a.u. of fluorescence. Fortification with VR showed reduced fluorescence of 3.3 a.u. indicating a lowered intracellular ROS level in *S. cerevisiae*. Subsequently, the cells stained with H_2DCF were observed under fluorescence microscope and it was observed that 97% of the MG exposed *S. cerevisiae* cells showed accumulation of ROS. On the other hand, control cells being unexposed to MG did show lowest accumulation of ROS which may be of metabolic origin. In case of cells treated with VR even in presence of MG showed reduction in intracellular ROS accumulation (30-35% of the cells) (Fig. 1).

Oxidative stress is known for inducing nuclear damage; hence, the effect of MG exposure on nuclear damage was studied using DAPI. Here, the cells exposed to MG alone showed 80% nuclear damage whereas the VR augmentation revealed only 25% nuclear damaged cells which was comparable with the control cells (Fig. 1). Apoptosis and necrosis are thought to be associated with oxidative stress. Staining with annexin V and PI revealed that the control cells did not show apoptosis and necrosis Fig. 2), whereas, 38 and 27% of the total MG exposed cells underwent apoptosis and necrosis, respectively (Fig. 2). Augmentation with VR on the other hand was observed to reduce apoptotic and necrotic cells to 8% of the total cells observed (Fig. 2).

Alterations in expression patterns of antioxidant and oxidoreductase enzymes as a function of MG exposure and VR augmentation

Tremendous generation of ROS as a result MG exposure was found to be responsible for induction of antioxidant and various oxido-reductases. The MG exposed cells showed 408, 144 and 140% induction in the activities of CAT, SOD and GPx, respectively when compared with control cells. Inclusion of VR however revealed noteworthy reduction in the activities of CAT (102%), SOD (57%) and GPx (111%) (Table 1). This indicated the decreased oxidative stress as a result of exogenous supply of antioxidants in the form of VR.

It was simultaneously observed that *S. cerevisiae* could utilize MG and therefore the involvement of several oxidoreductase enzymes was also studied. Inductions in the activities of lignin peroxidase,

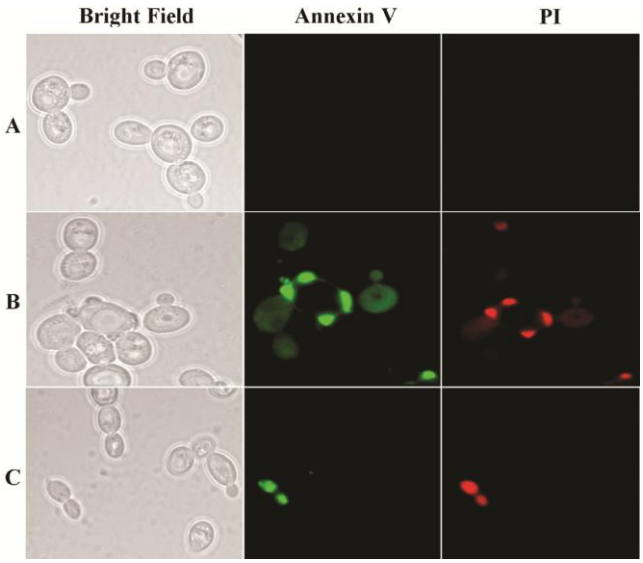

Fig. 2 — Effect on apoptosis and necrosis in *S. cerevisiae* (A) Control; (B) Malachite green exposed cells; and (C) MG exposed cells with VR augmentation

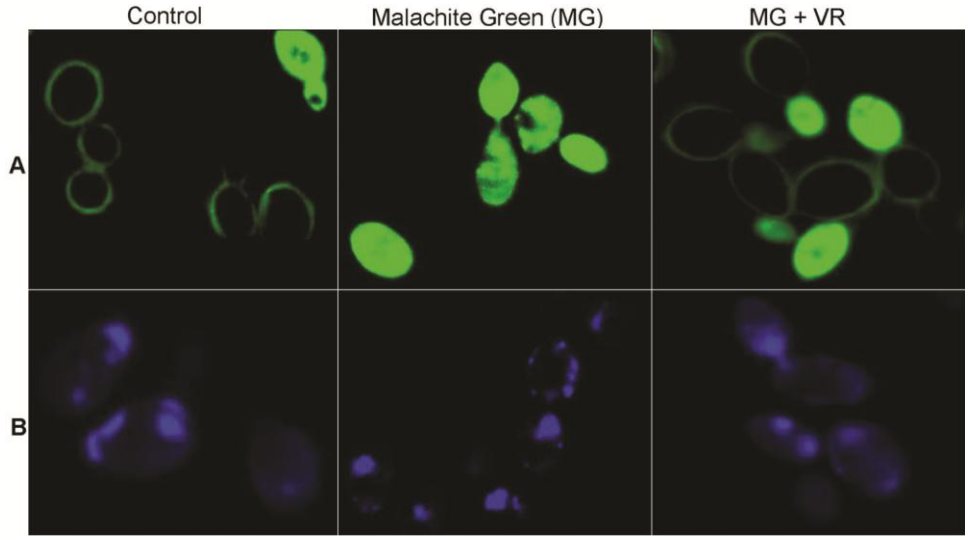

Fig. 1 — Effect of exposure of MG and supplementation with VR on (A) accumulation of ROS; and (B) Nuclear damage in *S. cerevisiae*

Table 1 — Activities of enzymes involved in antioxidant defense and MG utilization with and without VR fortification

Enzyme	Control		MG		MG + VR	
	0 h	2 h	0 h	2 h	0 h	2 h
CAT[a]	0.0022±0.0004*	0.0037±0.0008*	0.0059±0.0002*	0.0181±0.0006*	0.0045±0.0002*	0.0093±0.0006*
SOD[b]	0.007±0.002*	0.063±0.006*	0.022±0.002*	0.154±0.006*	0.007±0.002*	0.097±0.006*
GPx[c]	0.022±0.002*	0.050±0.004*	0.035±0.002*	0.122±0.004*	0.022±0.002*	0.057±0.004*
LiP[d]	0.025±0.005*	0.326±0.002*	0.457±0.002*	0.736± 0.003*	0.26±0.003*	0.403±0.001*
Laccase[d]	1.094±0.001*	1.618±0.003*	2.533±0.002*	4.975±0.004*	1.062±0.004*	2.633±0.005*
NADH-DCIP reductase[e]	0.277±0.002*	1.185±0.003*	1.354±0.001*	3.734±0.003*	0.293±0.003*	1.415±0.003*
MG reductase[f]	0.085±0.003*	0.278±0.002*	0.507±0.001*	1.426±0.002*	0.048±0.002*	0.629±0.004*

[Values are a mean of three experiments ± SEM, significantly different from control (0 h) at **P* <0.05, ***P* <0.01 and ****P* <0.001 by two-way ANOVA without replication using MS Excel. [a]nmol of H_2O_2 utilized (Units mL^{-1}); [b]50% inhibition of the NBT photoreduction rate (% Inhibition); [c]Activity in mmol min^{-1} mL^{-1}; [d]Activity in Units min^{-1} mL^{-1}; [e]µg of DCIP reduced min^{-1} mg $protein^{-1}$; and [f]µg of MG reduced min^{-1} mg $protein^{-1}$]

laccase, NADH-DCIP reductase and MG reductase by 125, 207, 215 and 415%, respectively was noted in presence of MG. VR augmentated *S. cerevisiae* cells nevertheless showed lesser induction in the activities of lignin peroxidase (82%), laccase (88%), NADH-DCIP reductase (163%) and MG reductase (126%) (Table 1). As evident from the oxidoredustase enzyme assays, the MG exposed cells were observed to show greater inductions in enzyme activities. However, beacause of VR augmentation and reduced oxidative stress levels, inductions in the activites of oxidoreductive enzymes were comparetively lowered. The utilization of MG by *S. cerevisiae* cells yet contineued showing involvement of oxidoreductive enzymes.

Inhibition of caspase 3/7 activity after VR supplementation

Apoptosis marker enzyme caspase was found to show lowered expression in terms of activity in the *S. cerevisiae* cells after VR supplementation to the culture media. On the contrary, the MG exposed cells showed enhanced expression of caspases 3/7. At 1 h, the enzyme activity in the MG exposed cells was found to be enhanced by 74%, however, the VR augmented cells gave only 56.85% elevated activity. Upon further exposure *viz*. 2h, the MG exposed cells showed 95.51% enhancement in the caspases 3/7 activity which was on the other hand only 30.12% after VR augmentation when compared to control (Fig. 3). The VR thus protected the *S. cerevisiae* cells from growth inhibition leading to apoptosis.

VR improves cell viability and growth dynamics of *S. cerevisiae* after MG exposure

Toxic effect of MG exposure substantially reduced the CFUs to 2 x 10^9 when compared to the control CFUs of 91×10^9. The augmentation of VR conversely showed the protective effect on *S. cerevisiae* achieving 38×10^9 CFUs. The inhibitory effect of MG

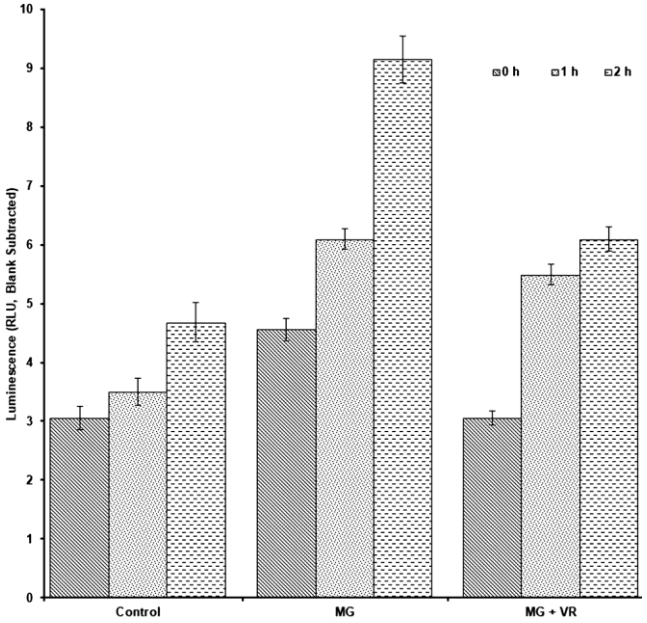

Fig. 3 — Inhibition of caspases 3/7 after VR supplementation during MG exposure in *S. cerevisiae*. [Data presented are mean ± standard deviation of three parallel experiments. **P* ≤0.05]

was evident from the extended log phase of *S. cerevisiae* growth. The cells were found to show a delayed initiation of log phase commencing from 32 h meaning that the lag phase was extended up to 32 h as compared to control cells which demonstrated the exponential growth after 4 h. Augmentation of VR to MG exposed cells was observed to protect the cells from toxic effect of MG and restored a normal initiation of log phase. Further, MG exposed cells continued log phase from 32 to 53 h and then entered the stationary phase. Control and VR augmented cells on the other hand showed the log phases in between 4-15 h and 6-25 h of growth (Fig. 4). The oxidative stress produced by MG must be responsible for the reduction of CFUs and alterations in the

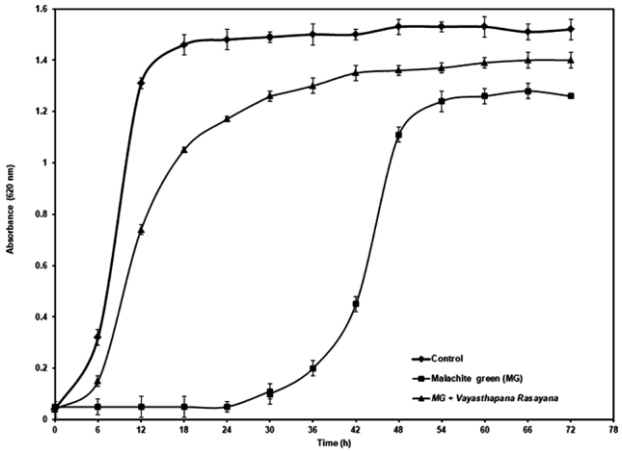

Fig. 4 — Growth dynamics of *S. cerevisiae* as function of exposure to MG and augmentation with VR. [Data presented are mean ± standard deviation of three parallel experiments. **P* ≤0.05]

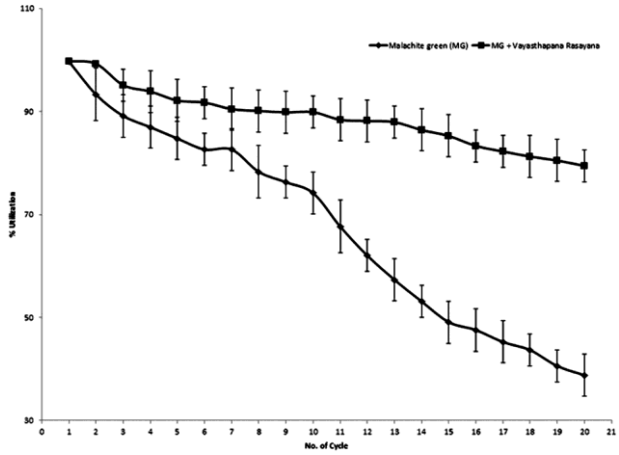

Fig. 5 — Effect of VR augmentation on MG utilization efficacy of *S. cerevisiae* over 20 utilization cycles. [Data presented are mean ± standard deviation of three parallel experiments. **P* ≤0.05]

growth patterns of *S. cerevisiae.* While antioxidants supplied in the form of VR extract may have played a crucial role in scavenging the produced ROS and protected the cells ultimately enhancing their survival.

VR supplementation enhances MG tolerance in *S. cerevisiae* upon repeated exposure

As VR fortification was observed to reduce the oxidative stress and ultimately protected cells restoring the CFUs, the same cells mass was investigated for stress tolerance upon repeated exposure of MG. The utilization of MG by *S. cerevisiae* was observed to be reduced from 99.68% to 78.26% at the end of 8th cycle in case of un-augmented cell mass. It was drastically reduced further during repeated exposure and at the end of 20th cycle only 38.77% MG utilization was noted. Further, the addition of VR revealed to MG exposed cells was found to enhance the tolerance achieving 90.10% utilization at the 8th cycle. At the end of 20th cycle of MG utilization, the VR supplemented cells could consume 79.44% which was superior to the un-augmented cells (Fig. 5).

Discussion

Malachite green (MG), a known antifungal is reported to be utilized up to 95.5% by *S. cerevisiae*[22] nonetheless; it has also been shown to undergo abiotic stress during this exposure[24]. As a toxic chemical, MG is also known to show deteriorative carcinogenic effects on reproductive and immune system of the rats[25]. MG being routinely utilized dye stuff and a major contaminant of water bodies has a potential to enter the human food chain[26]. In the light of these facts, it is imperative to look for an efficient remedy that can reduce the toxicity of MG. Generation of ROS is the main reason for oxidative stress which manifests in to disorders such as ageing and malignancy, heart diseases and memory disorders in human beings[19]. In the same work, VR, a poly herbal formulation has demonstrated strong ROS scavenging potential. The loss in cell mass was evident by the reduced CFUs after MG exposure. The VR supplementation on the other had was found to be restoring the normal CFUs.

S. cerevisiae being a unicellular eukaryote, can prove to be a suitable tool to study the oxidative stress induced apoptotic and necrotic effects of toxic substances[27,28]. Based on this notion, it was hypothesized to use MG challenged *S. cerevisiae* to scrutinize the ameliorative effects of a well known polyherbal formulation VR. Oxidative stress due to the presence of MG has been shown to be the key reason behind early apoptosis and necrosis in *S. cerevisiae*[24]. In this study, MG has been found to exert strong oxidative stress on *S. cerevisiae* due to generation of ROS and cause loss of cells viability, reduction of growth, nuclear damage and caspases mediated apoptosis. *S. cerevisiae*, although utilized MG remarkably but faced oxidative stress. Supplementation of VR did not alter the MG tolerance of cells but was found to reduce oxidative stress as evident by the decreased expression levels of antioxidant enzymes. This may have occurred because of the presence of phenolic compounds from each of the constituent plant from VR[19]. Muscadine grapes

and blueberry phenolics were shown to have protective effect on HepG2 cell, restoring 50% viability[29]. Similarly, polyphenols from apple proved to be efficient in increasing the cell viability and life span of yeast cells by countering oxidative stress[30]. Like MG furfural was also observed to be responsible for ROS accumulation and subsequent nuclear and cellular damages[14]. Managing oxidative stress can be a wise strategy to protect the cells from nuclear disorganization which may lead to cell death. Generation of ROS was reported to be a key modulator of apoptosis in yeasts like *S. cerevisiae*[31]. In the present study, VR augmentation was found to protect the cells and restored viability. Extracts of *Terminalia chebula*, *Clitoria ternatea* and *Boerhaavia diffusa* have been shown to restore cell viability in yeast by virtue of their phenolics[24].

Plant metabolites deliver straight protection against oxidative damage and help endogenous enzymatic antioxidants through synergism in ROS quenching[32]. The levels of antioxidant enzyme CAT, SOD and GPx were observed to be reduced after fortification with VR. Natural products of plant origin were found to be effective in increasing resistance of yeast towards H_2O_2 induced oxidative stress by preventing glutathione peroxidation with simultaneous reduction in CAT and SOD activities[32]. Findings of this work are well in agreement with the previous reports. Nuclear damage was the key reason behind the loss cell viability. Apoptosis in *S. cerevisiae* was proved because of DNA damage due accumulation of ROS[33]. Strong radical scavenging potential of VR has been reported by researcher[19].

Accumulation of ROS is known as the vital cause of cellular damages[34]. Important biomolecules like carbohydrates, proteins, lipids and DNA were oxidized by aggregated ROS and ultimately resulted in to irregular cellular membrane, reproductive functions and lethality in all aerobic prokaryotes and eukaryotes[35]. During experiments, it was observed that the MG exposed *S. cerevisiae* showed an elevated number of apoptotic and necrotic cells. Amassing of ROS in the cells of *S. cerevisiae* was shown to trigger the apoptosis cascade[36] Activated caspases are known to be associated with cellular apoptosis in mammals. Activation of initiator caspases (8, 9, 10 and 12) takes place in response to stimuli like ROS which in turn activates effector caspases (3, 6 and 7) leading to cell death[37]. In the light of this, it was imperative to study the effect of VR supplementation on inhibition of caspase 3/7 in presence of MG. This study shows a reduction in caspase 3/7 activity as a function of VR supplementation indicating reduced cell death.

A reduced number of apoptotic and necrotic cells after VR supplementation revealed its ROS scavenging potential. In this work, MG showed deteriorative effect on the cell growth of *S. cerevisiae*. Log phase of the growth was observed to be extended affecting the normal cell proliferation. The VR augmentation however restored the normal growth phase. Inclusion of major lipid peroxidation products like 4-hydroxy-2-nonenal, an aldehyde was shown to inhibit the cell cycle progression of *S. cerevisiae*[38]. A sequiterpene dialdehyde like polygodial was found to be responsible for enhanced ROS generation by reducing the glutathione content in *S. cerevisiae*[39,40]. All these studies are the evidences of oxidative stress dependent inhibition of *S. cerevisiae* cell growth.

Toxic effect of accumulated products after bio-catalysis generally inhibits the growth of cells leading to cell death in some cases[41,42]. MG tolerance in *S. cerevisiae* was evaluated after repeated exposure of MG over 20 cycles on same biomass of *S. cerevisiae*. Because of the eventual protection provided by VR augmentation, the cells demonstrated a prolonged resistance to MG induced oxidative stress. On the other hand, unaugmented *S. cerevisiae* cells after MG exposure could tolerate dye only for 8 utilization cycles. However, the VR fortification provided additional viability to cells achieving 20 cycles of tolerance. Supplementation with natural polyphenols from apple has shown an increase in life span of *S. cerevisiae*[30,43]. Findings of this work are well in accordance with the earlier reports. The approach of VR supplementation could be helpful in increasing life span of organisms exposed to toxic chemicals, and enhance health and efficiency against the stress. During utilization of MG, inductions in the enzyme activities of lignin peroxidase, laccase, NADH-DCIP reductase and MG reductase has earlier been reported[44,45]. The reduced stress was found to be responsible for lowering the expression levels of these enzymes after VR fortification.

Conclusion

The MG induced oxidative stress was observed to be detrimental to growth, viability and life span of *S. cerevisiae*. Accumulation of ROS led to nuclear damage and loss of CFUs decreasing the MG tolerance of cells. The supplementation of

Vayasthapana Rasayan (VR) which contains natural antioxidants from plants *viz*. *Terminalia chebula, Clitoria ternatea, Boerhaavia diffusa, Centella asiatica, Phyllanthus emblica, Asparagus racemossus and Tinospora cordifolia* showed protective effect on oxidatively stressed *S. cerevisiae.* VR fortification helped in scavenging ROS and significantly re-established the CFUs restoring normalized growth dynamics. Additionally, VR also proved ameliorative against nuclear damage; lowered the antioxidant enzymes and caspases 3/7 expression ultimately enhancing the MG utilization efficacy of *S. cerevisiae*. The protective effect displayed by VR on the model organism *S. cerevisiae* clearly demonstrated the efficacy of herbal medicine against oxidative damages.

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Conflict of interest

Authors declare no competing interests.

References

1 Mitrowska K, PosyniA & Zmudzki J, The effects of cooking on residues of malachite green and leucomalachite green in carp muscles. *Analytica chimica acta*, 586 (2007) 420.

2 Sudova E, Machova J, Svobodova Z & Vesely T, Negative effects of malachite green and possibilities of its replacement in the treatment of fish eggs and fish: *A review Vet Med* (*Praha*), 52 (2007) 527.

3 Bose B, Motiwale L & Rao KVK, DNA damage and G2/M arrest in Syrian hamster embryo cells during Malachite green exposure are associated with elevated phosphorylation of ERK1 and JNK1. *Cancer Lett*, 230 (2005) 260.

4 Majeed AS, Nambi KSN, Taju G, Vimal S, Venkatesan C & Hameed ASS, Cytotoxicity, genotoxicity and oxidative stress of malachite green on the kidney and gill cell lines of freshwater air breathing fish *Channa striata*. *Environ Sci Pollut Res Int*, 21 (2014) 13539.

5 Stammati A, Nebbia C, De Angelis I, Albo AG, Carletti M, Rebecchi C, Zampaglioni F & Dacasto M, Effects of malachite green (MG) and its major metabolite, leucomalachite green (LMG), in two human cell lines. *Toxicol In vitro*, 19 (2005) 853.

6 Dhamgaye S, Devaux F, Manoharlal R., Vandeputte P, Shah AH, Singh A, Blugeon C, Sanglard D & Prasad R, In vitro effect of malachite green on *Candida albicans* involves multiple pathways and transcriptional regulators UPC2 and STP2. *Antimicrob Agents Chemother*, 56 (2012) 495.

7 Hasanuzzaman M, Bhuyan MH, Zulfiqar F, Raza A, Mohsin SM, Mahmud JA, Fujita M & Fotopoulos V, Reactive oxygen species and antioxidant defense in plants under abiotic stress: Revisiting the crucial role of a universal defense regulator. *Antioxidants*, 9 (2020) 681.

8 Kumari S, Badana AK & Malla R, Reactive oxygen species: a key constituent in cancer survival. *Biomark Insights*, 13 (2018) 1177271918755391.

9 Maynard S, Schurman SH, Harboe C, de Souza-Pinto NC & Bohr VA, Base excision repair of oxidative DNA damage and association with cancer and aging. *Carcinogenesis*, 30 (2009) 2.

10 Ayer A, Fellermeier S, Fife C, Li SS, Smits G, Meyer AJ, Dawes IW & Perrone GG, A genome-wide screen in yeast identifies specific oxidative stress genes required for the maintenance of sub-cellular redox homeostasis. *PLoS ONE*, 7 (2012) e44278.

11 Ayer A, Sanwald J, Pillay BA, Meyer AJ, Perrone GG & Dawes IW, Distinct redox regulation in sub-cellular compartments in response to various stress conditions in *Saccharomyces cerevisiae*. *PLoS ONE*, 8 (2013) e65240.

12 Jarolim S, Ayer A, Pillay B, Gee AC, Phrakaysone A, Perrone GG, Breitenbach M & Dawes IW, *Saccharomyces cerevisiae* genes involved in survival of heat shock. *G3* (*Bethesda*), 3 (2013) 2321.

13 Thorpe GW, Reodica M, Davies MJ, Heeren G, Jarolim S, Pillay B, Breitenbach M, Higgins VJ & Dawes IW, Superoxide radicals have a protective role during H_2O_2 stress. *Mol Biol Cell*, 24 (2013) 2876.

14 Allen S, Clark W, McCaffery J, Cai Z, Lanctot A, Slininger P, Liu Z & Gorsich S, Furfural induces reactive oxygen species accumulation and cellular damage in *Saccharomyces cerevisiae*. *Biotechnol Biofuels*, 3 (2010) 1.

15 Cyrne L, Martins L, Fernandes L & Marinho HS, Regulation of antixodant enzymes gene expression in the yeast *Saccharomyces cerevisiae* during stationary phase. *Free Radic Biol Med*, 34 (2003) 385.

16 Ackerley DF, Barak Y, Lynch SV, Curtin J & Matin A, Effect of chromate stress on *Escherichia coli* K-12. *J Bacteriol*, 188 (2006) 3371.

17 Du L, Yu Y, Chen J, Liu Y, Xia Y, Chen Q & Liu X, Arsenic induced caspase and mitochondria mediated apoptosis in *Saccharomyces cerevisiae*. *FEMS Yeast Res*, 7 (2007) 860.

18 Mirek MK, Teeza RZ, Bednarska S & Bartosz G, Acrolein induced oxidative stress and cell death exhibiting feature of apoptosis in the yeast *Saccharomyces cerevisiae* deficient in SOD1. *Cell Biochem Biophys*, 71 (2015) 1525.

19 Mukherjee S, Pawar N, Kulkarni O, Nagarkar B, Thopte S, Bhujbal A & Pawar P, Evaluation of free-radical quenching properties of standard Ayurvedic formulation *Vayasthapana Rasayana*. *BMC Complement Altern Med*, 11 (2011) 38.

20 Parekh J & Chanda SV, In vitro Antimicrobial Activity and Phytochemical Analysis of Some Indian Medicinal Plants. *Turk J Biol*, 31 (2007) 53.

21 Jakubowski W, Biliński T & Bartosz G, Oxidative stress during aging of stationary cultures of the yeast *Saccharomyces cerevisiae*. *Free Radic Biol Med*, 28 (2000) 659.

22 Jadhav JP & Govindwar SP, Biotransformation of malachite green by *Saccharomyces cerevisiae* MTCC 463. *Yeast*, 23 (2006) 315.

23 Lowry OH, Rosebrough NJ, Farr AL & Randall RJ, Protein measurement with the folin phenol reagent. *J Biol Chem*, 193 (1951) 265.

24 Biradar SP, Rane NR, Patil TS, Khandare RV, Govindwar SP & Pawar PK, Herbal augmentation enhances malachite green bio degradation efficacy of *Saccharomyces cerevisiae*. *Biologia*, 71 (2016) 475.

25 Fernandes C, Lalitha VS & Rao VK, Enhancing effects of malachite green on the development of hepatic preneoplastic lesions induced by N-nitrosodiethylamine in rats. *Carcinogenesis*, 12 (1991) 839.

26 Srivastava S, Sinha R & Roy D, Toxicological effects of malachite green. *Aquat Toxicol*, 66 (2004) 319.

27 Duina AA, Miller ME & Keeney JB, Budding Yeast for Budding Geneticists: A Primer on the *Saccharomyces cerevisiae* Model System. *Genetics*, 197 (2014) 33.

28 Smith AM, Ammar R, Nislow C & Giaever G, A survey of yeast genomic assays for drug and target discovery. *Pharmacol Ther*, 127 (2010) 156.

29 Yi W, Akoh CC, Fischer J & Krewer G, Effects of phenolic compounds in blueberries and muscadine grapes on HepG2 cell viability and apoptosis. *Food Res Int*, 39 (2006) 628.

30 Palermo V, Mattivi F, Silvestri R, La Regina G, Falcone C & Mazzoni C, Apple can act as anti-aging on yeast cells. *Oxid Med Cell Longev*, 2012 (2012) 1.

31 Perrone GG, Tan SX & Dawes IW, Reactive oxygen species and yeast apoptosis. *Biochim Biophys Acta – Mol Cell Res*, 1783 (2008) 1354.

32 Balsano C & Alisi A, Antioxidant effect of natural bioactive compounds. *Curr Pharma Design*, 15 (2009) 3063.

33 Mendes TC, Cordeiro CAMM & Ferreira CEL, An experimental evaluation of macroalgal consumption and selectivity by nominally herbivorous fishes on subtropical rocky reefs. *J Exp Mar Biol Ecol*, 471 (2015) 146.

34 Burhans WC, Weinberger M, Marchetti MA, Ramachandran L, D'Urso G & Huberman JA, Apoptosis-like yeast cell death in response to DNA damage and replication defects. *Mutat Res Fundam Mol Mech Mutagen*, 532 (2003) 227.

35 Gopinathan R, Kanhere J & Banerjee J, Effect of malachite green toxicity on non target soil organisms. *Chemosphere*, 120 (2015) 637.

36 Rodriguez R & Redman R, Balancing the generation and elimination of reactive oxygen species. *Proc Natl Acad Sci USA*, 102 (2005) 3175.

37 Vigneswara V & Ahmed Z, The role of caspase-2 in regulating cell fate. *Cells*, 9 (2020) 1259.

38 Lavrik IN, Golks A & Krammer PH, Caspases: pharmacological manipulation of cell death. *J Clin Invest*, 115 (2005) 2665.

39 Kipanga PN, Demuyser L, Vrijdag J, Eskes E, D'hooge P, Matasyoh J, Callewaert G, Winderickx J, Van Dijck P & Luyten W, Investigating the Antifungal Mechanism of Action of Polygodial by Phenotypic Screening in *Saccharomyces cerevisiae*. *Int J Mol Sci*, 22 (2021) 5756.

40 Castelli MV, Lodeyro AF, Malheiros A, Zacchino SA & Roveri OA, Inhibition of the mitochondrial ATP synthesis by polygodial, a naturally occurring dialdehyde unsaturated sesquiterpene. *Biochem Pharmacol*, 70 (2005) 82.

41 Radi R, Oxygen radicals, nitric oxide, and peroxynitrite: Redox pathways in molecular medicine. *Proc Natl Acad Sci India USA B*, 115 (2018) 5839.

42 Falcone C & Mazzoni C. External and internal triggers of cell death in yeast. *Cell Mol Life Sci*, 73 (2016) 2237.

43 Stirpe M, Palermo V, Bianchi MM, Silvestri R, Falcone C, Tenore G, Novellino E & Mazzoni C, Annurca apple (M. pumila Miller cv Annurca) extracts act against stress and ageing in *S. cerevisiae* yeast cells. *BMC Complement Altern Med*, 17 (2017) 1.

44 Das N & Charumathi D, Remediation of synthetic dyes from wastewater using yeast—an overview. *Indian J Biotechnol*, 11 (2012) 369.

45 Nicolaou S, Gaida S & Papoutsakis E, A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: From biofuels and chemicals, to biocatalysis and bioremediation. *Metab Eng*, 12 (2010) 307.