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SYNERGISTIC EFFECT *OF MORINGA OLEIFERA* ATTENUATES OXIDATIVE STRESS INDUCED APOPTOSIS IN *SACCHAROMYCES CEREVISIAE* CELLS: EVIDENCE FOR ANTICANCER POTENTIAL.

V.PRASANNA AND S.SREELATHA *

Dept of Chemistry & Biosciences, SRC Campus, SASTRA University, Tamilnadu, India.

ABSTRACT

Moringa oleifera is a multipurpose medicinal food of dietary interest known to have health beneficial effects, and has been used as a possible ayurvedic medicine. This study was carried out to investigate the effects of H_2O_2 induced apoptosis, that measure cellular proliferation, viability, and morphological changes and to investigate if the dietary leaf extract could inhibit the oxidative stress events and the mechanism against the oxidative assaulted biomolecular targets, which simultaneously contributes to the understanding of cellular responses associated. H_2O_2 induced oxidative stress in yeast cells was assayed with parameters like cell viability, apoptotic morphological and nuclear changes and the antioxidant enzyme levels. The formation of TBARS was also assayed. H_2O_2 imposed oxidative damage in yeast cells resulted in a sequence of events characteristic of apoptosis, including loss of cell viability, morphological changes and apoptotic nuclear changes. There was a decrease in the levels of antioxidant enzymes, and an increase in the lipid peroxide formation. The presence of the leaf extracts attenuated the oxidative stress and significantly enhanced the levels of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT). The leaf extracts caused a very significant inhibition of the extent of lipid peroxide formation and further showed a significant hydroxyl radical scavenging activity. The leaf extracts were characterized for the active principles and quercetin glucoside and keampferol rhamnoglucoside were identified in the ethanol leaf extracts. Additionally the leaf extracts also suppressed the effects against H_2O_2 induced apoptotic cellular changes. These results suggest the valuable use of *Moringa oleifera* leaves, consumed as leafy vegetable, can provide potential functional nutrients that can help in the prevention of oxidative stress disorders.

KEYWORDS: Reactive Oxygen Species; Oxidative damage; Yeast cells; Apoptosis.

S.SREELATHA
Dept of Chemistry & Biosciences, SRC Campus, SASTRA University, Tamilnadu, India.

INTRODUCTION

Oxidative stress plays a major role in the development of chronic and degenerative ailments such as cancer, arthritis, aging, autoimmune disorders, cardiovascular and neurodegenerative diseases. In recent years there is an upsurge in the areas related to newer developments in prevention of disease especially the role of free radicals and antioxidants. . ROS are known to attack cellular biomolecules and are responsible for increased oxidation of protein, DNA, polyunsaturated fatty acids, and lipids. These oxidative stress-induced damages disrupt cellular function and membrane integrity, thereby leading to apoptosis[1-2]. As the major component of ROS, H2O2 has been extensively used as an inducer of oxidative stress in many *in vitro* models [3]. The apoptotic mode of cell death is an active and defined process in the development of multicellular organisms and in the regulation and maintenance of cell populations in tissues .Impairment of apoptosis , the physiological and pathological cell death process, is central to cancer development and renders tumor refractory to cytotoxic therapy[4].Cells in multicellular organisms commit suicide to achieve and maintain homeostasis by specifically ordered metabolic changes during normal development, environmental stress, or pathogen attack[5]. The implication of oxidative stress in the etiology of several chronic and degenerative diseases suggests that antioxidant therapy represents a promising avenue for treatment. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ, or externally supplied through foods and/or supplements. Endogenous and exogenous antioxidants act as “free radical scavengers” by preventing and repairing damages caused by ROS and RNS, and therefore can enhance the immune defense and lower the risk of cancer and degenerative diseases. Dietary antioxidants protect against oxidative damage to DNA, proteins, and lipids and have a significant impact on the regulation of gene expression. Intake or plasma concentration of dietary AOX has been associated with the low risk of chronic disease in healthy diets [6].

Moringa oleifera is an important food commodity which has enormous attention as the ‘natural nutrition of the tropics’. The leaves, fruit, flowers and immature pods of this tree are used as a highly nutritive vegetable in many countries, particularly in India, Pakistan, Philippines, Hawaii and many parts of Africa [7]. *Moringa oleifera leaves* have been reported to be a rich source of β-carotene, protein, vitamin C, calcium and potassium and act as a good source of natural antioxidants; and thus enhance the shelf-life of fat containing foods due to the presence of various types of antioxidant compounds such as ascorbic acid, flavonoids, phenolics and carotenoids. A number of medicinal properties have been ascribed to various parts of this highly esteemed tree .Almost all the parts of this plant, root, bark, gum, leaf, fruit (pods), flowers, seed and seed oil have been used for various ailments in the indigenous medicine of South Asia, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal, haematological and hepatorenal disorders [8-9]. In our previous study we have documented the *invitro* antioxidative potential of the *Moringa oleifera* leaf extracts [10]. *Moringa oleifera* leaves provide powerful benefits to anyone who is conscious of their nutrient intake. The leaves of the Moringa tree are among the most protein-dense leaves of any plant species.So far no comprehensive research has been compiled encompassing the efficacy of the leaf extract in oxidative stress induced programmed cell death. Its versatile utility as a medicine, functional food, nutraceutical and water purifying potential motivated us to bridge the information gap in this area.

MATERIALS AND METHODS

Plant material and preparation of extracts.

Moringa oleifera leaves were collected from the Horticulture Research Institute, Periyakulam, Tamil Nadu Agricultural University, India, and a voucher specimen was deposited in Botanical Survey of India, Coimbatore, India. The air dried leaves of

Moringa oleifera were made into a coarse powder. One gram of the powder was weighed , homogenized and extracted with 7:3 v/v ethanol- water mixture at room temperature. The extract was filtered, freeze dried and stored in a vacuum desiccator for further use. The yield of the extract was 32% with reference to the dry starting material. The concentration of *Moringa oleifera* leaf extract was determined based on its maximum protection against proliferation activity.

Phytochemical analysis

Moringa oleifera leaves were dried, powdered and extracted with ethanol for 24-36h.Extracts were filtered, dried in vacuo and evaluated for the presence of carbohydrates, proteins, flavonoids, tannins and alkaloids using standard procedures[11]. As the presence of phenolics and flavonoids in the leaf extracts were documented in our previous study [12] the leaves were extracted using the extraction protocols [13] specific for phenolics and flavonoids. Spectral analysis was conducted in order to identify the chemical nature of the active principles and to further confirm the active components possibly rendering antioxidant protection.

Determination of Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the *Moringa oleifera* leaf extract (MLE), which is generated by hydrogen peroxide was quantified by the method reported by [14]. The damage to deoxyribose was quantified as thiobarbituric acid reactive substances which are also taken as a measure of hydroxyl radical scavenging activity of the leaf extracts. The pink colour produced was measured at 535 nm in a spectrophotometer. Ascorbic acid was used as a positive control. Deoxyribose degradation was measured as TBARS and the percent inhibition was calculated.

Cell culture & Induction of apoptosis

When considering experimental systems for evaluating ROS-mediated toxicities, the yeast *Saccharomyces cerevisiae* is an excellent organism to study regulation of heterologous gene expression in response to oxidative stress .Yeasts have recently been used to study apoptosis, and a number of reports have provided evidence that *S.cervisiae* display several of the hall mark features associated with apoptosis and on examination of the completed genomic sequence of *S.cerevisiae*,yeast have no apparent homologous of major apoptotic regulators described in metazoan organisms. Radical oxygen species (ROS) trigger apoptosis in numerous mammalian cells and it has been demonstrated that ROS have a central role in all cases of yeast apoptosis known to date [15]. Commercially available wild –type Baker's strain, *Saccharomyces cerevisiae* was used throughout the study. This organism was maintained on YEPD (Yeast extract-peptone-dextrose) agar slants containing glucose 2%, yeast extract 1%, peptone 2%, and agar 2%. Cells from permanent culture were grown in YEPD liquid medium up to logarithmic phase. Cultures were maintained at 4°C for about 24 h before treatments and then transferred in YEPD liquid medium supplemented with 10mM KH_2PO4 [16] and shaken in semi anaerobic conditions for one night. Exponential growth-phase cells were harvested and suspended (10^7 cells /ml) in liquid YEPD medium containing 150mM H_2O_2.The treatments were carried out for 200min at 30°C with mechanical shaking (200rpm).

Cell proliferation assay.

In vitro cytotoxicity assay was carried out using trypan blue assay. The cell lines in concentration 5×10^5 cells/ml were treated with different concentrations of testing extracts and incubated 24 h at 37 °C in air atmosphere humidified 5% CO_2. At the end of this period, the medium from each plates was removed by aspiration. Next, the cells were washed with PBS and centrifuged at an 800 rpm for 10 min, and then PBS was removed by aspiration. Then 10 µl suspension cells were incubated for 5 min with the 10 µl 0.4% trypan blue solution (Sigma). Thereafter, an Olympus BX41 microscope was used to analyse the presence of nonviable cells, which were dark blue and viable cells, which excluded the dye[17].

Cellular apoptotic analysis

Apoptotic morphological changes of cells.

Overnight grown cultures of *Saccharomyces cerevisiae* were spun down and the cell pellet washed twice with saline and resuspended in saline. Cells ((1 × 10^6 cells) were incubated for 200min with H_2O_2 and /or leaf extracts. After incubation, the medium was removed and cells were collected by centrifugation resuspended in few drops of diluted Giemsa Stain. They were then observed by phase contrast inverted microscope (Zeiss, Germany) at1000× magnification [18].

Apoptotic nuclear changes

PI can stain the nuclear changes of living and apoptotic cells. Briefly, cells (1×10^6 cells/well) were incubated for 48 h with H_2O_2 and plant extracts. After incubation, cells were permeabilized with a mixture of acetone, methanol (1, 1) at −20 ◦C for 10 min after treating with extract. Cells were washed with HBSS, then, 200µl of 5µg/ml PI was added into each well and incubated at 37 ◦C for 30 min in the dark. Cells were detected by green filter of fluorescence inverted microscope (Zeiss, Germany) at 400× magnification [19].

Determination of Apoptotic Index.

Acridine orange is a fluorescent dye which gives fluorescent green colour with non apoptotic cells (viable) and red colour with apoptotic cells (non-viable) based on nuclear changes. Adherent cells were washed twice with PBS and stained with a mixture of 4 µg/ml acridine orange for 5 minutes. Cells were fixed with 1% formaldehyde/0.2% glutaraldehyde for 5min at room temperature [20] and visualized by epifluorescence microscopy (LEICA DM IL FLUO, Wetzlar, Germany) at a magnification of 40×. Viable (normal, green nuclei), early apoptotic (condensed, green nuclei), late apoptotic (condensed, red nuclei), and necrotic (normal, red nuclei) cells were counted. At least 400 cells were scored in triplicate for each sample.

Lipid per oxide Formation.

Lipid per oxidation (LPO) has been considered as one of the best known manifestations of oxidant-induced cell injury. Lipid per oxidation was followed by measuring the TBARS formed [21] with minor modifications and the result expressed as mole of MDA /mg protein. Equal volumes of leaf extract and 200µM H_2O_2 in FBS free DMEM were added to each well and the cell plate was incubated for 24 hr. Briefly, yeast cells were lysed using a freezing- thawing method. To the lysate added thiobarbituric acid reagent and it was incubated at 90ºC for 1h and then cooled and further added a mixture of n-butanol –pyridine (15:1,v/v) and centrifuged for 15mins. The absorbance of the upper phase was measured at 532nm.

Antioxidant Enzyme assay

The SOD activity was assayed according to the method described by [22] in yeast cells with modifications. Briefly, 20 µL of samples with the same amount of protein were mixed with 870 µL of solution A (50 mmol phosphate buffer (pH 7.8) with 0.1 mol ethylenediaminetetraacetic acid, 0.001 N NaOH with 5 µmol xanthine, and 2 µmol cytochrome C) and 20 µL of solution B (50 mmol phosphate buffer [pH 7.8] and 0.2 µmol xanthine oxidase). Enzyme activity in the sample was calculated from a standard curve with the range from 0.05 to 12.5 units/mg protein using SOD enzyme (Sigma, Saint Louis, MO, USA). One unit of SOD activity is defined as the enzyme concentration required to inhibit chromogen production by 50% in 5 min under the assay conditions. CAT activity was assayed by the method of[23] in yeast cells with modifications. Briefly, 50 µL of samples with the same amount of protein were mixed with 1 mL of 0.01 mol phosphate buffer (pH 7.0) and H_2O_2. The mixture was immediately read at 240 nm for 1 min on a spectrophotometer. Changes in absorbance were taken to be proportional to the breakdown of H_2O_2. Enzyme activity was expressed as units of CAT/mg protein.

Statistical analysis.

All the parameters studied were subjected to statistical treatment using Sigma Stat statistical package (Version 3.1). The data were expressed as mean ±S.D (n=6) where 'n' represents the no of samples. One-way ANOVA, followed by post-hoc analysis using Fischer's LSD was adopted to all the parameters under study to test the level of

statistical significance. The difference was considered significant if $p<0.05$.

RESULTS & DISCUSSION

Moringa oleifera is a widely consumed traditional plant food used in the treatment of various ailments [9].The present study was designed to investigate the possible molecular mechanism in the oxidative stress condition.

Bioactive Components

As the leaves when subjected to phytochemical screening revealed the presence of various phytoconstituents the leaf extracts were then examined for the specific phenolic composition by the spectral analysis.The structure of the bioactive compounds present in the leaf extract was elucidated after analyzing the data obtained by spectral analysis.The result graph was compared with the reference chart and possible functional group present in the plant were determined[24]. Therefore, ethanol extract of the leaves contain chlorogenic acid, quercetin glucoside and keampferol rhamnoglucoside which reiterates that these polyphenolic compounds can prevent radical mediated cell damage.

Hydroxyl radical scavenging activity

The extracts of *Moringa oleifera* leaves decreased the harmful effects of H_2O_2 by effectively scavenging hydroxyl radicals Fig-1(a). Hydroxyl radical is an extremely reactive species that readily oxidizes all cellular macromolecules including proteins, sugars, lipids and DNA .It has been demonstrated that ROS have a central role in all cases of yeast apoptosis known to date [25]. Naturally occurring antioxidants have been shown to inhibit ROS generation, scavenge free radicals and initiate alteration of intracellular redox potential [26]. This study demonstrates the potential scavenging effect of the leaf extracts on the intracellular oxidative damage induced by H_2O_2 which in turn, lead to an improvement in cell viability. . There are sufficient evidences revealing that polyphenolic antioxidants prevent radical mediated cell damage and indirectly suppress apoptosis[27]. Our findings are in agreement with those obtained with hydroxyl radical scavenging activity indicating major active principles responsible for the regulation of apoptotic cell death through the antioxidant mechanism.

Cellular Proliferation assay

Oxidative stress is the development that happens at close by cell-membranes, setting off a ripple effect of free-radical formation, which eventually leads to damage of the cell [28].. The response of yeast when treated with ROS –generating agents is consistent with this observation, as the exposure of H_2O_2 resulted in a clear apoptotic morphology and subsequent cell death. Oxidative damage caused by H_2O_2 administration can induce cell damage and eventually cell death through iniation of free radicals and lipid per oxidation chain reactions [1]. The results of the trypan blue assay indicate that H_2O_2 exposure inhibits the proliferation of yeast cells during the active phase of the cell cycle. In the present study, the effect of *Moringa oleifera* leaf extracts on the extent of survival of yeast cells challenged with H_2O_2 was followed using the trypan blue exclusion assay. The cell viability decreased significantly with H_2O_2 treatment, but enhanced, markedly upon co-treatment with the leaf extract of *Moringa oleifera* Fig-1 (b). H_2O_2 induced cell –growth suppression had been reported in many cell types. Furthermore it has been suggested that cellular proliferation may be inhibited in the event of attack of free radicals on membrane lipids and ultimate cell death [1].Therefore the results obtained are consistent with these reports and reiterates the cytotoxicity by H_2O_2 exposure. The results also reiterated that natural compounds present in the extracts exhibited cytoprotective effect on the yeast cells.

Cellular apoptotic changes

The Morphological changes in the yeast cells observed by phase contrast microscope revealed changes in the refractive index of cell , followed by a series of changes like cytoplasmic membrane shrinkage, loss of contact with neighboring cells, membrane blebbing and apoptotic body. Fischer et al [29] have reported that the key morphological alterations during apoptosis including

chromatin condensation, nuclear remodeling and membrane blebbing are determined by interplay of caspase substrate cleavage. In tune with these studies, the results obtained in our investigation confirm that yeast cells when treated with H_2O_2 showed apoptotic body formation as a morphological mark of apoptosis. Membrane blebbing is considered to be one of the key morphological alterations of apoptotic cells [30].Several studies have characterized membrane blebbing as a feature to quantitate apoptotic death of cells.H_2O_2 induced oxidative damage was further supported by the results of the morphological changes in yeast cells. Morphologically, apoptosis is first characterized by a change in refractive index of the cell, followed by a series of changes like cell shrinkage, deformation and loose contact with its neighboring cells, chromatin condensation and membrane blebbing and budding[31].In the present study, there was a steep increase in the number of cells undergoing apoptosis when subjected to oxidative stress with H_2O_2 as presented in Fig-1 (c). This result clearly demonstrated that yeast cell apoptosis by oxidative stress was suppressed by pretreatment with the leaf extracts, suggesting that H_2O_2 -induced oxidative stress increased plasma membrane damage and the leaf phenolics protected the cells from membrane toxicity.

H_2O_2 exposure caused a very high number of yeast cells to become permeable to PI, indicating oxidation-induced apoptosis. The number of cells undergoing apoptosis induced by H_2O_2 decreased sharply by the presence of the leaf extracts. H_2O_2 induced oxidative damage was supported by the results of Propidium Iodide (PI) staining and acridine orange (AO) staining Fig -1 (d, e). Reactive oxygen species (ROS) production has been observed concomitant with increased apoptosis, as measured by PI permeability, in yeast cells [32].The formation of acidic vesicular organelles (AVOs) is also related to cell autophagy[33]. Acridine orange stained cells showed diffused green fluorescence where as the acidic compartments, including (AVOs) showed bright red fluorescence. Propidium iodide (PI) is a fluorescent molecule that intercalates into nucleic acids and can be used to visualize the nuclear changes in apoptotic cells. Cells undergoing apoptosis become increasingly permeable to propidium iodide (PI) which is too large a molecule to enter live and active cells. PI is membrane impermeant that is commonly used for identifying cells. The antioxidant N-acetyl –L-cysteine inhibited etoposide –induced apoptosis in HL-60 tumor cells as seen by PI staining. Therefore, PI staining and acridine orange staining is taken as an index of the extent of apoptosis in the cells [34]. Plant extracts and plant-derived products have been reported to modulate the process of apoptosis in several cells, which is indicative of their pharmacological efficiency. It was also has reported that the chemicals that can modify programmed cell death are likely to be potentially useful drugs [35]. In light of the above reports, our results suggest that H_2O_2 –induced apoptosis in yeast cells can be effectively counteracted by the leaf extracts of *Moringa oleifera*. Phenolic compounds possess diverse biological properties such as scavenging of free radicals, anticancer activity, antimutagenic and anti-apoptotic activity [36]. The phytochemicals present in plant tissues responsible for the antioxidant capacity can be largely attributed to the phenolics and flavonoid compounds [37].Thus the polyphenolic antioxidants show significant cell protecting activity and inhibit apoptosis. Again, our findings are in agreement indicating major active principles responsible for anti-apoptotic activity against free radicals. Therefore, it reiterates that consumption of the dietary leaf extract with the high phenolics content will supply beneficial antioxidative phytochemicals which plays an important role in reducing oxidative stress disorders.

Lipid peroxidation & Antioxidant Enzyme Response

Reactive oxygen species damage membrane phospholipids and results in lipid peroxidation [38].The levels of MDA concentration in H_2O_2-induced cells were significantly increased compared to control cells. The treatment of cells with the leaf extract significantly reduced the MDA production as shown in Fig 2(a). LPO has been used as a reliable marker of oxidative stress, both *in vitro* and *in vivo*. Several plant extracts have been shown to inhibit LPO as measured by the levels of

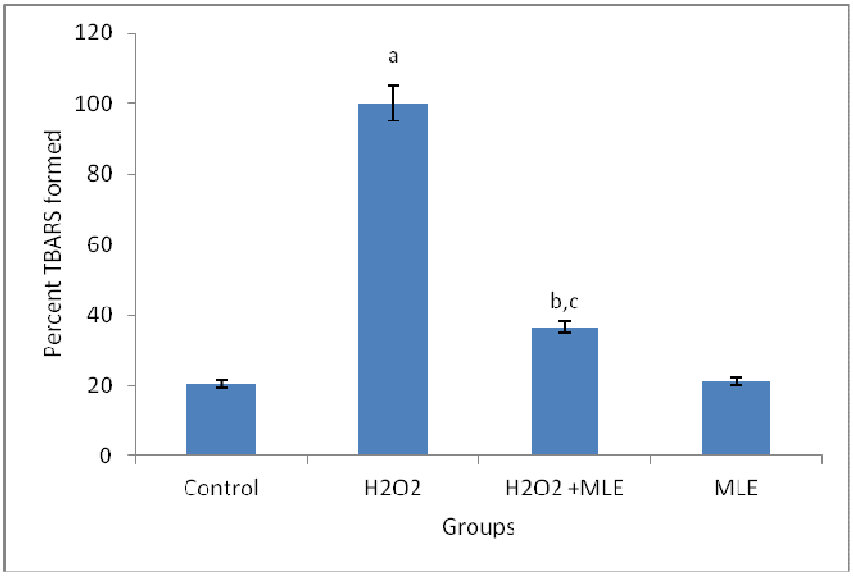
TBARS. ROS and RNS may initiate and propagate lipid per oxidation, of which, the end product malondialdehyde possesses genotoxic and mutagenic properties [38]. Lipid per oxidation is a key process in many pathological events and is one of the reactions induced by oxidative stress. Increased MDA accumulation has been noted in response to H_2O_2 and the cytotoxic effects of H_2O_2 on yeast cells were shown by its strong inhibition of cell growth and MDA formation. These results indicate that the leaf extract is capable of reducing H_2O_2-induced cytotoxicity and lipid per oxidation. Thus the prevented lipid per oxidation may explain its cytoprotective property of the leaf extracts on the cell membrane damage caused by the free radicals.

The protective effect of the leaf extract on the levels of the antioxidant enzymes as shown in Fig-2 (b,c) reveals that the levels of SOD and CAT in H_2O_2 – induced cells were significantly reduced compared to the control group. However, treatment of the cells with the leaf extract increased the levels of these antioxidant enzymes significantly. The antioxidant system includes several enzymes which are capable of removing oxygen radicals and their products and/or repairing the damage caused by oxidative stress. SOD and CAT play a major role in defence mechanism and extensively used as biochemical indicators of pathological states, associated with oxidative stress[39]. Superoxide dismutase and catalase can act as anticarcinogens and inhibitors at initiation and promotion / transformation stage in carcinogenesis and have a vital antioxidant role in human health, conferred by their scavenging of the free radicals[40]. Treatment with the leaf extract significantly increased the levels of the antioxidant enzymes SOD and CAT. Earlier studies have also shown strong antoxidant and free radical scavenging activities of the leaf extracts [41-42]. The result of this study state that *Moringa oleifera* leaf extracts can act as effective modulators in reducing the toxicity by enhancing the stimulation of enzymes in the cells under stress. In conclusion the results demonstrated that oxidative damage caused by H_2O_2 can be reduced by *Moringa oleifera* extracts iterates the scavenging capacity of the extracts. The assay on yeast cells demonstrates its promising activity against oxidative damage and thus these findings suggest *Moringa oleifera* leaf extract may be useful as a potential agent against oxidative stress.

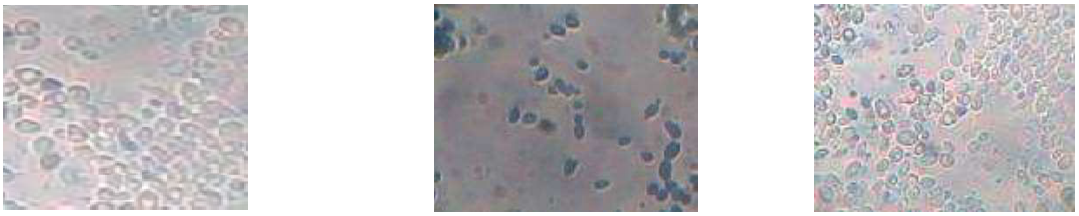
Figure-1

Figure –1 Effect of Moringa oleifera leaf extract (MLE) on radical Scavenging activity and apoptotic cellular changes.

a)

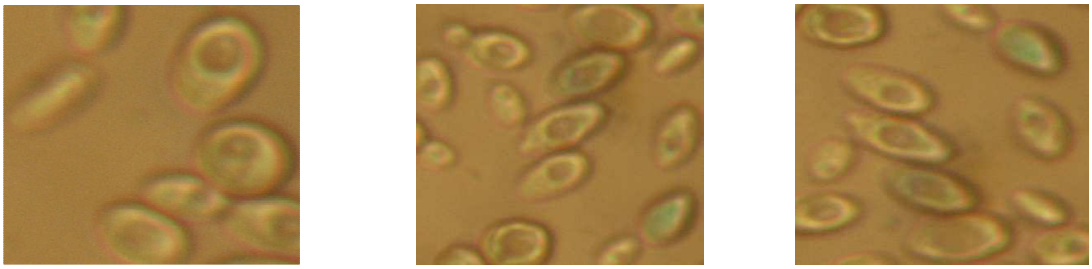


b)



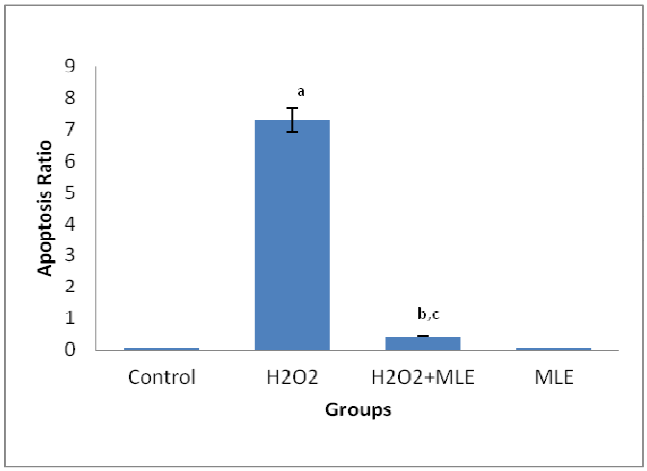
Viable cells | Non-viable cells | H_2O_2 + Leaf extract

c)

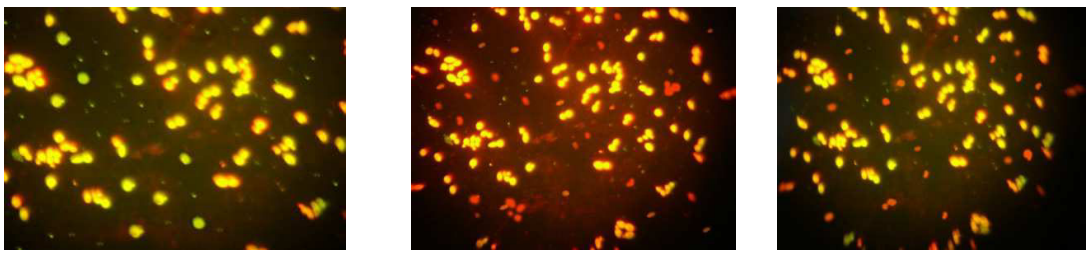


Control | H_2O_2 | H_2O_2 + Leaf extract

d)



e)



Control | H_2O_2 | H_2O_2 + Leaf extract

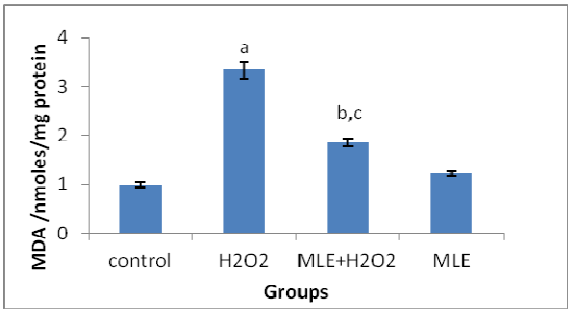
a) Percent hydroxyl scavenging activity. Results are expressed as mean ± SD,n=6. [a] p<0.05 compared to control, [b] p< 0.05 compared to plant control, [c] p< 0.05 compared to cells exposed to H_2O_2 alone.

b) Morphological changes in yeast cells with membrane blebbing. c) Trypan blue assay with viable and non-viable cells. d) & e) PI and acridine orange staining with apoptosis and nuclear changes.

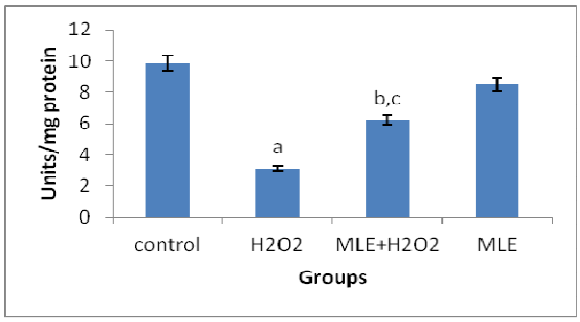
Figure 2

Figure- 2 Effect of Moringa oleifera leaf extract (MLE) on a) Lipid peroxidation b) SOD activity c) CAT activity

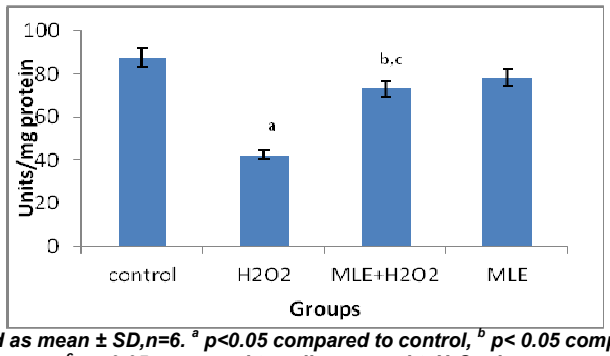
a)



b)



c)



Results are expressed as mean ± SD,n=6. [a] p<0.05 compared to control, [b] p< 0.05 compared to plant control, [c] p< 0.05 compared to cells exposed to H_2O_2 alone.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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